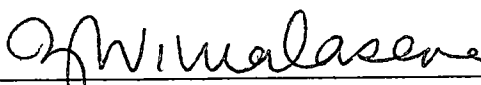
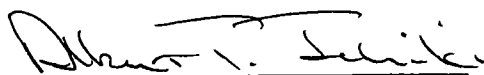


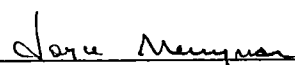
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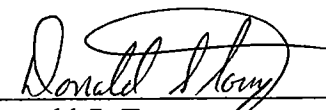
I am submitting herewith a dissertation written by Tzu-Hao Wang entitled "The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) in paclitaxel-induced apoptosis of cancer cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

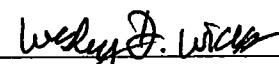

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**THE C-JUN N-TERMINAL KINASE/STRESS-ACTIVATED
PROTEIN KINASE (JNK/SAPK) PATHWAY IN
PACLITAXEL-INDUCED APOPTOSIS OF CANCER CELLS**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**Tzu-Hao Wang
May 1999**

DEDICATION

This dissertation is dedicated to:

my parents

Mr. Chung-Ling Wang

(王鍾靈)

Mrs. Kuan Cheng Wang

(王蔓欸)

my wife

Mrs. Ching-Ling Wang

(王靜玲)

and my daughters

Shih-Tien (Tien-Tien) Wang

(王師恬)

Shih-Yee (Mimi) Wang

(王師怡)

ACKNOWLEDGMENTS

I am very grateful to my Major Professor, Dr. Jay Wimalasena, and my Dissertation Committee, Drs. Albert T. Ichiki, Joyce Merryman, Donald S. Torry, and Wesley D. Wicks, for their encouragement and advices during the last five years of this study.

I am most thankful to my brother, Dr. Hsin-Shih Wang, who is also my research collaborator during the past years and in the future, and Diana M. Popp and Dr. Jane Mural for their collaboration of flow cytometric analyses.

I would like to thank many people in the University of Tennessee Medical Center Research for their technical help and Dr. Michael R. Caudle, Dean of the Graduate School of Medicine, for financial support.

The greatest debt is owed to my wife, Ching-Ling, and my daughters, Tien-Tien and Mimi. Without their understanding, encouragement, and support, I would never have accomplished this work.

ABSTRACT

The essential cellular functions associated with microtubules have led to a wide use of microtubule-interfering agents in cancer chemotherapy with promising results. Although the most well-studied effect of microtubule-interfering agents is an arrest of cells at the G₂/M phase of the cell cycle, other effects may also exist. I have observed that paclitaxel (Taxol), docetaxel (Taxotere), vinblastine, vincristine, nocodazole and colchicine activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway in a variety of human cells. Activation of JNK/SAPK by microtubule-interfering agents is dose-dependent and time-dependent and requires interactions with microtubules. Functional activation of the JNKK/SEK1-JNK/SAPK-c Jun cascade was demonstrated by cotransfection with a TPA-response element reporter construct and dominant negative (dn) signal transducers followed by chloramphenicol acetyl-transferase assays. Microtubule-interfering agents also activate both Ras and apoptosis signal-regulating kinase (ASK1), and coexpression of dn Ras and dn ASK1 exerted individual and additive inhibition of JNK/SAPK activation by microtubule-interfering agents. These findings suggest that multiple signal transduction pathways are involved with cellular detection of microtubular disarray and subsequent activation of JNK/SAPK.

To further examine the role of JNK/SAPK signaling cascades in apoptosis resulting from microtubular dysfunction induced by paclitaxel, I have coexpressed dn signaling proteins of the JNK/SAPK pathway (Ras, ASK1, Rac, JNKK, JNK) in human ovarian cancer cells with a selectable marker to analyze the apoptotic characteristics of cells expressing dn-vectors following exposure to paclitaxel. Expression of these dn signaling proteins had no effect on Bcl-2 phosphorylation, yet inhibited apoptotic changes induced by paclitaxel up to 16 h after treatment. Coexpression of these dn-signaling proteins had no protective effect after 48 h of paclitaxel treatment. These data indicate that: (i) activated JNK/SAPK acts upstream of membrane changes and caspase-3 activation in paclitaxel-initiated apoptotic pathways, independently of cell cycle stage, (ii) activated JNK/SAPK is not responsible for paclitaxel-induced phosphorylation of Bcl-2, and (iii) apoptosis resulting from microtubule damage may comprise multiple mechanisms, including a JNK/SAPK-dependent early phase and a JNK/SAPK-independent late phase.

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LIST OF ABBREVIATIONS

7-AAD: 7-amino actinomycin-D.

Ab: antibody,

ACTD: actinomycin-D,

AIF: apoptosis-inducing factor,

AP-1: activating protein-1,

Apaf: apoptosis-associated factor,

ARIA: activated Ras interacting assay,

ASK1: apoptosis signal-regulating kinase,

ATF: activating transcription factor,

AV: annexin V,

BH: bcl-2 homology,

BMP4: bone morphogenic protein 4,

bZIP: basic region leucine zipper,

ced: cell death abnormal gene of *C. elegans*,

ces: cell death specification gene of *C. elegans*,

CAD: caspase-activated Dnase,

CARD: caspase recruiting domain,

CBP: CREB-binding protein,

CAT assay: chloramphenicol acetyl-transferase assay,

CDDP: cis diamminedichloroplatinum (cisplatin),

CDK: cyclin-dependent kinase,

CKI: CDK inhibitor,

CRE: cAMP response element,

CREB: CRE-binding protein,

CMV: cytomegalovirus,

COL: colchicine,

DAB: deacetylchalcin,

Daxx: Fas death domain-associated protein,

DeR: decoy receptor,

DD: death domain,

DED: death effector domain,

DFF: DNA fragmentation factors,

DL: death ligand,

DMEM: Dulbecco's modified Eagle's medium,

DMSO: dimethyl sulfoxide,

dn: dominant negative,

DR: death receptor,

DTX: docetaxel (Taxotere),

EGF: epidermal growth factor,

EGFP: enhanced green fluorescent protein,

EGFR: EGF receptor,

ELISA: enzyme-linked immunosorbant assay,

ERK: extracellular signal-regulated kinase,

FADD: Fas associated protein containing death domain,

FDA: fluorescein diacetate,

FBS: fetal bovine serum,

FC: flow cytometry or flow cytometric,

FITC: fluorescein isothiocyanate,

GCK: germinal center kinase,

GSH: glutathione,

GST: glutathione S-transferase,

h: hour,

HA: hemagglutinin epitope of influenza virus,

HPK1: hematopoietic progenitor kinase 1,

hsp: heat shock protein,

IAPs: inhibitors of apoptosis,

ICAD: inhibitor of caspase-activated DNase,

ICE: interleukin-1 β -converting enzyme,

IP: immunoprecipitate,

JAB1: Jun activation domain binding protein 1,

JIP-1: JNK interacting protein-1,

JNK: c-Jun N-terminal kinase.

JNKK: JNK kinase (=SEK1, MKK4),

kD: kilodalton,

MAP: microtubule-associated protein,

MAPK: mitogen-activated protein kinase,

MAPK-APK: MAPK-activated protein kinase,

MBP: myelin basic protein,

MC540: merocyanine 540,

MEF2: myocyte-enhancer factor 2,

MEKK: MEK kinase,

MEK/MKK: MAP Erk kinase / MAPK kinase,

MIAs: microtubule-interfering agents,

min: minute,

MLK: mixed lineage kinase,

MP1: MEK partner 1,

MT: microtubule,

NBS: newborn bovine serum,

NGF: nerve growth factor,

NOC: nocodazole,

PAK: p21-activated kinase,
PARP: poly (ADP-ribose) polymerase,
PBS: phosphate-buffered saline,
PCD: programmed cell death,
PE: phycoerythrin,
PH: pleckstrin-homology,
PI: propidium iodide,
PKA: protein kinase A (=cAMP-dependent protein kinase),
PKB: protein kinase B (=Akt),
PKC: protein kinase C (=Ca²⁺- and phospholipid-dependent protein kinase),
cPLA2: cytosolic phospholipase A2,
PS: phosphatidylserine,
PTPC: permeability transition pore complex,
PTX: paclitaxel (Taxol),

RBD: Ras binding domain of Raf,
RIP: receptor interacting protein,
RT: room temperature,

s: second,
SAPK: stress activated protein kinase,
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis,
SEK1: SAPK or ERK kinase-1 (=JNKK, MKK4),
SH: Src homology,
SRE: serum response element,
SRF: serum response factor,

TAK-1: TGF- β -activating kinase-1,
TCF: ternary complex factor,
TGF: transforming growth factor,
TPA: 12-*O*-tetradecanoylphorbol-13-acetate,
TNF: tumor necrosis factor,
TNFR: tumor necrosis factor receptor,
TRAF-2: TNFR-associated factor 2,
TRADD: TNFR-associated death domain protein,
TRE: TPA response element,
TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick end labeling,

UV: ultraviolet,

VBL: vinblastine (Velban),
VCR: vincristine (Oncovin),

WB: Western blot analysis,
wt: wild type,

PART I.

LITERATURE REVIEW

APOPTOSIS

Cell division and cell death are the ultimate differential decisions made by the cell (410). Cell death occurs throughout the life span of all multicellular organisms as a basic mechanism in tissue kinetics, from the formation of limbs during embryogenesis to the peeling of sunburned skin (539). Apoptosis is the most prevalent type of cell death. The machinery for apoptosis in all cell types of multicellular organisms is genetically encoded and readily expressed. Characteristic apoptotic morphology has been detected even in unicellular organisms: the kinetoplastid parasites *Trypanosoma cruzi* and *Trypanosoma brucei rhodensiense*, the free-living slime mold *Dictyostelium discoideum* (135), and the free-living ciliate *Tetrahymena thermophila* (15). These discoveries indicate apoptotic machinery of the cell may have developed for more than 2 billion years, whereas multicellular animals and plants appeared around 0.7 billion years ago (15).

In contrast to necrosis (Table 1), apoptosis is an energy-consuming (ATP-dependent) process (315)(426) and may involve a series of well-regulated synthetic events (261), which earns apoptosis the reputation as a suicidal process (539)(630). During the last two decades, this tightly-confined, cellular self-destruction has been recognized as the major mode of cell death. Till the end of 1998, more than twenty thousand apoptosis-related papers have been compiled in the MEDLINE database.

HISTORICAL REVIEW

In Greek, *apó* means "from", *ptósis* means "a fall", and *apoptosis* is used to describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees (406)(501). This word was first adopted by Kerr *et al.* in 1972 to depict the phenomenon of controlled cell deletion as an antithesis to mitosis in the regulation of cell populations (406). The same authors had previously named this mode of cell death as "~~shrinkage~~ necrosis", describing that cells undergoing apoptosis become condensed and compact (402). Key discoveries on apoptosis are summarized in Table 2.

PHYSIOLOGY, PATHOLOGY AND CLINICAL SIGNIFICANCE

Apoptosis has wide-ranging implications in tissue kinetics. All multicellular organisms

Table 1
Comparison between apoptosis and necrosis

Features	Apoptosis	Necrosis
<i>Nature of stimuli</i>	Physiological or pathological	Pathological (usually accidental)
<i>Susceptibility</i>	Tightly regulated	Unregulated or poorly regulated
<i>Energy requirement</i>	ATP-dependent	None
<i>Histology</i>	Chromatin condensation, budding of apoptotic bodies, death of single isolated cells	Cellular swelling, disruption of organelles, death of patches of tissue
<i>Plasma membrane</i>	Intact, but with molecular "eat-me" signals	Lysed
<i>Phagocytosis of dead cells</i>	By neighboring cells	By immigrant phagocytes
<i>Tissue reaction</i>	No inflammation	Inflammation
<i>DNA breakdown pattern</i>	Ladder of fragments in internucleosomal multiples of 185 base pairs	Randomly sized fragments
<i>Activation of caspases</i>	Yes	No
<i>Involvement of mitochondria</i>	Yes	Yes
<i>Mitochondrial changes</i>	No swelling	Swelling

References: (315)(426)(686).

Table 2

Milestone studies of apoptosis

Year	Discovery or Event	Reference
1885	"Chromatolysis" was used by Walther Flemming to describe the disappearing process of nuclear debris.	(501)
1951	A form of "physiological cell death" was described by A. Glucksmann.	(501)
1964	"Programmed cell death" was proposed by R. A. Lockshin.	(484)
1971	"Shrinkage necrosis" was used to differentiate a type of cell death from the classical necrosis.	(402)
1972	"Apoptosis" was used to describe a type of cell death with a characteristic morphology.	(406)
1980	"Ladder" pattern of DNA fragmentation in apoptosis	(839)
1988	Bcl-2 is anti-apoptotic	(779)
1991	<i>ced</i> (cell death abnormal) genes in <i>Caenorhabditis elegans</i>	(182)
1991	The viral protein BHRF1 was recognized to be anti-apoptotic, resembling Bcl-2.	(264)
1992	Fas/APO-1/CD95	(362)(586)
1993	The role of p53 in regulation of apoptosis	(121)(489)
1993	Fas ligand	(735)
1993	Interleukin-1 β converting enzyme (ICE) is the mammalian homolog of <i>ced-3</i>	(873)
1993	IAPs (inhibitors for apoptosis)	(138)(47)
1994	Mitochondria was proposed to be a primary target during apoptosis.	(781)
1996	AIF (apoptosis-inducing factor)	(739)
1996	"Caspase" was coined to represent a group of proteases that are activated during apoptosis.	(10)
1996	Leakage of cytochrome c from mitochondria during apoptosis	(478)
1997	Apafs (apoptosis-associated factors)	(898)(460)
1997	DFF (DNA fragmentation factor)	(480)
1998	CAD (caspase-activated DNase) and ICAD (inhibitor for CAD)	(185)(677)

physiologically use apoptosis and/or programmed cell death (PCD) for development, homeostasis, defense, and aging (780). It has been observed in processes of development, metamorphosis of insects and amphibians, terminal differentiations, immune responses, cellular responses to hormones and growth factors, injuries, and neoplastic diseases (698)(801). The magnitude of apoptosis could be enormous in given organs, for example, more than 95% of thymocytes die in the thymus during maturation (570). Apoptosis is also prevalent in diseases, such as the insufficient apoptosis in tumorigenesis and the excessive apoptosis in neurodegeneration, autoimmunity, heart diseases, and infectious diseases including AIDS (25)(315)(668)(757).

Physiological roles of apoptosis

Apoptosis is a self-destructive process. Why have unicellular organisms capable of this self-destruction survived the selection of evolution (15)(135)? For a group of unicellular organisms, apoptosis allows constant selection for the best-fit cell in a colony and optimal adaptation of cell numbers to the environment (15)(630). In multicellular organisms, the general purposes of apoptosis (or PCD in ontogeny) are summarized in **Table 3**.

Developmental plasticity and selection for the best-fit cells

A common developmental scheme of ontogeny is that more-than-needed number of cells are originally generated, then many original cells die through PCD, so that only a fraction of the total number of original cells make the final organs. For instance, only approximately 50% of the motor neurons produced during chick embryogenesis survive to function prior to birth (283)(593). An intriguing question therefore is “why should these cells die before they have had a chance to function?” (365). The answers could be two fold. First, PCD may be a mechanism providing a developmental plasticity in addressing a wide range of developmental problems by using a limited number of genes. For example, given about 100,000 genes in the human genome and about 10 billion cells in the human brain, it is impossible to assign each individual cell a unique phenotype. A general strategy of neurogenesis is to generate many more cells than will be needed for a particular function, then to trim this group of cells by a common set of instructions regarding differentiation, migration, and target specificity. Only those cells functionally interacting with their natural targets receive a retrograde trophic factor to survive (539). This strategy of “system matching” ensures the functional integrity of the central nervous systems where given patterns of intercellular connection are essential. Second, PCD may be the selecting mechanism in the developmental

Table 3

Physiological roles of apoptosis

Functions	Examples	References
Developmental plasticity and selection for the best-fit cells	Brain development, development of immune system	(593) (301)
Sculpting the body	Disappearance of the human tail, digit formation of limbs,	(195) (235)
Homeostasis	Hematopoietic system, ovary, endometrium	(523) (388) (742)
Protection of an organism from dangers created by its own deleterious cells	Inactivation of active immune cells after an immune response, host defense against tumorigenesis and viral infections	(776) (802)(613)

scheme where developing cells constantly compete for a limited amount of survival signals. Only the best-fit cells, *i.e.*, the most healthy, fastest growing cells toward the correct direction, are able to access the survival signals from their microenvironment (365)(630).

Sculpting the body

Massive loss of cells through PCD and/or apoptosis is the chisel to sculpt the body during embryogenesis, metamorphosis, and in adult life across species. The elimination of surplus cells is necessary for the formation of digits of the limbs (15)(235)(538), hollowing out solid structures to create lumina (365), and disappearance of the human tail (195). The cells that have outlived their usefulness, such as tadpole tails (748) and the human endometrium at the end of each menstrual cycle (596) should be eliminated during metamorphosis and preparation for the next cycle, respectively.

Homeostasis

Homeostasis of cell numbers requires a delicate balance between input (proliferation) and output (apoptosis) processes (96). Hence the sizes of both organs and organisms are maintained by regulation of cell division (629) and cell death (365). Apoptosis is essential for size control, renewal of cell populations, and maintenance of functions. How proliferation and apoptosis are coordinated to achieve a balance within each cell lineage is not completely clear. These two processes might be controlled by independent genetic pathways with regulation dependent on the level of extracellular signals (96). Alternatively, proliferation and apoptosis may be controlled by overlapping genetic pathways and the cell may employ the same mechanistic components to achieve each process.

During fetal development, apoptosis accounts for the regression of Müllerian ducts in males and Wolffian ducts in females (623). Apoptosis is also responsible for both the ovarian follicle development from the fetal period through the reproductive ages (388)(759) and the renewal of endometrium during each menstrual cycle (332)(742). Both the ovary and endometrium requires hormones and growth factors for development and functions, hence, they are the unique systems for examining the hormonal regulation of apoptosis.

Protection of organisms from deleterious cells

PCD and/or apoptosis also play an important role in protecting organisms from deleterious cells as in the “Better dead than wrong” maxim pointed out by Cohen *et al.* (131). For example, elimination of an auto-reactive cells that bear self-reactive T cell receptors to avoid auto-immune response is the purpose of the negative selection of thymocytes (301)(566). Equally importantly, upon encountering adverse insults such as UV or ionizing irradiation, one safeguarding mechanism for the organism against potential

tumorigenesis is to trigger apoptosis of genome-damaged cells, mainly through the p53-dependent pathways (802)(895). In short, organisms employ apoptosis to delete unwanted, injured, or virus-infected cells (613).

Apoptosis-associated pathology

Homeostasis of cell numbers is tightly balanced between proliferation and apoptosis. Either inadequate or excess apoptosis may result in disease (Table 4).

Induction of apoptosis as the end point of antineoplastic therapy

Accumulating evidence indicates that induction of apoptosis is the common result of different types of antineoplastic therapy (206)(254)(317)(780)(802). Both successful chemotherapy and radiation therapy induce apoptosis of tumor cells rather than killing them as the result of a direct insult to DNA (521)(757). The apoptotic response correlates well with the efficacy of antineoplastic treatment, hence the propensity to apoptosis of tumor cells has been proposed to be a candidate predictor for tumor treatment response (537).

One potential advantage of setting therapy-induced apoptosis as a goal of antineoplastic treatment is that critical concentrations of many anticancer agents may induce apoptosis only in tumor cells but not in normal cells (317)(802). This also suggests that the threshold for the onset of cell death can be determined by the relative expression levels of genes which promote or suppress apoptosis (318). The foremost example is the gestational trophoblastic diseases (GTD), a group of neoplasms (hydatidiform mole, invasive mole, choriocarcinoma) originating from the placenta (801). GTD may become fatal, however, proper combined chemotherapy has brought the cure rate up to 94% (496). Both the embryonic origin and the good response to chemotherapy earn GTD the reputation as “God’s first cancer, man’s first cure.” commented by A. T. Hertig (252). Since the placenta functions as a “disposable” organ after delivery, trophoblasts may be destined to cell death at the end of pregnancy. Their propensity to apoptosis may be the fundamental explanation for the good response of GTD to chemotherapy (801).

How to increase the apoptotic response of tumors to anticancer therapy has been enthusiastically studied. In addition to conventional radiation therapy and chemotherapy, newer approaches include enhancement of pro-apoptotic pathways and inhibition of anti-apoptotic signaling cascades in cancer cells.

More than 60% of human cancer contain mutations in the p53 genes (291)(326)(456) and p53

Table 4

Apoptosis-associated diseases

Diseases associated with Inhibition of Apoptosis	Diseases associated with Increased Apoptosis
<i>Autoimmune diseases:</i> Autoimmune diabetes Autoimmune lymphoproliferative syndromes (ALPS) Immune-mediated glomerulonephritis Systemic lupus erythematosus (SLE)	<i>Acquired immunodeficiency diseases (AIDS)</i>
<i>Cancer:</i> Bax mutation Bcl-2 overexpression Hormone-dependent tumors (breast, ovary, prostate) p53 mutation pRb mutation	<i>Chemically induced:</i> Alcohol-related liver disorders
<i>Endometriosis</i>	<i>Decreased blood production:</i> Myelodysplastic syndromes Aplastic anemia
<i>Viral infections:</i> Adenovirus Epstein-Barr virus Herpesvirus Poxvirus	<i>Heart diseases:</i> Idiopathic dilated cardiomyopathy (IDCM)
	<i>Ischemic injuries:</i> Myocardial infarction Reperfusion injury Stroke
	<i>Neurodegenerative disorders:</i> Alzheimer's disease Amyotrophic lateral sclerosis Cerebellar degeneration Parkinson's disease Retinitis pigmentosa
	<i>Physically induced:</i> Sunburn

References: (25)(40)(41)(96)(173)(243)(315)(497)(523)(524)(526)(528)(539)(802)(831)(757)(869).

mutations have been correlated to poor prognoses in a variety of tumor types (1)(455)(521). Both radiation and chemotherapy can induce apoptosis that depends on the function of wt p53, perhaps through both downregulation of Bcl-2 and upregulation of Bax (282)(549)(550)(702)(741). Therefore, restorations of wt p53 function in tumor cells to enhance the efficacy of antineoplastic therapy have been extensively tried with some encouraging results (122)(174)(224)(255)(706)(743)(808)(865)(867).

From the clinical viewpoint, however, induction of p53-independent apoptosis may be a more important strategy to treat cancers with mutant p53 (802). Various chemotherapeutic agents have been demonstrated to induce p53-independent apoptosis, such as cisplatin, camptothecin, etoposide, vincristine, paclitaxel (Taxol®) (58)(714)(802). A study further indicates that loss of wt p53 function confers cell susceptibility to paclitaxel (PTX) by increasing the number of cells arrested at G₂/M and subsequent apoptosis (788).

The advances in apoptosis research also pave the avenue for development of novel strategies for fighting cancers by targeting the apoptotic pathways of tumor cells. The anti-apoptotic Bcl-2 is found at abnormally high levels in more than half of all human cancers (640). Anti-sense technology has been used to reduce the Bcl-2 protein levels in tumor cells with the effects of inducing tumor cell apoptosis and/or increasing sensitivity to chemotherapy (41)(77)(412)(639)(751)(814).

Other potential targets for augmentation of treatment-induced apoptosis may reside in signal transduction pathways mediating apoptosis (32)(302)(457). Many cell types requires the presence of specific growth factors for survival. A proto-oncogene, the serine/threonine protein kinase Akt (also known as protein kinase B, PKB), may play a critical role for the growth factor-dependent cell survival by sensing the survival signals from growth factor receptors and inhibiting apoptosis by inactivating the pro-apoptotic BAD (140)(152)(176)(210)(211)(212)(302)(306)(305). Our understanding of this growth factor-dependent survival pathway reveals a series of potential targets for therapeutic intervention (302).

APOPTOTIC CHARACTERISTICS

Apoptosis can be detected morphologically and biochemically. Two apoptotic characteristics commonly used to document apoptosis are the apoptotic morphology (20)(404) and “ladder” pattern of DNA fragmentation (839)(841). The characteristic apoptotic morphology recognized by electron or light microscopy is consistently similar across cell types and species and is believed to be the most unequivocal

experimental proof of apoptosis (324). On the other hand, DNA fragmentation is not always found together with morphological changes in cells undergoing apoptosis. The characteristic apoptotic morphology has been found in the absence of typical DNA fragmentation (129)(583)(762) and it even could be induced in a nucleus-free cytoplasm system (364). Many other biochemical characterizations of apoptosis, such as externalization of phosphatidylserine and activation of caspases, as well as identification of various substrates for caspases, have facilitated advances in apoptosis research. Equally importantly, rapid clearance of apoptotic cells without inflammation is also a unique feature of apoptosis.

Morphology

Morphological changes (**Figure 1**)(**Table 5**) (802) in apoptotic cells include compaction of nuclear chromatin (*pyknosis*) which is packed against the nuclear membrane (margination of chromatin), condensation of cytoplasm, convolution of nuclear and cell outlines, followed by rupture and fragmentation of nucleus (*karyorrhexis*), and formation of apoptotic bodies (budding) (20)(404). The descriptive terms budding and blebbing have been used interchangeably to describe the formation of apoptotic bodies and their separation from the original cell (86)(405)(408)(677)(786). The interchangeable usage may be inappropriate, however, because apoptotic bodies contain any type of organelles including nuclear fragments while blebs are fluid-filled structure, typically devoid of organelles (501).

To study effects of particular genes in regulation of apoptosis in transfection experiments, expression vectors for genes of interest and for β -galactosidase (such as pCMV-lacZ) have been simultaneously delivered into cells. Cells coexpressing β -galactosidase become blue when stained with X-gal. Thus occurrence of apoptosis among cells expressing genes of interest can be evaluated by counting the number of cells with apoptotic morphology among the whole population of blue cells (483).

Biochemistry

Biochemical features of apoptosis include externalization of phosphatidylserine exerting high affinity to annexin-V (AV), activation of caspases, cleavage of 112-kD poly ADP-ribose polymerase into a 86-kD species, and DNA fragmentation (128)(509)(786).

Membrane changes of apoptotic cells

Apoptotic cells have to express some “eat-me” signals (686) that can be recognized by



(Figure 1) Characteristic morphology of an apoptotic cell. Apoptosis of ovarian cancer BR cells was induced by treatment with 0.1 μ M paclitaxel for 24 h. Compaction of nuclear chromatin (*arrowheads*), convolution of nuclear and cell outlines, formation and budding of apoptotic bodies (*arrows*) are characteristic for apoptosis (*H & E staining, 1,000X*).

Table 5

Morphological characteristics of apoptosis

Pyknosis: compaction of nuclear chromatin which is packed against the nuclear membrane (*margination* of chromatin),

Condensation of cytoplasm,

Convolution of nuclear and cell outlines,

Rupture and fragmentation of nucleus,

Formation of apoptotic bodies (*budding*).

macrophages or neighboring cells, facilitating clearance of apoptotic cells through phagocytosis (Table 6). Teleologically, these “eat-me” signals on the cell membrane should be expressed as early as possible by an apoptotic cell, when its membrane still remains intact, to ensure it to be phagocytosed before noxious cell content in the apoptotic cell leaks and initiates inflammatory reactions. Several “eat-me” signals have been discovered, including anionic thrombospondin-1 binding sites (215)(688)(687), carbohydrate modification (178)(228)(559), and externalization of phosphatidylserine (PS) (68)(193)(783)(878).

PS exposed in apoptotic cells exerts high affinity to AV, hence labeling PS on apoptotic cells with fluoro-chrome-conjugated AV is a sensitive identification for apoptotic cells (417)(509). Furthermore, since apoptotic cells, but not dead cells, are still able to exclude nucleotide intercalating dyes such as propidium iodide (PI) and 7-amino actinomycin D (7AAD), the combined applications with FITC-conjugated AV with PI or PE-conjugated AV with 7-AAD for flow cytometric analyses can quantitatively differentiate dead cells (PI or 7AAD positive) from apoptotic cells (AV positive / PI or 7AAD negative) (Figure 2:A-C). To study effects of particular genes in regulation of apoptosis in transfection experiments, expression vectors for genes of interest (either wild type or dominant negative) and for an enhanced green fluorescent protein (pEGFP, Clontech, Palo Alto, CA) can be simultaneously delivered into the cell. At the time of sufficient expression of transfected genes, apoptosis in transfected cells (coexpressing green fluorescent protein) can be evaluated by staining with PE-conjugated AV (Figure 2:D-F). Furthermore, apoptosis and cell death can be differentiated by simultaneous staining with the PE-conjugated AV and 7-AAD and analyzed with flow cytometry. Fluorochromes such as green fluorescent protein expressed by pEGFP, PE-AV, and 7-AAD can be excited by laser tuned to 488 nm and emissions can be detected at 507 nm, 575 nm, and 650 nm, respectively (800).

Activation of caspases results in apoptotic characteristics

In an *in vitro* model of apoptosis, exposure to cytosol extracted from apoptotic cells induces apoptotic characteristics in normal nuclei. The apoptotic features include condensation and margination of chromatin, DNA fragmentation, and proteolysis of lamins, which are the main proteins of nuclear envelope (443)(444). Members of the caspase family have been identified to be responsible for these changes (128)(408)(577)(786) and many apoptosis-related cellular targets for caspases have been identified by using caspase inhibitors (184)(487). Proteolysis of cellular targets for caspases account for most of apoptotic characteristics.

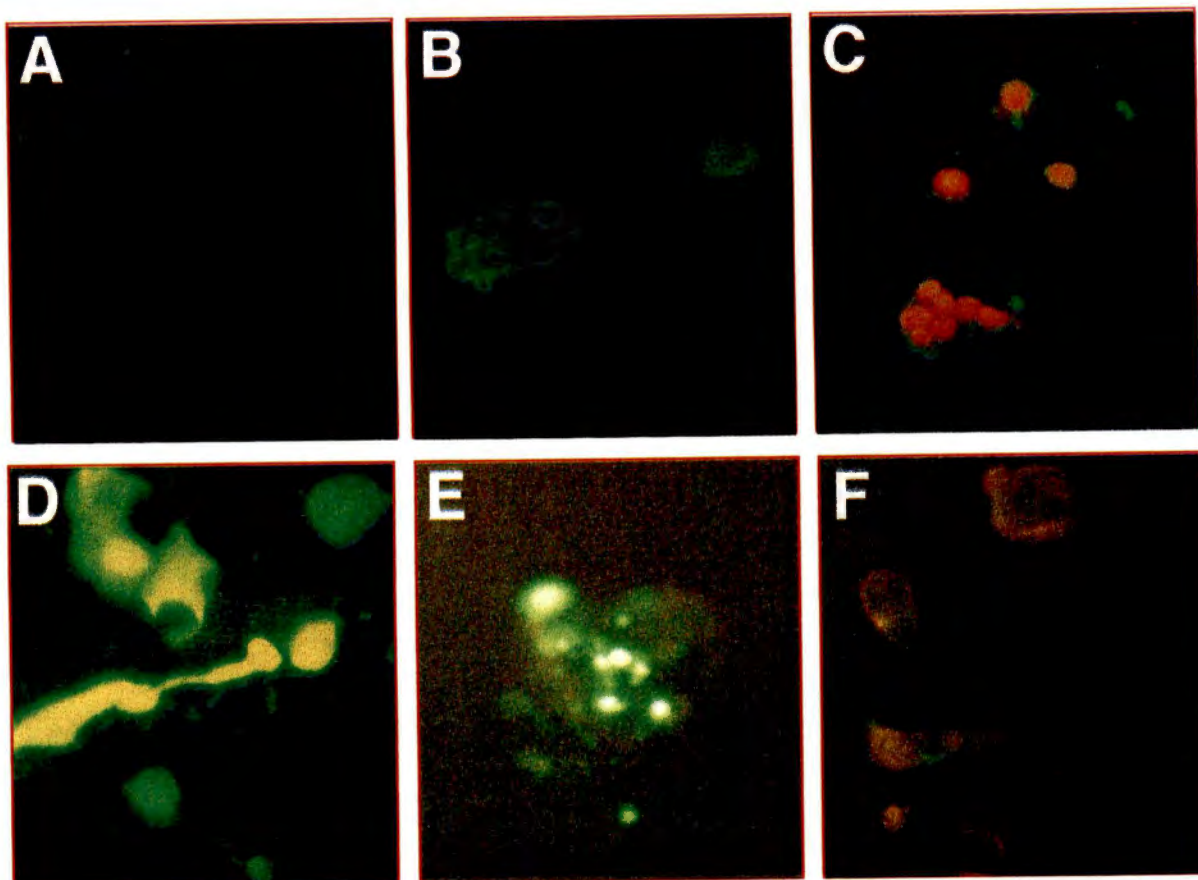
Cellular targets for caspases fall into three categories: (i) procaspases, (ii) cellular proteins that

Table 6

Components associated with elimination of apoptotic cells

"Eat-me" signals on the membrane of apoptotic cells	Membrane components of neighboring cells for interaction with apoptotic cells	Cellular components required for engulfment of apoptotic cells
Anionic thrombospondin 1 binding sites	ATP-binding cassette transporters	CDM (CED-5, DOCK180, MBC) family
Modified carbohydrates	CD14	
Phosphatidylserine	Class A scavenger receptors	
	Lectin	
	Phosphatidylserine receptors	
	Thrombospondin receptors (integrin $\alpha v \beta 5$, CD36)	

References: (685)(686).



(Figure 2) Detection of apoptotic cells with fluorochrome-conjugated annexin-V. *A-C*, Ovarian cancer BR cells growing on glass slides were treated with 1 μ M paclitaxel for 8 h followed by simultaneous staining with FITC-conjugated annexin-V (green) and propidium iodide (red). *A*, no staining of either annexin-V or PI were detected in live cells. *B*, early apoptotic cells were only stained with annexin-V. *C*, dead cells were stained with both FITC-conjugated annexin-V and propidium iodide. *D-F*, BR cells were cotransfected with expression vectors for the gene of interest and for the green fluorescent protein (pEGFP), treated with paclitaxel, and stained with phycoerythrin (PE)-conjugated annexin-V (orange color). *D*, green fluorescence served as an indicator for expression of transfected genes. *E*, apoptosis in cells expressing the gene of interest was detected by identification of PE-annexin-V in the green cells. *F*, apoptosis in non-expressing cells was detected by identification of PE-annexin-V in the non-green cells (*fluorescent microscopy*, 200X).

need to be inactivated for cell death to occur, and (iii) cellular proteins whose activation is required for the apoptotic process (**Table 7**)(408). It is noteworthy that some of the target proteins for caspases listed in **Table 7** may not always be cleaved during apoptosis. Furthermore, none of the cleavage events compiled in **Table 7** has been proven to be absolutely required to kill cells (745).

DNA fragmentation

One biochemical hallmark of apoptosis (133) commonly used to document apoptosis is the "ladder" pattern of DNA fragmentation (**Figure 3**)(802), which results from chromatin cleavage at internucleosomal sites at about 180-200 bp intervals by activated DNases (839)(841). Since this internucleosomal DNA cleavage is distinct from the nonspecific DNA degradation present in cell necrosis, such DNA fragmentation has also been used as the basis for several histochemical techniques for identification of apoptotic cells: such as the TdT-mediated dUTP nick end labeling (TUNEL) assay (**Figure 4**)(240)(802)(821). Although the role of DNA destruction during apoptosis is not entirely clear, it has been speculated to play a role in suppressing inflammatory responses (60).

Various DNases, such as DNase I, DNase II, and cyclophilins, have been proposed to be the executor for DNA fragmentation (34)(552)(611). However, whether they are really apoptosis-specific had been controversial, until the human DNA fragmentation factor (DFF) was identified in 1997 (480). DFF is a heterodimer of the 40-kD DNase (DFF40) and the 45-kD inhibitor of DNase (DFF45). DFF45 is cleaved by caspase-3 (CPP32), suggesting that DFF functions downstream of caspases.

Several months later in 1998, a caspase-activated DNase (CAD) and its inhibitors (ICAD) were identified in murine cells (185). In addition to being an inhibitor of CAD, ICADs also functions as chaperon proteins, stabilizing CAD during its synthesis (185). Apoptotic cells from DFF45 knock-out mice lacked DNA fragmentation, however, homozygous DFF45/ICAD null mice were healthy and fertile, indicating that apoptosis required for ontogenic development is intact despite lack of DNA fragmentation (888).

In apoptotic cells, activated caspases (mainly the caspase-3) cleave ICAD/DFF45 and release it from CAD/DFF40, resulting in activation of CAD/DFF40 and internucleosomal DNA degradation (185)(677). The role of caspase-3 in induction of apoptotic DNA fragmentation was supported by the observation of suppression of DNA fragmentation during apoptosis of breast cancer MCF-7 cells, which lose caspase-3 due to a 47-bp deletion within exon 3 of the *CASP-3* gene (367).

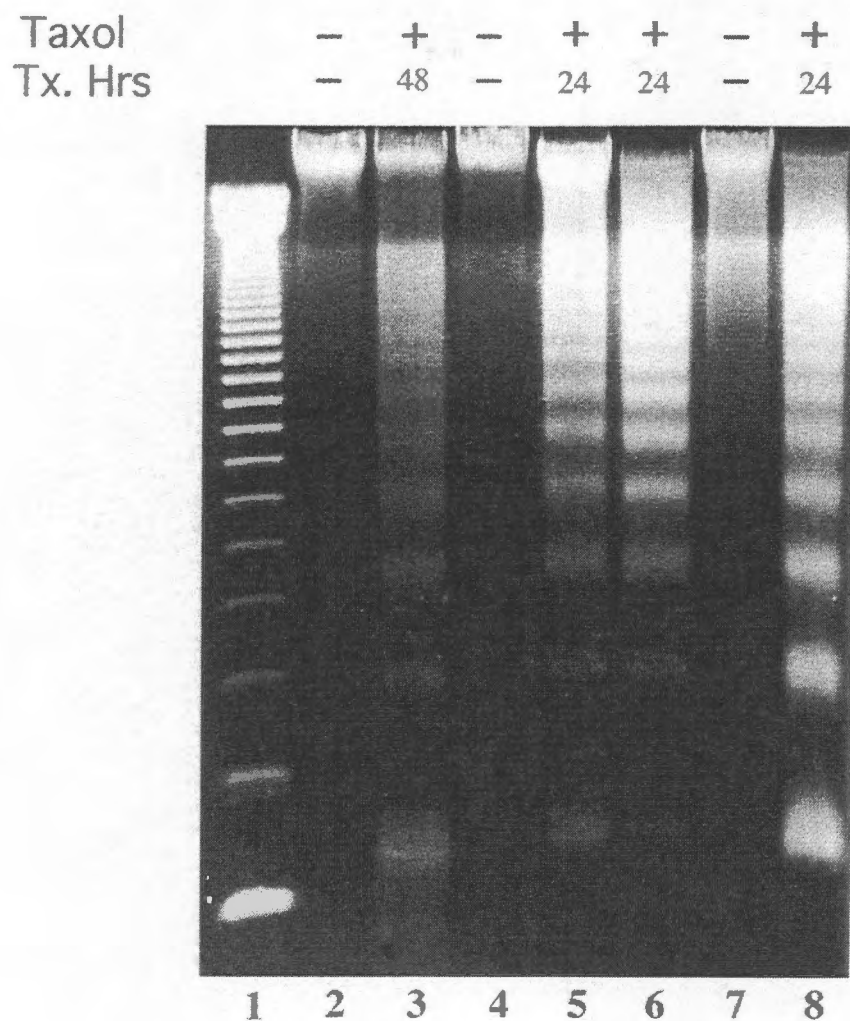
In summary, (i) activation of CAD/DFF40 by caspases is a late event in the apoptotic process,

Table 7

Caspase activation resulting in apoptotic characteristics

	Group	Target Proteins	Functions of Target Proteins
I	Procaspases	Procaspases	Precursor of caspases
II	Proteins needing to be inactivated	Actin Bcl-2, Bcl-X _L CDK27, p21, p27 DFF-45kD/ICAD DNA-PK FAK Fodrin GAS2 Gelsolin Keratin Lamins MDM2 PARP pRB Rho-GDI 70kD-U1-RNP	Cytoskeleton components Anti-apoptotic proteins Inhibitors of some CDKs Inhibitor of caspase-activated DNase DNA repair Protein kinase Cytoskeleton components Cytoskeleton components Actin-severing protein Intermediate filament components Nuclear envelope components Inhibitor of p53 DNA repair Assembly of repressor complex Inhibitor of small G-protein Pre-mRNA splicing
III	Proteins needing to be activated	MEKK1 PAK2 PKC δ PKC θ PITSLRE kinase c-PLA2 SREBPs	Protein kinase Protein kinase Protein kinase Protein kinase Protein kinase Phospholipid metabolism Cholesterol-regulating factors

References: (106)(123)(128)(408)(458)(480)(677)(745)(898).



(Figure 3) The ladder pattern of DNA fragmentation in apoptotic cells. Apoptosis of four human cancer cell lines was induced by treatment with paclitaxel (0.1 μ M) for 24 or 48 h. *Lane 1*: 123 bp ladder DNA marker; *lanes 2 and 3*: breast cancer MCF-7 cells; *lanes 4 and 5*: ovarian cancer BR cells; *lane 6*: ovarian cancer BG1 cells; *lanes 7 and 8*: osteosarcoma SAOS-2 cells.



(Figure 4) Terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labeling (TUNEL) assay. Human ovarian cancer BR cells were treated with 0.1 μ M paclitaxel for 24 h and assay with an in situ DNA fragmentation detection kit, Apoptag (Oncor, Bethesda, MD). Fragmented DNA in condensed chromatin of apoptotic cells is labeled in dark brown (arrowheads) (*methyl green counterstaining, 1,000X*).

(ii) DNA fragmentation may be dispensable during apoptosis (771), explaining the observations where DNA fragmentation was not detectable in some cell types exhibiting characteristic apoptotic morphology (583)(762), (iii) the DNase activity of purified DFF40/CAD is stimulated by chromatin-associated histone H1 and high mobility group proteins, and (iv) both DFF40/CAD and DFF45/ICAD are localized in the nucleus, and DFF40/CAD also induces chromatin condensation (479).

Summary (how to document occurrence of apoptosis?)

In addition to morphological identifications, various methods are commonly used to identify the apoptotic process at different stages in cultured cells (**Figure 5**). The early apoptosis is characterized by externalization of phosphatidylserine on the intact cell membrane that is still able to exclude viability dyes such as PI, 7-AAD, and trypan blue. AV exerts high affinity to phosphatidylserine, therefore, early apoptotic cells can be labeled as AV (+) and viability dye (-). Ongoing apoptosis is characterized by activation of caspases, which can be measured *in vitro* by using colorimetric or fluorometric substrates. Consequences of caspase activation, such as PARP cleavage and DNA fragmentation (detected by the ladder pattern of electrophoretic DNA or the TUNEL assay for cells or tissues), have been used for documentation of apoptotic process. The severity of DNA fragmentation can be quantified by ELISA-based methods, such as the Cell Death Detection ELISA (Boehringer Mannheim, Indianapolis, IN). At the end stage of apoptosis, dead cells and apoptotic bodies can no longer exclude viability dyes and are stained by DNA-targeting dyes such as PI and 7-AAD.

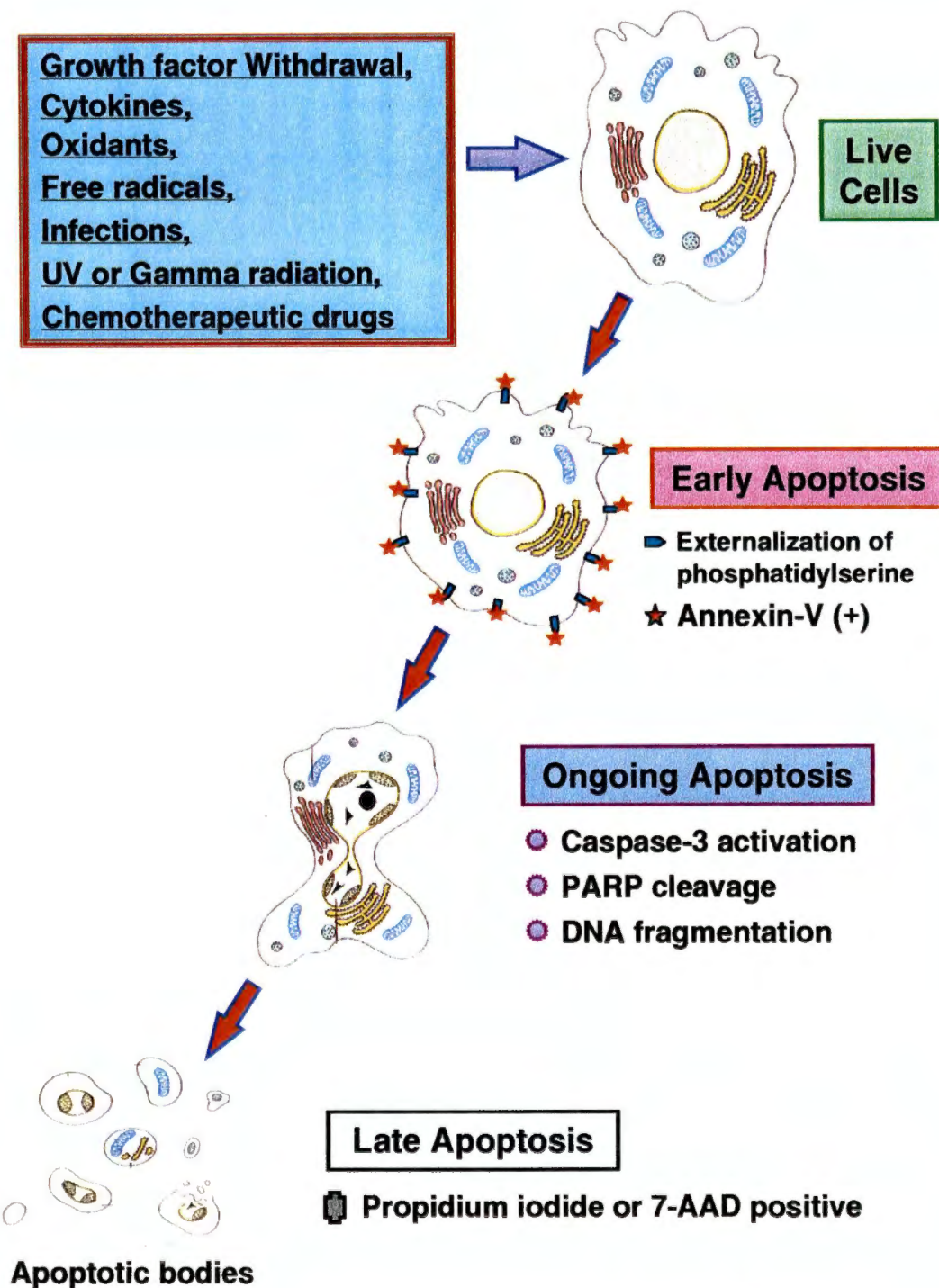
Elimination of apoptotic cells

Elimination without inflammation

The third unique characteristic of apoptosis is the minimal, if any, involvement of inflammation in the rapid elimination of apoptotic cells by phagocytosis (406)(630). The absence of inflammation minimizes tissue injury and scar formation, hence apoptosis is the ideal shaping mechanism during ontogenic development. Two factors may contribute to the absence of inflammation during apoptosis. (i) The membrane integrity of apoptotic cells and apoptotic bodies prevents leakage of cell contents into environment (315)(630). (ii) The apoptotic cell itself may have immunosuppressive effects on phagocytes (787).

Phagocytes require some membrane components to interact with apoptotic cells

A variety of membrane components on phagocytes may be required for recognition and



(Figure 5) Some detection methods for apoptosis. Abbreviations used are: PARP, poly (ADP-ribose) polymerase; and 7-AAD, 7-amino actinomycin D.

phagocytosis of apoptotic cells (**Table 6**). The abundance of phagocyte receptors for apoptotic cells apparently reflects the vital importance of the clearance process, since secondary necrosis and catastrophic damage to tissues may occur in the absence of clearance or when apoptosis is overwhelmed (587).

Cellular components required for engulfment of apoptotic cells

Phagocytes, both the "professional macrophages" and "semi-professional" cells neighboring apoptotic cells, employ various membrane "tethering" components (**Table 6**) to interact with apoptotic cells. The interactions, through yet to be defined mechanisms, trigger transmembrane signaling which leads to activation of the CED-5, DOCK180, MBC (CDM) family members and reorganization of the cytoskeleton, ultimately engulfing the apoptotic cell (685).

At least six *ced* genes: *ced-1*, *-2*, *-5*, *-6*, *-7*, and *-10*, of the nematode *C. elegans* regulate the engulfment of apoptotic cells (**Table 8**)(182). Mutations of these genes inhibit extension of the membranes of engulfing cells around the dying cells. The amino acid sequence of the human DOCK180 shares 26% homology with CED-5. Both CED-5 and DOCK180 also share significant homology with the protein product of the *Drosophila* gene *mbc* (837). The mutation of *ced-5* in *C. elegans* results in defects of engulfment of cell corpses and migration of the distal tip cells. The latter defect can be rescued by expression of the human DOCK180 (837). DOCK180 is a cytoplasmic protein with an SH3 domain which allows it to interact with various signal transduction pathways (294). Furthermore, the farnesylated DOCK180 drives cell spreading, suggesting its involvement in the regulation of cell movement by tyrosine kinases (685). Collectively, from the rescue of cell migration in *ced-5* mutant and the functions of MBC (Myoblast City) in myoblast fusion, Wu *et al.* suggest a potential role of CDM family members in the cytoskeletal reorganization required for engulfing the apoptotic cell.

Summary

The abundance and redundancy of phagocyte receptors for apoptotic cells apparently reflect the absolute importance for efficient elimination of apoptotic cells (**Table 6**). Possible answers for the question "why so many mechanisms?" are discussed as the followings. (i) Phagocyte receptors may need cooperation to exert optimal phagocytosis. (ii) Some of the cooperative phagocyte receptors may elicit essential immunosuppressive signals to avoid the harmful inflammatory responses from the macrophage. (iii) Redundancy of the phagocyte receptors ensures the critical functions as each receptor backs up the others. (iv) Recognition of apoptotic cells may be only the "part-time" functions of some phagocyte receptors (such as $\alpha v\beta 5$, CD36, class A scavenger receptors).

Table 8

Nematode gene products associated with apoptosis and their mammalian homologs

Stages	Functions	<i>C. elegans</i>	mammalian
Decision to die	Pro-apoptotic	<i>ces-2</i> <i>egl-1</i>	
	Anti-apoptotic	<i>ces-1</i>	
Execution of death	Pro-apoptotic	<i>ced-3</i> <i>ced-4</i> <i>ced-8</i>	ICE Apaf-1
	Anti-apoptotic	<i>ced-9</i>	Bcl-2
Engulfment	Required for engulfment	<i>ced-1</i> <i>ced-2</i> <i>ced-5</i> <i>ced-6</i> <i>ced-7</i> <i>ced-10</i>	DOCK180
Degradation	Required for degradation of engulfed cells	<i>nuc-1</i>	

Reference: (239)

COMPONENTS OF THE APOPTOTIC PATHWAY

Depending on the cell type, a variety of stimuli may initiate apoptosis. The apoptotic process can be arbitrarily divided into three phases: detection and activation, regulation, and execution by effectors (Table 9).

STIMULI

Genomic damage

Apoptosis is the major mode for an organism to remove genome-damaged cells. Genomic assaults include: UV irradiation (67)(539)(895), ionizing irradiation (245)(357), and DNA-targeting chemicals including many anticancer chemotherapeutic agents such as *cis*-platinum, 5-fluorouracil, 5-fluorodeoxyuridine, and others (33). Genomic damage initiates apoptosis mainly through p53-dependent pathways (121)(489)(490)(741)(884). Hence, p53 earns the reputation as the “guardian of the genome” (438).

Cells also employ additional pathways to stop or eliminate genome-damaged cells. A comparison of activation of p53, c-Abl, and JNK/SAPK in murine fibroblasts treated with *cis*-platinum, ionizing radiation, methyl methanesulfonate (MMS), mitomycin C, or UV irradiation indicates that p53 is induced by all of these tested genotoxic stresses; c-Abl is activated by most stimuli except UV; and JNK is only strongly activated by UV and MMS (79)(482).

Withdrawal of survival signals

Survival signals can be provided by: (i) growth factors, such as colony stimulating factor (823) and nerve growth factor (94), (ii) hormones, such as steroid hormones (651), prolactin (791), and ACTH (840), (iii) interactions between integrin system and the extracellular matrix (64)(631), or (iv) the cell shape (669)(98).

Exposure to apoptotic stimuli

Apoptotic stimuli may act systemically or locally. Systemically, glucocorticoids trigger apoptosis in thymocytes (130)(839), eosinophils (398), and leukemia cells (518), whereas thyroid initiates apoptosis

Table 9
Components of apoptosis

Category	Examples
Stimuli	Genomic damage, Withdrawal of survival signals, Exposure to apoptotic stimuli, Viral infections
Detection and activation	Apoptosis-related proto-oncogene and tumor suppressor gene products, Apoptosis-related signal transduction pathways
Regulators	Bcl-2 family Apoptosis-associated factors (Apafs) Inhibitors of apoptosis (IAP) family Mitochondria and ATP status
Effectors	Caspase family Cyclin-dependent kinases (CDKs)

during metamorphosis of the tadpole (403).

Most daily apoptotic events in the body are more likely regulated by locally acting stimuli, such as TNF- α , TGF- β , granzyme B, Fas ligand (FasL) and others. Examples include TNF- α in the regulation of cyclic renewal of endometrium (742), TGF- β in the peri-implantation decidua (562), and bone morphogenetic proteins (BMPs) in interdigital apoptosis (899). Cytotoxic T-lymphocytes (CTL) use multiple local factors to execute their killing: (i) CTL express the ligand (FasL) for Fas/CD95 and thus kill Fas-bearing target cells, and (ii) CTL also produce the granzyme B and deliver it into target cells through the help by another protein, perforin (387)(491)(710). Fas-mediated apoptosis also accounts for maintenance of immune privilege sites such as the testis and the anterior chamber of the eye. Cells of immune privilege tissues naturally produce FasL and kill infiltrating Fas-bearing lymphocytes and inflammatory cells (37)(263)(265)(570). Some tumors may also express FasL and evade immune surveillance by killing the infiltrating CTL of the host (584)(671).

Viral infections

Apoptosis may be a defense mechanism of the host to control infection of viruses (125)(126)(780). Viruses would perish if all infected host cells die before viruses have enough time to replicate. Thus, evolution has selected for those viruses equipped with gene products capable of counteracting apoptotic processes of the host cell (520). Such viral anti-apoptotic proteins include BHRF1 (Epstein Barr virus) (264)(307), crmA (cowpox virus) (227)(636), E1B19k (adenovirus) (148), LML5-HL (African swine fever virus) (7), p35 (baculovirus) (73)(851), and IAPs (baculovirus) (47)(138).

DETECTION AND ACTIVATION OF APOPTOSIS

Apoptosis-related proto-oncogenes and tumor suppressors

Several proto-oncogenes and tumor suppressors are involved in the detection and activation of apoptotic process (410)(729)(802). They fall into three groups: pro-apoptotic (Bax, p53, p73), anti-apoptotic (Akt, Bcl-2, pRb), and dual-functional (E2F-1, c-Myc) (Table 10).

Akt

Many cell types requires specific growth factors for survival. A proto-oncogene, the serine/threonine protein kinase Akt (36), also known as protein kinase B (PKB) (127)(377), may play a

Table 10**Apoptosis-related proto-oncogenes and tumor suppressors**

Functions	Proto-oncogenes	Tumor suppressors
Anti-apoptotic	Akt Bcl-2	pRb
Dual-functional (anti- or pro-apoptotic depending on the cellular context)	c-Myc E2F-1	E2F-1
Pro-apoptotic		Bax p53 p73?

critical role for the growth factor-dependent cell survival (140)(176)(306)(305). Activation of a tyrosine kinase growth factor receptor by binding of growth factor results in receptor tyrosine phosphorylation, which serves as a docking site for the Src-homology 2 (SH2) domains of several proteins, including GRB2 and the p85 subunit of phosphoinositide 3-kinase (PI 3-K). GRB2, through SOS, interacts with Ras that in turn activates the Raf-MEK-ERK cascade which may promote cell proliferation. Additionally, both Ras (647)(648) and the p85 subunit of PI 3-K are capable of activation of p110, the catalytic subunit of PI 3-K. Active PI 3-K at the membrane converts phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) into PI-3,4,5-P₃, which can be further converted into PI-3,4-P₂ by inositol 5'-polyphosphatase (302). The PI-3,4-P₂ recruits Akt to the membrane and activates it (213)(210)(211)(212). Activated Akt may phosphorylate the pro-apoptotic BAD and release BAD from the formation of heterodimers on the mitochondrial membrane with other anti-apoptotic Bcl-2 family members (140). Phosphorylated BAD may be further sequestered in the cytoplasm in a complex with 14-3-3 proteins (152)(302)(306)(305).

Akt also inhibits apoptosis by direct regulation of caspase activity. Overexpression of either active Ras or Akt abolishes cytochrome *c*-induced proteolytic processing of pro-caspase-9. Akt phosphorylates caspase-9 on Ser196 and inhibits its protease activity. Mutant procaspase-9 (Ser196 -Ala) results in apoptosis that is resistant to Akt-mediated phosphorylation and inhibition (83). In short, the Akt may sense the survival signals from activated growth factor receptors and inhibit apoptosis through inactivation of the pro-apoptotic BAD and caspases.

E2F-1

The E2F transcription factor family consists of eight members in two subfamilies: E2F (-1, -2, -3, -4, -5, -6) and DP (-1, -2) (375). All of these eight members share homology in the DNA-binding domain and dimerization domain, but only E2F-1, -2, and -3 contain the N-terminal nuclear localization signals (375). E2F and DP form heterodimers (303)(832) and these heterodimers can further form heterotrimers with members of the RB pocket protein family (pRB, p107, p130) (375)(424).

The functions and regulations of the pRB/E2F/DP complex in the progression of the cell cycle are summarized as: (i) pRB/E2F/DP heterotrimers function as transcription repressors in the G₀ and early G₁ phase; (ii) during the late G₁ and early S phase, cyclin D/CDK4,6 phosphorylate pRB and thus release E2F/DP, which function as transcription activators; and (iii) during the late S and G₂/M phases, E2F/DP are inactivated through phosphorylation of DP by cyclin A/Cdc2 (375)(423).

While the other E2Fs only induce S-phase entry, E2F-1 can both promote S-phase entry and

induce apoptosis (151)(705)(835). E2F-1 has been considered as an oncogene because of *in vitro* evidence that overexpressed E2F-1 induces anchorage-independent growth in immortalized fibroblasts (718)(845) and, when in cooperation with an activated *ras* gene, it leads to oncogenic transformation (700). On the other hand, E2F-1(-/-) knockout mice are viable and fertile, but exhibit defective apoptosis and develop a wide range of tumors at old ages (205)(853)(854), suggesting that E2F-1 functions as a tumor suppressor (314).

Indeed, accumulating evidence has indicated that E2F-1 promotes apoptosis (205)(705)(835). E2F-1-induced apoptosis can be either p53-dependent or p53-independent. (i) E2F-1 can induce accumulation of p53 (319), perhaps through binding to Mdm2 and blocking its downregulating effects on p53 (295)(429)(508). (ii) Induction of p53-independent apoptosis by E2F-1 has been demonstrated in cultured cells (339)(615), in transgenic animals (327), and in tissues after adenoviral delivery of E2F-1 (8). E2F-1 induces apoptosis which cannot be rescued by coexpression of E2F-2 and E2F-3, suggesting that E2F-1 induces apoptosis through induction of an apoptosis-promoting activity rather than the lack of induction of a survival activity (151). Although the exact mechanism remains unclear, E2F-1-mediated CDK activation, may be also responsible for apoptosis, since catastrophic CDK activities have been suggested to be the actual effector of apoptosis (293)(458)(894).

c-Myc

The proto-oncogene product c-Myc is a 64-67 kD protein with a short half-life 30 min. Myc may exist as homodimers, however, it preferentially forms heterodimers with Max that is a 21-22 kD protein with a longer half life (758). Myc/Max heterodimers transactivate many genes including Cdc25A (230), cyclins A and E (370), Cdc2 (57), dihydrofolate reductase (500), ornithine decarboxylase (598), and heat shock protein 70 (384). It also can repress expression of various genes including β -integrin (382) and cyclin D1 (370)(614). The homozygous null mutation of *myc* in mice is embryonically lethal (142).

Myc apparently promotes cell growth (13): it is expressed during proliferation in a variety of cells (314) and it promotes cell progression through G₁/S phases and G₂ phase of the cell cycle (704). The dual mechanisms for Myc to move cells through the cell cycle are believed to be: (i) that Myc transactivates components required for the cell cycle such as Cdc25A, cyclins E and A, and Cdc2, and (ii) that Myc promotes pRb phosphorylation, which is an essential step in the G₁ phase, through the dissociation of an inactive complex formed by cyclins/CDKs/p27^{Kip1} (314). The most intriguing role of Myc, however, is the induction of apoptosis: both decreased and increased levels of Myc have been shown to cause apoptosis (758).

Paradoxically, apoptosis has been shown to be induced by either downregulation of *c-myc* (392)(519)(828) or upregulation of *c-myc* (191)(262)(349)(656)(712). The mechanisms for the dual functions of Myc remain unclear. Myc may be required for transactivation of survival genes and/or for repression of lethal genes, explaining apoptosis induction by downregulation of *c-myc* (758).

On the other hand, upregulated c-Myc may promote apoptosis through two mechanisms described below. (i) Myc activates cyclin-dependent kinases (CDKs) (13) and accumulating evidence suggests that various CDKs may be the common components required for both cell cycle and apoptosis (226)(293)(345)(458)(529)(711)(894). (ii) Myc may sensitize cells to Fas-mediated apoptosis (348), perhaps by inhibition of Saf (suppressor of apoptosis by Fas) (260). The identity of Saf remains unclear but one candidate is FLIP (359). To avoid the fate of apoptosis, cells need survival signals including those from activated Akt (302)(306)(305) and Bcl-2 (50)(96).

p53

The tumor suppressor p53 can regulate both the cell cycle and apoptosis. When the genome is damaged, p53 can either slow down the cell cycle allowing the DNA repairing mechanisms to fix the damage, or trigger the apoptotic process to eliminate the genome-damaged cell (563)(802). How the cell decides to repair its DNA or undergo apoptosis remains unclear. Transactivation of target genes by p53 appears to be important for both slowing down of the cell cycle and triggering apoptosis (594). To become a more efficient transcription factor, p53 may have to form a complex with other gene products, such as p33 encoded by the *ING1* gene (237)(238)(236).

p53 may control the cell cycle at both G₁/S phases and G₂/M phase. (i) Progression through the G₁/S phases can be inhibited by p53 transcriptional activation of the CDK inhibitor p21^{WAF1/CIP1} (179)(267)(290) and GADD45 (396)(885)(883). (ii) The G₂/M phase progression may be blocked by p53 (9)(728) through transactivation of the protein 14-3-3 σ that sequesters phosphorylated Cdc25C, preventing it from dephosphorylating Cdc2 (312). Additionally, the p53-dependent G₂/M arrest may result from the as-yet unclear spindle checkpoint control mechanism (139) and centrosome duplication control mechanism (225).

Results from p21 knockout animals and other studies indicate that p21 is not required for induction of apoptosis (24)(153). Nevertheless, both transactivator and non-transactivator functions of p53 have been shown to be involved in apoptotic regulation (288)(563). In a systematic approach toward understanding how increased p53 expression leads to apoptosis, Vogelstein's group infected a human colorectal cancer cell line DLD-1, which has no functional p53, with an adenoviral vector carrying wt

human p53, then analyzed the changes of gene transcription resulting from overexpression of p53 (617). Among more than 7,000 transcripts analyzed by the serial analysis of gene expression (SAGE), 34 transcripts differed in expression by more than ten fold after p53 expression: 14 transcript increased (resulting from p53 transactivation) while 20 decreased (repression by p53) (617)(838). Among the top 14 transactivated transcripts is the CDK inhibitor p21^{WAF1/CIP1}, whereas intriguingly, Bax and GADD45 are not on this list. In this p53 restoration study, no additional genome-damaging treatment was administered, which may account for the lack of Bax and GADD45 transactivation.

The promoter of the pro-apoptotic *Bax* gene contains p53-binding sequences (550) and induction of p53 by DNA-damaging agents increases Bax expression (549)(884). The sole upregulation of Bax by p53, however, cannot explain all p53-dependent apoptosis, as shown in studies (i) that the *Bax* transgene in p53 null mice does not increase DNA damage-induced apoptosis compared with p53 null thymocytes not carrying Bax transgene (66) and (ii) that transfection of wt p53 into Saos-2 and H1299 tumor cell lines does not increase Bax protein levels compared with nontransfected controls (657). Therefore, additional factors may be required and/or tissue-specificity may also account for these observations (563). Other transcriptional targets for p53 include Fas/CD95 (597), the insulin growth factor I-binding protein 3 (IGF-BP-3) (72)(216)(492) and the protein p85, a regulator of the PI 3-K (866).

The non-transactivator role of p53 has been shown by studies that inhibition of RNA or protein synthesis cannot inhibit UV-induced, p53-dependent apoptosis (75) and that overexpression of a transactivation-defective mutant of p53 still induces apoptosis (296). To promote apoptosis, p53 functions as a transcriptional repressor in downregulation of *Bcl-2* (282)(548), *IGF-1R* (625)(816), and the microtubule associated protein 4 (*MAP4*) genes (567).

p73

The protein p73 is originally isolated from the chromosomal locus 1p36 that is a region frequently deleted in neuroblastoma (386). p73 shares high homology to p53, especially in the p53 DNA-binding domain; it activates p53-responsive promoters; and it induces apoptosis in tumor cells lacking functional p53; suggesting that p73 is another tumor suppressor related to p53 (166)(381)(385)(386)(622). The putative p53 family also include p40 (769) and p51 (595), both share protein sequence homology with p53 and p73. Unlike p53, however, no mutations of the *p73* gene have been identified in human cancers (385)(737). Thus, it does not conform to the classical Knudson's "two-hit" model. Other results also argue against the tumor suppressor role of p73: (i) mice lacking p73 are viable and do not develop tumors (385), and (ii) activation of a silent allele and overexpression of wild-type p73 are associated with lung

tumorigenesis (499). In short, the role of p73 as a tumor suppressor and its effect on apoptosis regulation remain to be clarified.

pRb

Like p53, the retinoblastoma protein pRB also regulates both the cell cycle and apoptosis. Unphosphorylated pRB arrests cells in the early G₁ phase of the cell cycle; unlike p53 however, pRB protects cells from apoptosis (170)(410)(745).

The anti-apoptotic role of pRB has been extensively demonstrated. (i) The *Rb*(-/-) knockout mutation causes embryonic lethality in the mouse with widespread apoptosis in the peripheral and central nervous systems (120)(363)(445). (ii) Transfection of hepatocytes with *Rb* antisense oligonucleotides results in apoptosis (196). (iii) The human papilloma virus (HPV) E7 binds to and inactivates pRB (798), inducing apoptosis (817)(602). (iv) Co-expression of the wt *Rb* gene inhibits E2F-mediated apoptosis (420).

During the apoptotic process, pRB is both dephosphorylated (171)(554)(795) and cleaved (102)(368)(744). Inactivation and elimination of pRB during apoptosis may result from at least two types of caspase-dependent cleavages: the initial C-terminal cleavage and the latter cleavage and degradation (170)(745). The C-terminal cleavage of pRB at the peptide bond between Asp₈₈₆-Gly₈₈₇ results in the truncated RB (Δ RB) and a 5-kD fragment (102)(368)(744) whereas the latter cleavage may further produce p68 and p48 fragments (200).

Activation of p53 and E2F (and c-Abl) has been proposed to be the mechanism for pRB to lose anti-apoptotic functions after caspase-dependent cleavage (745). (i) The truncated Δ RB loses the binding to Mdm2 (368) yet remains able to bind E2F and c-Abl tyrosine kinase as wild-type pRB (744). Mdm2 protects the cell from p53-dependent apoptosis (100)(416) but Mdm2 is also a substrate of caspase (188). Thus the release of Mdm2 from pRB may increase the susceptibility of Mdm2 to caspase cleavage (745). (ii) The latter degradation of pRB would release its binding partners E2F (205)(835) and c-Abl (755)(874), two proteins when overexpressed promote apoptosis.

Apoptosis-related signal transduction pathways

The cell constantly responds to its microenvironment through various signal transduction pathways and decides whether to divide, stay in the G₀ phase of the cell cycle, or undergo apoptosis. Some signal transduction pathways important in initiating apoptosis are discussed below.

Ceramide

Ceramide, a sphingosine-based lipid, plays important roles not only in the metabolism of sphingolipids but also as a second messenger molecule (287)(415). Although ceramide can be synthesized de novo from serine and palmitoyl-CoA after a series of reactions catalyzed by ceramide synthase and dihydroceramide reductase (61)(532), the major production of ceramide in response to stimuli is the sphingomyelinase-catalyzed catabolism of sphingomyelin into ceramide (590). Sphingomyelinases, the sphingomyelin-specific forms of phospholipase C, exist in several isoforms that are distinguished by different pH optima (415)(820). Acid sphingomyelinase (A-SMase) prefers pH 4.5-5.0 and is localized in acidic compartments such as lysosome or endosome (118)(476)(696)(820). Neutral sphingomyelinase (N-SMase) may reside in the plasma membrane (476)(820). Activation of A-SMase and N-SMase may respond to stimulus within seconds to minutes whereas activation of ceramide synthase may take hours (415), hence SMase activation is the main response to stress-related stimuli.

Increased levels of ceramide suppress cell growth and promote apoptosis. Ceramide-mediated cell cycle arrest in the G₁ phase is accompanied by dephosphorylation of pRB and cell cycle arrest is not elicited in response to ceramide in pRB-deficient cells (144)(253)(371)(635). Although pRB appears to be critical for ceramide-mediated suppression of cell growth, it is not required for the ceramide-mediated apoptosis (887). The role of ceramide in apoptosis has been demonstrated in many studies (415). (i) Activation of Fas (268) or treatments with TNF- α (582), ionizing radiation, UV radiation, heat shock or oxidative stress (277)(782) induces rapid generation of ceramide, and the ED₅₀ for ceramide formation correlates well with the LD₅₀ for induction of apoptosis. (ii) Specific cell-permeable ceramide analogues induce apoptosis (268)(277)(582)(782). (iii) Cells derived from patients with Niemann-Pick disease, an inherited deficiency of A-SMase, and from A-SMase-deficient mice show a defective apoptotic response (683).

How do these stress-related stimuli activate SMases? In the cells treated with TNF- α , both N-SMase and A-SMase are activated and likely to exert opposite effects toward cell activation and apoptosis, respectively (415). Two motifs in the intracellular regions of the p55 TNF receptor account for the differential activation of SMases: the NSD motif through interaction with a WD-repeat protein FAN activates N-SMase (2); the death domain through interaction with the adaptor proteins TRADD and FADD activates A-SMase (697)(820). Downstream of N-SMase may be the ERK pathways leading to cell growth, whereas downstream of A-SMase may be the JNK/SAPK pathway and mitochondrial regulation leading to apoptosis (415). On the other hand, ionizing radiation, UV-irradiation, and other stresses may

act directly on membranes and activate A-SMase (161)(649).

Like all apoptotic processes mediated by other signaling pathways, regulation of mitochondrial permeability transitions (607)(738) and caspase activation (183)(887) have been shown to be involved in ceramide-mediated apoptosis. The exact role of ceramide in the induction of apoptosis, however, remains unclear due to the lack of mechanistic evidence for the interactions among ceramide, its direct target ceramide-activated protein phosphatase (826), mitochondria, and caspases.

It is noteworthy that ceramide may initiate apoptosis but it does not commit the cell to the death process. Two models have been proposed to explain the role of ceramide in apoptosis. (i) Ceramide may function as a “biostat” that measures cellular stress and initiates either growth arrest or apoptosis (287). All ceramide inputs from de novo synthesis and catabolic pathways contribute to the final ceramide concentration. Hence, the final ceramide levels may reflect the effects of multiple stimuli and the overall amount of stress or injury to which the cell has been exposed. (ii) Another model emphasizes the topology of ceramide generation. In an attempt to explain how increased levels of ceramide may lead to different cellular responses, this model proposes that selective activation of various signaling pathways is determined by its co-localization with a given sphingomyelinase in the same subcellular compartment (415). Both models, however, remain to be tested.

Death receptors

Certain cell types are equipped with “death receptors (DRs)” on their surface to initiate the apoptotic pathway in response to “death ligands (DLs)” (23). Some of the best characterized DRs and DLs are summarized in **Table 11** and the downstream proteins interacting with them are summarized in **Table 12**. DRs are members of the tumor necrosis factor receptor (TNFR) superfamily. In addition to the extracellular cysteine-rich domains shared by all TNFR family members, DRs contain the death domain (DD) in the cytoplasmic region (571)(570). The DDs, which are conserved protein-protein interaction sequences of about 80 amino acids, are also found in various adaptor proteins interacting with death receptors (**Table 12**) (793). Since DD have a propensity to associate with one another perhaps via electrostatic interactions (793), the binding of DLs to DRs leads to clustering of the death receptors’ DDs that further interact with the DDs of other downstream interacting proteins (23). Some proteins interacting with DRs also have the death effector domains (DED). The DED functions as the caspase recruiting domain (CARD), which is also found in the caspases with a large prodomain (caspases-2, -8, -9, -10) (23).

Table 11

Death receptors and ligands

Type	Name	Other names	Tissue Distribution	Functions
<u>Receptors</u>	TNFR1	p55, CD120a	Ubiquitous expression	Activation of NF- κ B and AP-1; apoptosis when protein synthesis is blocked.
	Fas	Apo1, CD95	Many tissues	Apoptosis
	DR3	Apo3, WSL-1, TRAMP, LARD	Spleen, thymus, peripheral blood, stimuli-activated transcription in T cells	NF- κ B activation, apoptosis
	DR4	Apo2, KILLER, TRAIL-R2,	Many tissues	Apoptosis
	DR5	TRICK2	Many tissues	Apoptosis
<u>Ligands</u>	<i>TNF</i>	<i>Apo1L, CD95L</i>	Activated m ϕ and T cells	Binds TNFR1
	<i>FasL</i>		Activated T cells and NK cells, immune-privileged tissues (eye, testis, etc.)	Binds Fas
	<i>Apo3L</i>	<i>TWEAK</i>	Many tissues	Binds DR3
	<i>Apo2L</i>	<i>TRAIL</i>	Constitutive in many tissues, stimuli-activated transcription in T cells	Binds DR4, DR5, DcR1, and DcR2

Reference: (23)

Table 12

Downstream proteins interacting with death receptors

Proteins	DD*	DED/CARD*	Interact with**:
FADD (Fas-associated death domain)	+	+	Fas (U), TRADD (U) Caspase-8 (D)
RAIDD/CRADD (RIP-associated ICH-1/CED3 homologous protein with a death domain)	+	+	TNFR1 (U) RIP (D), Caspase-2 (D)
TRADD (TNFR-associated death domain protein)	+		TNFR1 (U) FADD (D), RIP (D), TRAF2 (D)?
RIP (Receptor-interacting protein)	+		RAIDD (U), TRADD (U)
Daxx (Fas death domain-associated protein)	-		Fas (U) ASK1 (D)
TRAF2 (TNFR-associated factor 2)			TRADD (U) ASK1 (D), NIK (D)
Caspases-2, -8, -9, -10	-	+	FADD (U) Substrates (D)
cFLIP (FLICE-inhibitory protein)	-	+	FADD (U)?

* DD: death domain; DED: death effector domain; CARD: caspase recruitment domain.

** U: upstream; D: downstream.

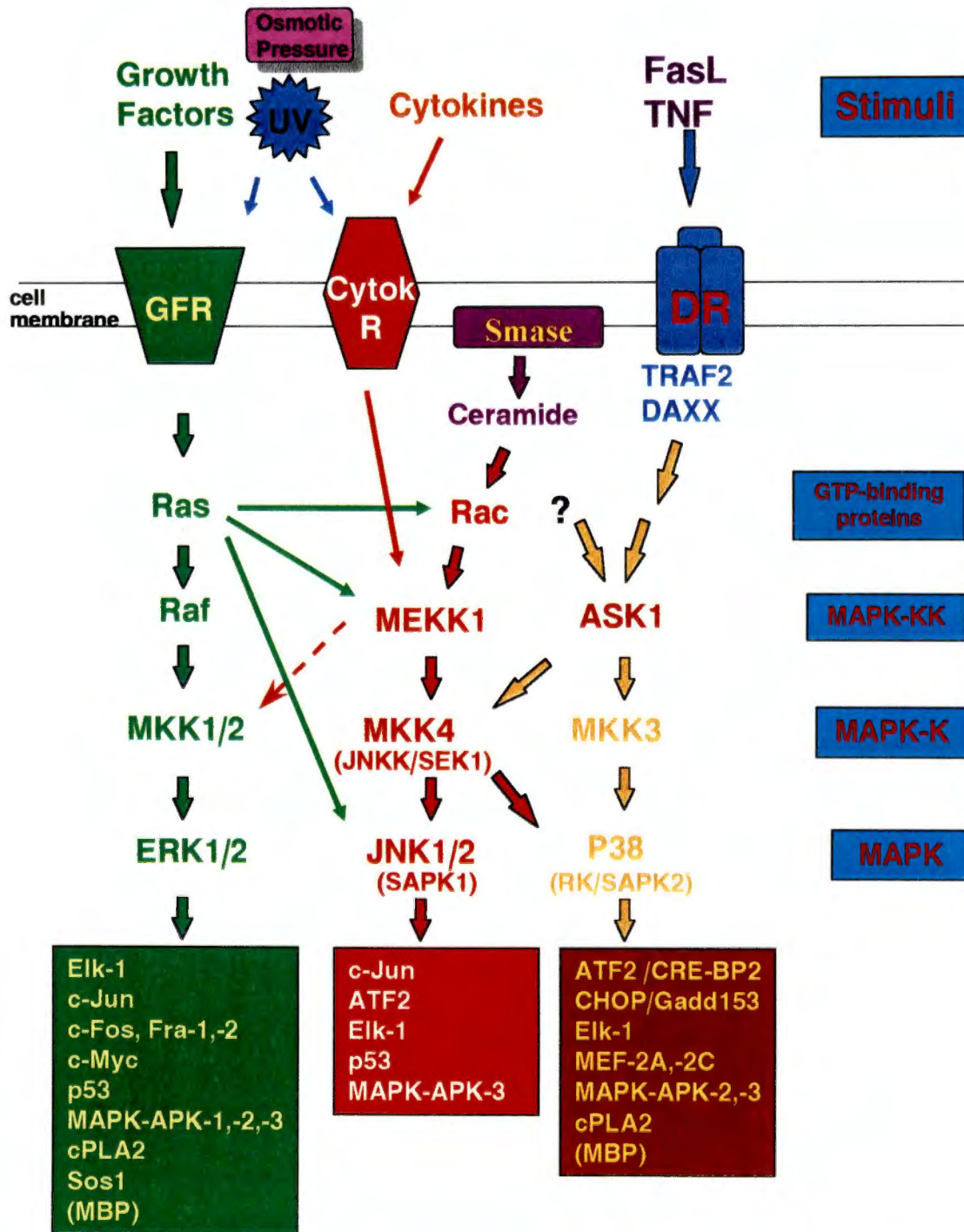
References: (23)(93)(581)(859)

The binding of FasL, TNF, and Apo3L induces clustering of Fas, TNFR1, and DR3, respectively (Table 11). These clustered DRs interact with downstream adaptor proteins (Table 12), which further recruits caspase-8 through the DED-DED interaction. Upon induction of proximity, caspase-8 may oligomerize and activate one another through self-cleavage (569), initiating apoptosis. Initiation of apoptosis through DRs requires no protein synthesis (793), thus DRs seem to be the express ways leading to apoptosis. Nevertheless, binding of these DLs to DRs not only leads to activation of caspase-8 but also recruits other adaptor proteins and activates some signal transduction pathways, such as the NF- κ B and the JNK pathways, exerting additional functions (Table 12). For instances, Daxx (93) and TRAF2 (581) may activate the apoptosis signal-regulating protein (ASK1), which is an upstream regulator of JNK/SAPK (356)(676)(803).

Binding of Apo2L(TRAIL) to DR4 or DR5 leads to apoptosis; however, DR4, DR5 and Apo2L are surprisingly expressed in many tissues. It turns out to be that signaling through DR4 and DR5 is modulated by a unique set of “decoy” receptors (DcRs), DcR1 (TRID/TRAIL-R3/ LIT) and DcR2 (TRAIL-R4/TRUNDD), both efficiently binding Apo2L (601)(708). Since DcR1 lacks the cytoplasmic region and DcR2 has a loss-of-function deletion on its DD, binding of Apo2L to these DcRs transmits no death signals. The genes encoding DcRs, DR4, and DR5 map together to the human chromosome 8p21-22, suggesting they may originate from a common ancestral gene (150)(507). Interestingly however, expression of DcR1 and DcR2 in normal tissues are more frequently than in tumor cells, suggesting the potential use of Apo2L in cancer therapy (23)(274). To prevent spontaneous clustering of DRs and subsequent apoptosis, cells widely express an cytoplasmic protein, silencer of death domain (SODD), to inhibit the interaction between DRs and TRADD (373).

JNK/SAPK

c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), are involved in a signal transduction pathway parallel to that of mitogen-activated protein kinases (MAPKs) (Figure 6) (156)(389)(436). This highly conserved cascade is responsive to stress-related stimuli such as UV irradiation, ionizing radiation, ischemia and reperfusion, and inflammatory cytokines, eliciting phosphorylation and activation of JNK/SAPKs (393)(394)(435)(558). Activated JNK/SAPKs phosphorylate a variety of transcription factors including c-Jun, leading to transcriptional activation through interactions with c-Jun responsive DNA elements such as TPA-response element (TRE)(459). In addition to responding to extracellular stimuli, the JNK/SAPK pathway is also activated by intracellular



(Figure 6) A simplified scheme of three mitogen-activated protein kinase (MAPK) pathway. Abbreviations used are: GFR, growth factor receptors; Cytok R, cytokine receptors; Smase, sphingomyelinase; DR, death receptors; ASK1, apoptosis signal-regulating kinase-1.

stresses including inhibition of protein synthesis, treatment with antimetabolites, DNA damage, or microtubule dysfunctions induced by chemotherapeutic agents (105)(393)(435)(803).

Activation of the JNK/SAPK signaling pathways has been mechanistically implicated in regulation of apoptosis (263)(358)(394)(415)(435), however, the roles of JNK/SAPK in promoting (104)(531)(879) or preventing apoptosis (579)(621) differ, depending on both cell type and apoptosis-triggering stimuli (358)(394). Furthermore, in addition to apoptosis, JNK/SAPK activation may be involved in proliferation (63)(720) and oncogenic transformation (646).

JNK/SAPK has been shown to be involved in activation of caspases that are required for execution of the apoptotic process (76)(84)(104)(666)(701). The role of JNK/SAPK in activation of caspases is, however, not straightforward. JNK/SAPK could be either upstream (104)(701) or downstream of (76)(84)(666) caspase activation, depending on the cell type and apoptosis-initiating agents. We have demonstrated that inhibition of the JNK/SAPK cascade transiently inhibits paclitaxel (Taxol®)-induced caspase-3 activation, PARP cleavage, and DNA fragmentation, indicating that JNK/SAPK is upstream of caspase-3 activation in paclitaxel-initiated apoptosis (800). Collectively, it is tempting to speculate that JNK/SAPK may act upstream of caspase activation in chemotherapy-initiated apoptosis.

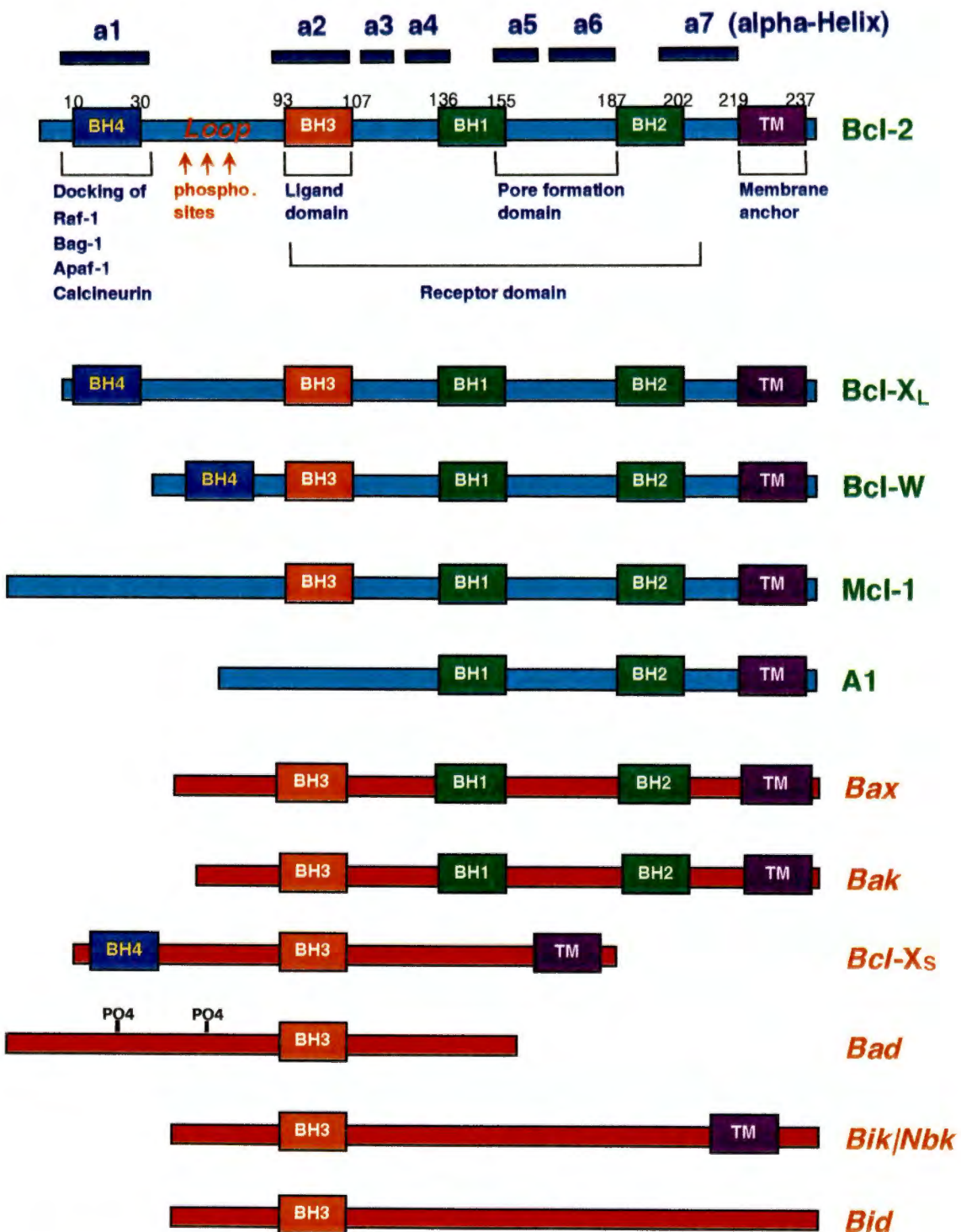
REGULATORS

Apoptosis is regulated by a variety of cellular proteins, including Bcl-2 family members, apoptosis-associated factors (Apafs), and inhibitors of apoptosis (IAPs) family members, perhaps through either changing the permeability transitions of mitochondria, regulation of caspase activation, or both.

Bcl-2 family members

The Bcl-2 family represents a class of proto-oncogenes that block cell death without promoting cell proliferation (418). Furthermore, Bcl-2 overexpression has been shown to delay cell entry to S phase and promote cells back to G₀ phase (773).

Members of Bcl-2 family can be either anti-apoptotic (Bcl-2, Bcl-XL, etc.) or pro-apoptotic (Bax, Bak, Bcl-Xs, etc.) (**Figure 7**) (3)(55)(96)(401)(425)(638). They can form either homodimers or heterodimers and the ratio of Bcl-2 to Bax has been shown to be important in determining the susceptibility to apoptosis (591)(96). Nevertheless, studies of transgenic and knock-out mice indicate that,



(Figure 7) **Representative Bcl-2 family members.** The anti-apoptotic members are drawn with green bars, while the pro-apoptotic members are with red bars and labeled by italic legends. The numbers represent the number of amino acid residues. Abbreviations used are: BH, Bcl-2 homology; and TM, transmembrane domain.

despite the *in vivo* competition between Bcl-2 and Bax, each is capable of regulating apoptosis independently of the other molecule (414). Bax can directly cause release of cytochrome *c* from mitochondria (383) and cytochrome *c* can initiate apoptotic characteristics *in vivo* and *in vitro* (599)(893). In co-transfection studies, Bcl-2 co-localizes with Bax to mitochondria but does not prevent Bax-induced cytochrome *c* release, yet Bcl-2 inhibits apoptosis initiated by cytochrome *c* release (650). Collectively, these results suggest that (i) dimerization between Bax and Bcl-2 does not necessarily counteract the functions of each molecule, and (ii) Bcl-2 has anti-apoptotic functions beyond the regulation of mitochondrial release of cytochrome *c*, probably through direct inhibition of caspase activation.

Structures and functions

Bcl-2 is a 239-residue protein and contains many structural motifs, which account for three distinct functions: (i) dimerization, (ii) adaptor function, and (iii) channel formation. From the N-terminus to the C-terminus, there are four Bcl-2 homology (BH) domains (BH4, BH3, BH1, BH2) and a transmembrane (TM) domain, which anchors Bcl-2 to the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope (**Figure 7**) (3)(96)(401)(425)(638). In Bcl-2 and Bcl-X_L, there is a loop domain between BH4 and BH3. This loop is the target site for phosphorylation and caspase-mediated cleavage, hence it is generally considered as a site for negative regulation (i.e., inhibition of protective effects of Bcl-2 and Bcl-X_L) (401). BH1 and BH2 domains are required for dimerization (864) while the BH3 is essential for pro-apoptotic functions (111)(350). Mammalian anti-apoptotic Bcl-2 family members generally harbor at least three BH domains (BH3, BH1, BH2) and a transmembrane domain. On the other hand, the minimum requirement for a pro-apoptotic member is a BH3 domain (401). Some pro-apoptotic members, such as Bik, Blk, Hrk, BNIP3, Bim_L, Bad, and Bid, only have BH3 domain (3). On the other hand, most viral anti-apoptotic Bcl-2 homolog, such as BHRF1, LMW5-HL, ORF16, KS-Bcl-2, and E1B-19K, do not have BH3 domain (3). BH3 is therefore believed to be the death domain of Bcl-2 family members.

The three-dimensional structure of Bcl-X_L indicates that BH3, BH1, and BH2 cooperatively form a hydrophobic groove on the surface of Bcl-X_L, which serves as the “receptor” during dimerization (564). The inserting “ligand” is an amphipathic helix BH3 provided by the dimerizing partner (164)(684). BH3 binds with high affinity to the hydrophobic pocket (“receptor”) created by BH3, BH1, and BH2 domains through hydrophobic and electrostatic interactions (684).

The N-terminal BH4 domain is not required for heterodimerization but is likely involved in

protein-protein interactions with other regulatory proteins outside the Bcl-2 family (401). BH4 is the docking site for Raf-1 (796), Bag-1 (a Bcl-2 binding protein) (797), calcineurin (a calcium-dependent protein phosphatase) (713), and Apaf-1 (apoptosis-associated factor-1) (600). Inactivation of Bad by Raf-1 exemplifies how interactions between Bcl-2 and other proteins through BH4 regulate Bcl-2 functions. The pro-apoptotic Bad has no membrane anchoring TM region yet inhibits the cytoprotective functions of Bcl-2 and Bcl-X_L by heterodimerizing with them. Bcl-2 has been shown to target the protein kinase Raf-1 to mitochondria. Once there, Raf-1 phosphorylates Bad and releases it from Bcl-2 (796).

X-ray and NMR data indicate structural similarity between Bcl-X_L and the membrane translocation domain of bacterial toxins, such as diphtheria toxin and the colicins (564). The structural analyses of Bcl-X_L reveal a seven α -helical bundle, at the center of which lies a hairpin comprised of two hydrophobic core helices, α 5 and α 6. Undergoing profound conformational changes, these two α -helices presumably insert perpendicularly across the lipid bilayer (564). Indeed, Bcl-X_L and Bcl-2 can form ion channels in planar lipid bilayers (546)(692) and the removal of α 5 and α 6 from Bcl-2 abrogates channel formation *in vitro* and abolishes its anti-apoptotic functions in cells (692). The pro-apoptotic Bax also can form ion channel (695).

The mechanism how ion-conductive pores regulate apoptosis, however, remains unclear. Speculations include: (i) Bcl-2 or Bax may regulate an electrochemical gradient, altering some substrates or products residing in the intermembrane space of the mitochondria, and/or (ii) the Bcl-2 family members may regulate other ion channels or transport other unidentified molecules (96). Alternatively, the Bcl-2 family members may participate in the formation of mitochondrial megachannel (also called permeability transition pore complex, PTPC) that accompanies apoptosis (259). The mitochondrial megachannel functions as a calcium-, voltage-, pH-, and redox-gated channel but with little, if any, ion selectivity (259). Intriguingly, only Bax and Bag-1, but not Bcl-2 or Bcl-X_L, have been co-purified in the megachannel complex (513), although Bcl-2 has been shown to antagonize the channel-forming activity of Bax (18). Bax has recently been shown to cooperate with adenine nucleotide translocator (ANT) within the PTPC to increase mitochondrial membrane permeability and to trigger cell death (512).

Regulation of Bcl-2 by phosphorylation

Phosphorylation is involved in the regulation of Bcl-2 functions (92). The potential phosphorylation sites are multiple serine residues that reside in the loop region between the α 1 (BH4) and α 2 (BH3) helices (92)(279)(516). The roles of Bcl-2 phosphorylation in promoting (51)(92)(99)(281)(652) or inhibiting apoptosis (334)(360), however, remain controversial.

Phosphorylation of Bcl-2 has been shown in cells treated with microtubule-active agents (paclitaxel, vinblastine, vincristine) and is thought to promote cell death (52)(51)(280). Raf-1 activation has been suggested to be required for Bcl-2 phosphorylation in paclitaxel-treated cells (52)(51), however, opposite observations have been reported (355). Bcl-2 phosphorylation can be induced by a phosphatase inhibitor okadaic acid (279) and cAMP-dependent protein kinase (PKA) has been shown to be required for paclitaxel-induced Bcl-2 phosphorylation (725). When apoptosis is induced by the simultaneous PKC inhibition and v-Ras overexpression, Ras may account for phosphorylation of Bcl-2 (99).

On the other hand, Bcl-2 phosphorylation has been noted to occur only in the cells arrested at the G₂/M phase and Bcl-2 therefore is proposed to be "the guardian of the microtubule integrity" (278). A recent study also indicates that Bcl-2 phosphorylation is a marker of M phase of the cell cycle and argues against its role as a determinant of apoptosis (471).

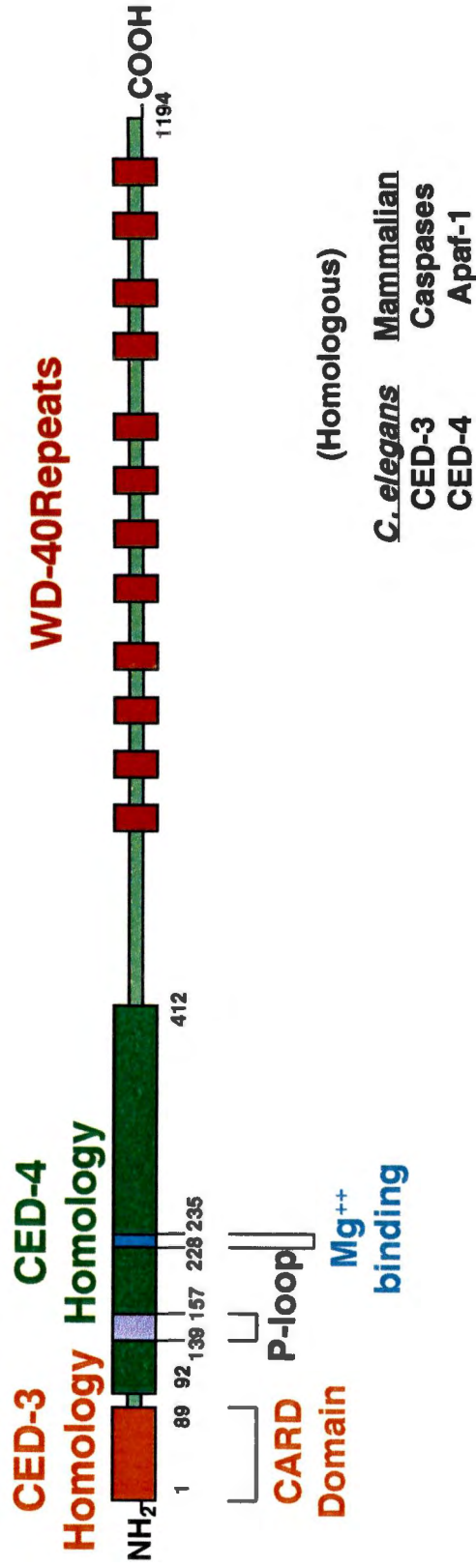
Cleavage of Bcl-2 and Bcl-X_L to ensure apoptosis

Bcl-2 family members regulate apoptosis by at least two ways: (i) regulation of mitochondrial release of cytochrome *c* and other apoptosis-inducing factors (AIFs) and (ii) direct inhibition of caspase activity (383)(546)(564)(650)(692)(695)(893). Likewise, CED-9 (the *C. elegans* Bcl-2 homolog) (Table 8) has been shown to be a bifunctional cell-death inhibitor (850). More intriguingly, CED-9 is also a substrate for CED-3 (the *C. elegans* caspase homolog) (850) and Bcl-2 and Bcl-X_L can be cleaved by caspases (106)(123)(257). Caspases cleave the 26-kD Bcl-2 into the 23-kD C-terminal fragment at the Asp31 and Asp34 residues in the loop region of Bcl-2 (Figure 7) (106)(257). The consequent C-terminal fragment of Bcl-2 or Bcl-X_L, which lacks BH4, promotes apoptosis (106)(123)(257). In summary, during the apoptotic process, activated caspases convert the anti-apoptotic Bcl-2 and Bcl-X_L into the pro-apoptotic fragments, ensuring the irreversibility of cell death. This also help to explain the paradoxical observations that higher Bcl-2 expression in breast cancers is correlated with better prognosis (304)(374)(473).

Apoptosis-associated factors (Apafs)

Three identified Apafs are: Apaf-1, a 130-kD cytosolic protein, the human homolog to *C. elegans* CED-4 (Table 8); Apaf-2 which turns out to be cytochrome *c* (898); and Apaf-3 which is identical to caspase-9 (460). From the N-terminus to the C-terminus, Apaf-1 contains regions homologous to CED-3 (caspases) and CED-4, and twelve Walker's domain (WD) repeats (Figure 8) (600)(898). Apaf-1 is not a

Apoptosis associated factor-1 (Apaf-1)



(Figure 8) Apoptosis associated factor-1 (Apaf-1). Abbreviations used are: CARD, caspase recruiting domain; and WD, Walker's domain. The numbers represent the number of amino acid residues.

caspase. Instead, it functions as an adaptor protein through interacting with caspases using the CED-3 homolog as the caspase recruitment domain (CARD) and associating with other proteins through multiple WD repeats that are potential sites for both protein-nucleotide and protein-protein interactions (573)(790). Indeed, Apaf-1 has been shown to associate with caspases-4, -8, and -9 (343). For interaction between Apaf-1 and the Bcl-2 family members, both the CED-4 homologous domain and the WD repeats-rich C-terminal region can bind Bcl-X_L (343).

The discovery of Apafs and subsequent studies again confirm the evolutionary conservation of apoptotic components among species from the nematode *C. elegans* to the human (309). Three key cell death proteins that have been identified in *C. elegans* are CED-4, CED-9, and CED-3; while the mammalian equivalents are Apaf-1, Bcl-2 or Bcl-X_L, and caspase-9 (Apaf-3), respectively (**Table 8**). In *C. elegans*, interaction of CED-4 and proCED-3 is necessary and sufficient for activation of CED-3. The nematode Bcl-2 homolog, CED-9, prevents apoptosis by binding CED-4 in an inactive conformation, thereby preventing CED-4-mediated activation of proCED-3 (309). Likewise, Apaf-1 (the human homolog of CED-4), Bcl-X_L (a human homolog of CED-9), and caspase-9 (Apaf-3, the human homolog of CED-3) have been found to form a ternary complex (600), suggesting that Bcl-X_L regulates caspase-9 through Apaf-1 (343). The complex has been designated as the mammalian "apoptosome" (460)(898).

How does CED-4 (Apaf-1) activate pro-CED3 (pro-caspase-9) and how does CED-9 (Bcl-2) inhibit this activation? A current model is that CED-4 (Apaf-1) may function as the "apoptotic chaperone" which holds multiple inactive pro-CED3 (pro-caspase-9) molecules together to increase their local concentration and may also arrange them into the conformation that promotes their activation (308). Indeed, point mutations that abolish CED-4 oligomerization inhibit its pro-apoptotic activity (858). This model also explain the role of CED-9: CED-9 (Bcl-2) may bind CED-4 (Apaf-1) and interfere with the CED-4-mediated oligomerization of pro-CED-3 (pro-caspase-9), inhibiting the activation of CED-3 (caspase-9). Supporting this model, (i) CED-9-bound CED-4 cannot oligomerize (858), and (ii) deletion of the Apaf-1 WD-40 repeats region (**Figure 8**), which has been shown to bind Bcl-X_L (343), makes the truncated Apaf-1 constitutively active and capable of activating pro-caspase-9 independent of cytochrome *c* (724). Although the "induced proximity" of pro-CED-3 (pro-caspase-4) by CED-4 (Apaf-1) appears to be required for activation of CED-3 (caspase-9), this may not be the only role for CED-4 (Apaf-1). Both the presence of nucleotide-binding domain in CED-4 (Apaf-1) and the requirement of ATP suggest that CED-4 (Apaf-1) should have ATPase activity and likely other functions too (308).

Inhibitors of apoptosis (IAPs) family

IAPs (inhibitors for apoptosis), although unrelated to p35, have also been discovered in the baculovirus (47)(138). Human homologs of IAP include NAIP (neuronal apoptosis inhibitor protein) (474), c-IAP1/hIAP-2/MIHB (mammalian IAP homolog-B), c-IAP2/hIAP-1/MIHC, hILP (human IAP-like protein)/X-IAP (X-linked IAP) /MIHA (653)(772) and survivin (4)(14).

The conserved structures of the IAP family members are the multiple N-terminal BIRs (baculovirus IAP repeats) and a C-terminal RING finger, which is a type of zinc finger (124). The unique BIRs are required for the anti-apoptosis activity of IAPs and contain conserved residues, Cys-XX-Cys and Cys-X₆-His motifs, which suggest zinc-binding ability (124). The human IAPs, c-IAP1 and c-IAP2, use BIRs to interact with the TRAF-N domain of TRAF-2 (TNF receptor associated factor-2) (653). TRAF-2 is an upstream regulator of NF- κ B, suggesting IAPs may regulate apoptotic pathways via NF- κ B. In summary, the mechanisms of anti-apoptosis by IAPs may be through (i) direct inhibition of caspases-3 and -7 by hILP/X-IAP (162), c-IAP1 and c-IAP2 (664), and (ii) indirect modulation of other signaling proteins, such as NF- κ B (727) or JNK1 (682).

EFFECTORS

Caspase family members

Members of the cysteine protease family (**the caspase family**) play the executioner role in apoptosis (**Table 13**) (31)(128)(408)(577)(786). The term “caspase” was coined to highlight the general properties of these protease: the “c” in caspase denotes the presence of cysteine at the active site, a pentapeptide QACR(or Q/G)G, and the “aspase” denotes the principle cleavage activity adjacent to aspartate (10).

Knock-out mice studies have demonstrated the essential roles of caspase in apoptosis. Caspase-3 null mice, born at a frequency lower than expected by Mendelian genetics, are smaller than their litter mates and die at 1-3 weeks. Decreased apoptosis and defective brain development are detected by embryonic day 12 (430), suggesting that each caspase may have tissue-type specific functions. Signal transducer and activator of transcription 1- (STAT1) null mice are resistant to apoptosis by TNF- α and reintroduction of STAT1 restores both TNF- α -induced apoptosis and the expression of caspases-1, -2, and -3 (431), suggesting that STAT1 is required for efficient constitutive expression of these caspases.

Table 13
Summary of Caspases

Caspase	Other names	Recognition site	Inhibitors (Peptide or chemical*)	Viral Inhibitors	Cellular substrates
1	CED-3 ICE	YVAD ↓ G YVPD ↓ S	DEVD YVAD zVAD	CrmA E8-FLIP p35	Actin, CED-9, PARP, PITSLRE, Pro-caspase-1, -2, -3, Pro-IL-1β
2	ICH-1 NEDD-2		zVAD	p35	Pro-caspase-2, PARP
3	Apopain CPP32 Yama	DEVD ↓ G DMQD ↓ N YVPD ↓ S	DEVD-CHO TPCK* zVAD	CrmA p35	Bcl-2, Bcl-X _L , DFF _{45kd} , DNA-PK, Fodrin, GAS2, ICAD, MDM2, PAK2, PARP, PKC, PITSLRE, pRB, Pro-caspase-3, -6, -9, RNP, Rbo-GDI, SREBP
4	ICE-II ICH-2 TX		DEVD YVAD zVAD	p35	PARP, Pro-IL-1β, Pro-caspase-1, -4
5	ICE-III ICH-2 TY			CrmA	
6	Mch2	VEID ↓ NG	TLCK* zVAD	CrmA p35	Lamins, PARP
7	Mch3 ICE-LAP3	DEVD ↓ G	DEVD-CHO EVD	CrmA	PARP, Pro-caspase-6, RNP
8	FLICE1 MACH1 Mch5		DEVD IETD zVAD	CrmA E8-FLIP	Pro-caspase-3, -4, -7, -9
9	ICE-LAP6 Mch6			CrmA	PARP
10	FLICE2 Mch4		DEVD	p35	PARP, Pro-caspase-3, -7

References:(10)(106)(123)(128)(408)(480)(677).

Structure-functional considerations

Caspases are synthesized as procaspases that can be activated through cleavage by proteases, including itself and other caspases. A procaspase contains an N-terminal prodomain structure of variable length and two enzyme subunits. During its activation, the prodomain of the procaspase is removed and two subunits are released but further heterodimerize into an active enzyme (**Figure 9**). According to the different lengths of prodomains of procaspases and preferences for substrates, caspases are further categorized into two classes: regulatory caspase (e.g., caspase-1, -2, -4, -5, -8, -9, -10) and effector caspases (e.g., caspase-3, 6, 7) (128)(786). Through the long prodomains, regulatory caspases may interact with regulators, such as Apaf-1, FADD, and RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain)/CRADD (caspase and RIP adapter with death domain) (109)(128)(786).

Chemical and peptide inhibitors and substrates

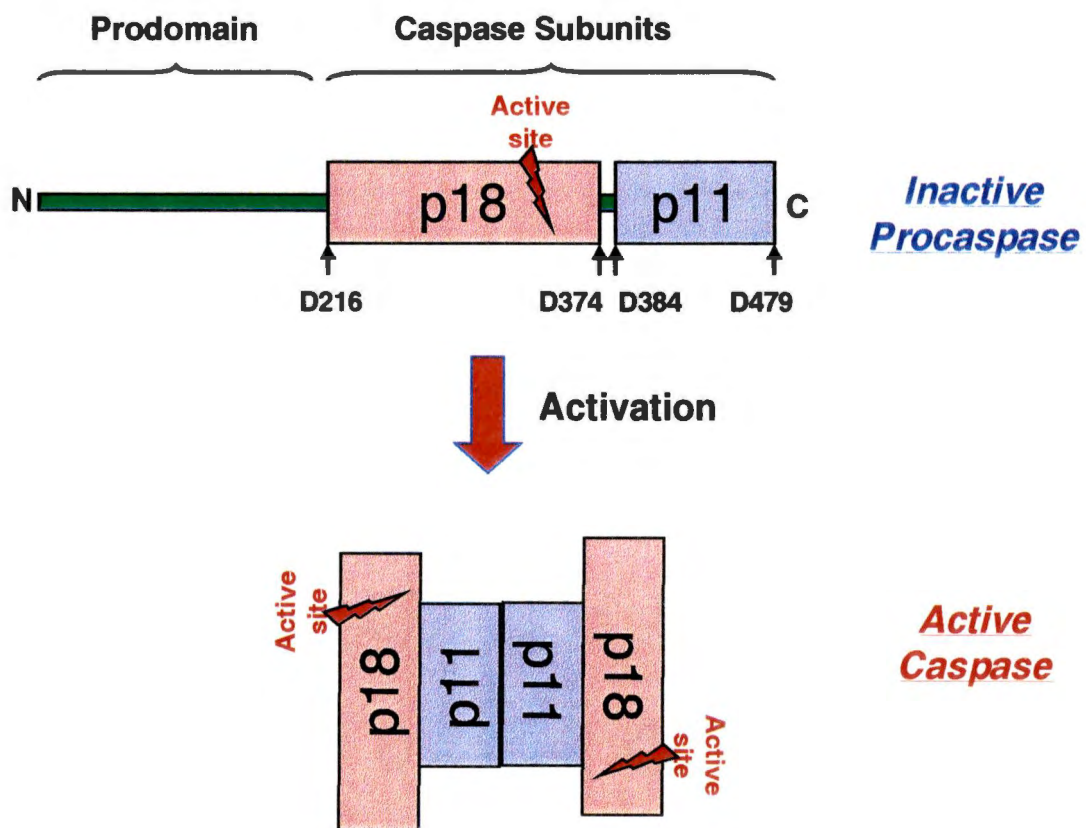
The serine protease inhibitors, N-tosyl-L-phenyl-alanylchloromethyl ketone (TPCK) and N-tosyl-L-lysylchloromethyl ketone (TLCK) (71)(206), and several short peptides of three to four amino acid residues have been used in studies of caspase functions. Synthetic peptide inhibitors include DEVD-fmk (acetyl-Asp-Glu-Val-Asp-fluoromethylketone), VEID-fmk (acetyl-Val-Glu-Ile-Asp-fluoromethylketone), YVAD-fmk (acetyl-Tyr-Val-Ala-Asp-fluoromethylketone), zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone)(**Table 13**)(22)(184)(487). Synthetic substrates include DEVD-AMC (acetyl-ASP-Glu-Val-Asp-aminomethylcoumarin) and YVAD-AMC (acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin). The hydrolysis of these substrates by caspases produces the fluorescent AMC product (22)(511).

Cellular targets for caspases

Cellular targets for caspases fall into three groups: (i) procaspases, (ii) cellular proteins that need to be inactivated for cell death to occur, and (iii) cellular proteins whose activation is required for the apoptotic process, as summarized in **Table 7**.

Cellular and viral inhibitors for caspases

Apoptotic functions of caspases are regulated at least by the Bcl-2 family members and the inhibitor for apoptosis (IAP) family members. Bcl-2 has been suggested to protect cells from apoptosis through its inhibition on caspase activation (22)(110). Supporting the role of Bcl-2 in inhibition of caspase activation, the *C. elegans* CED-9 (Bcl-2 homolog) has been shown to interact with CED-4 and prevent the ability of CED-4 to activate the CED-3 (the homolog of caspase-1) (833)(834). Likewise in mammalian cells, Bcl-2 or Bcl-X_L inhibits apoptosis through its interaction with the apoptosis-initiating



(Figure 9) Activation of procaspase by cleavage at the aspartate residues. The cleavage sites of the procaspase are labeled as D (aspartate) followed by the number of amino acid residues.

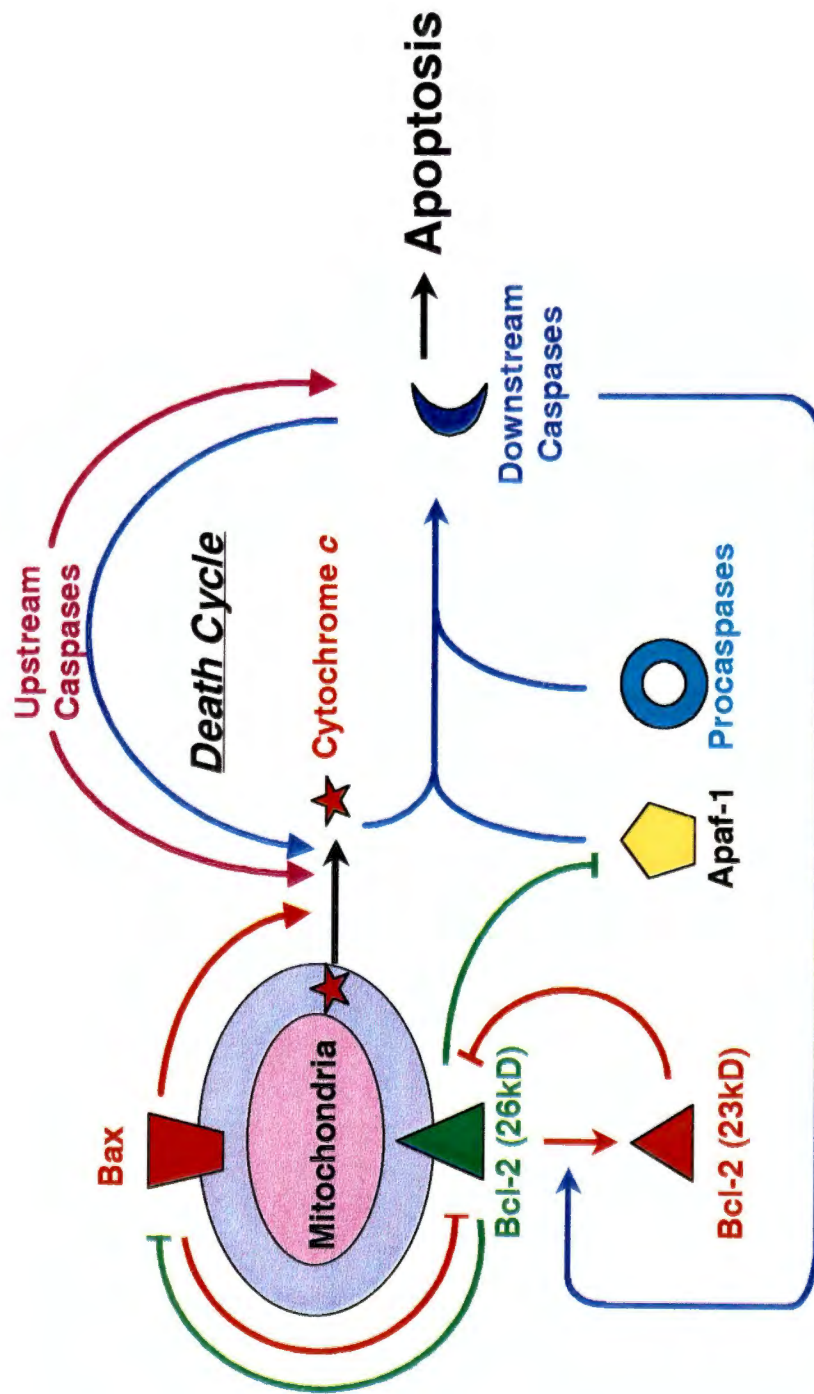
complex apoptosome, which is comprised of Apaf-1 (the mammalian homolog of CED-4), cytochrome *c* (Apaf-2), and caspase-9 (Apaf-3) (Figures 10, 11) (343)(460)(599)(650)(898). Among members of the inhibitors for apoptosis (IAP) family, hILP/X-IAP has been shown to directly inhibit caspases-3 and -7 (162).

Various viral proteins have been shown to inhibit caspase activity, including the cowpox virus cytokine response modifier A (CrmA) (227)(636), the baculovirus p35 (73)(851), the baculovirus inhibitors for apoptosis (IAPs) (47)(138), and the viral FLICE-inhibitory proteins (FLIPs) (44)(342)(756). CrmA is a 38-kD serine protease inhibitor (serpin) that preferentially inhibits caspase 1-like proteases by forming a stable complex with the caspase. p35 has a broader target range than CrmA and can inhibit both caspase 1-like and caspase 3-like proteases. The viral IAPs, although unrelated to p35, are also isolated from the baculovirus. FLIPs are viral proteins containing sequences related to the death effector domain (DED), which is found in the intracellular part of Fas and TNFR1 (408). Viral FLIPs have been isolated from the equine herpesvirus type 2, bovine herpesvirus-4, herpesvirus saimiri (HVS), human herpesvirus-8 (HHV-8), and the human molluscum contagiosum virus (MCV). Through interactions between DED-like domain with the prodomain of FLICEs (caspase 8 and 10), FLIPs effectively inhibit apoptosis mediated by FLICEs.

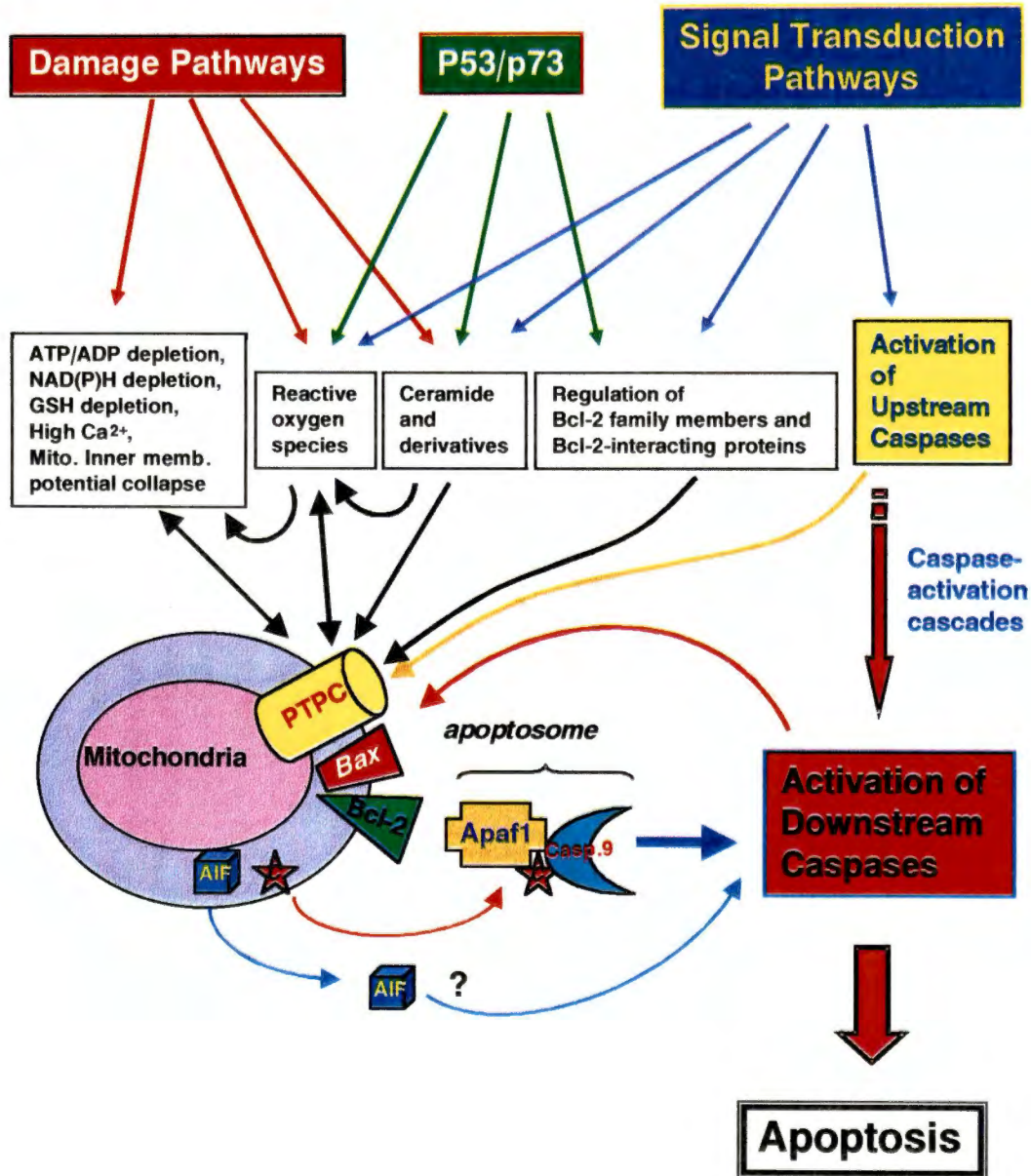
Cyclin-dependent kinases (CDKs)

Functions and regulations

The eukaryotic cell cycle is regulated by the periodic activation of various cyclin-dependent kinases (CDKs). The CDK holoenzymes are heterodimeric complexes consisting of a catalytic subunit (kinase) and an activating subunit (cyclin). A series of different cyclin-CDK complexes are required to catalyze the phosphorylation of various protein substrates important for the cell to progress through the cell cycle. To ensure perfect timing and coordination of cell-cycle events, activities of cyclin-CDK complexes are tightly regulated by the following mechanisms. (i) The monomeric kinase subunit of CDK alone is inactive and requires association with a cyclin subunit and subsequent phosphorylation of a key threonine residue, e.g., T160 in CDK2, to become fully active. (ii) Activity of a cyclin-CDK complex can be reversibly inactivated either by phosphorylation on a tyrosine residue, such as Y15 located in the ATP-binding domain of the CDK2, or by association with cyclin-kinase inhibitory proteins (CKIs). (iii) After completion of the cell-cycle transition, the cyclin-CDK complex is irreversibly inactivated by the



(Figure 10) **The death cycle model of apoptosis.** In this model, Bcl-2 can act both upstream and downstream of the cytochrome c release from the mitochondrion.



(Figure 11) **The circular model of apoptosis.** In this model, there are multiple points of entry to the mitochondria-caspase activation circle, explaining why this circle is vulnerable to inhibition by either caspase inhibitors or mitochondria-stabilizing agents. Abbreviations used are: GSH, glutathione; Mito, mitochondria; PTPC, permeability transition pore complex (also known as “megachannel”); AIF, apoptosis-inducing factors; and C, cytochrome *c*.

degradation of the cyclin subunit (510).

There are two families of CDK inhibitors (CKIs): the INK4 family (p15, p16, p18, p19) and the CIP/KIP family (p21, p27, p57) (709). Members of the INK4 family specifically bind to CDK4 and CDK6, acting as inhibitors of cyclin Ds. On the other hand, members of the CIP/KIP family form heterotrimeric complexes with the CDK and the cyclin. The CIP/KIP family members bind to most CDK-cyclin complexes to some extent but have the highest affinity for G₁ CDK-cyclin complexes (cyclin D-CDK4, cyclin D-CDK6, cyclin E-CDK2) (510).

CDKs in apoptosis

The discoveries of apoptosis in unicellular organisms (15)(135) indicate that apoptotic machinery may be as ancient as 2 billion year-old, perhaps appearing only a little later than, if not at the same time as the cell replication machinery (15). It also raises a possibility that it would be economical if both cell replication and apoptosis employ the same machinery. Furthermore, the co-existent apoptosis may contribute to the well-being of multicellular organisms in reducing the risk of tumorigenesis accompanying cell proliferation because an additional set of survival signals are required for counteracting apoptosis (260). In favor of this hypothesis, many genes that control the cell cycle and cell differentiation including proto-oncogenes, tumor suppressors, cyclins, and CDKs, have been demonstrated to participate in the control of apoptosis (191)(300)(494)(822).

Recent data have indicated an important role for CDK activation in apoptosis. Premature activation of p34^{cdc2} has been shown to be required for apoptosis (711)(714), and apoptosis has been shown to be inhibited either by expression of dominant negative (dn)-CDKs (529) or by butyrolactone I, a specific inhibitor of Cdc2 (226). Activated CDK7 has also been associated with activation of p34^{cdc2} in paclitaxel-treated cells (345).

Furthermore, some regulators for CDK activity have been shown to be substrates of caspases. For instances, caspase-3 or caspase-3-like enzymes can cleave CDK inhibitors p21^{Cip1/Waf1}, p27^{Kip1} (458), as well as Wee1 and CDK27, the inhibitory kinases for Cdc2 and CDK2 (894). Catastrophic CDK activities therefore have been proposed to be the actual effector of cell death (293)(458)(894).

MITOCHONDRIA AND ATP STATUS: CURRENT MODELS

Two fundamental decisions of cell fate are: (i) life versus death, and (ii) apoptosis versus necrosis. What event determines whether the cell passes "the point of no return" toward death has been

long under debate. DNA fragmentation and caspase activation, have turned out to be consequences, but not decision makers of apoptosis. The Bax/Bcl-2 ratio has been proposed as a determinant for life or death (591), however, the observations that caspase inhibitors block Bax-induced apoptosis (650) suggest that, although Bcl-2 family members may regulate apoptosis, they are not the ultimate decision makers.

Increasing amount of data demonstrate the involvement of mitochondrial changes in cell death, both necrosis and apoptosis (426)(428). Disruption of mitochondrial transmembrane potential has been shown to precede nuclear DNA fragmentation (89)(781)(877)(878). Bcl-2 may regulate apoptosis through preservation of mitochondrial functions (89)(505)(739)(857)(877). Activation of caspases and mitochondrial changes represent two distinct yet interacting events, demonstrated by the observations (i) that caspase inhibition may delay and/or prevent mitochondrial dysfunction (62)(323)(738), (ii) that caspases cause mitochondrial dysfunction (513)(738), and (iii) that mitochondrial dysfunctions cause caspase activation (181)(413)(460)(478)(738)(857)(898). Caspases can be classified into the upstream regulatory (caspases-2, -8, -10) and the downstream effector (caspases-3, -6, -7) (259). Activation of the downstream effector caspases often occurs with a several-hours lag phase after activation of upstream regulatory caspases (184)(738), and Bcl-2 can prevent activation of downstream effector caspases without effects on the upstream regulatory caspases (527). Collectively, these studies suggest the existence of a link between the upstream and downstream caspases and the link is likely to be the mitochondria, whereas Bcl-2 may regulate apoptosis through inhibiting mitochondrial dysfunction. A linear model of "upstream caspase activation → mitochondrial dysfunctions → downstream caspase activation", however, cannot explain the recent observations that Bcl-2 protects cells from apoptosis induced by either injected cytochrome *c* (893) or Bax-induced cytochrome *c* release from mitochondria (650).

A "death cycle" and a "circular" model have been independently proposed (259)(310), both characterized by a self-amplifying vicious cycle that eventually leads to a large-scale caspase activation and apoptosis. The death cycle model emphasizes that mitochondria act as the apoptotic amplifier and that Bcl-2 can act both upstream and downstream of cytochrome *c* release from the mitochondria (**Figure 10**) (310). The circular model highlights multiple points of entry to the mitochondria-caspase activation circle, which explains why this circle is vulnerable to the inhibitory effect of caspase inhibitors and of agents that stabilize the mitochondrial membranes (**Figure 11**) (259). Both models indicate the decision-maker role of mitochondria, dysfunctions of which could be the point of no return (259)(310) and after amplification, irreversible mitochondrial disruption commits the cell to die irrespective of subsequent

apoptotic or necrotic processes (259).

The second decision for cell death through either apoptosis or necrosis may be dictated by whether the downstream caspases can be activated. Despite the historical use of necrosis in contrast to apoptosis (**Table 1**) (315)(406)(426), accumulating data argue against the antithesis between apoptosis and necrosis (338)(361)(427)(441)(587). Since functions of the "apoptosome" (the complex of Apaf-1, cytochrome *c*, caspase-9, Bcl-2 or Bcl-X_L) require ATP, abundant cytosolic levels of ATP would allow cells undergo apoptosis, while cells with depleted ATP levels would die of necrosis (259)(426). This model is supported by the observations that ATP depletion (451) or caspase inhibition (145)(323)(843) cause cells to die through necrosis in response to classical apoptotic stimuli.

JNK/SAPK PATHWAY

OVERVIEW OF KINASES

The cell uses a complex signal transduction network not only to respond to the changes in its microenvironment but also to monitor and integrate all intracellular signals. One important part of the signaling network is composed of a variety of protein kinases. The protein kinase superfamily is the largest protein family of the cell: they are encoded by approximately 1,000 protein kinase genes, about 1% of the whole mammalian genome (289).

The basic function of a protein kinase is to transfer the γ -phosphate of ATP (or GTP) to the protein alcohol groups on serine (Ser) and threonine (Thr) and/or to the protein phenolic groups on tyrosine (Tyr). To counteract the cellular functions of protein kinases, members of the phosphatase superfamily catalyze hydrolysis of phosphoesters, removing phosphate from the Ser, Thr, and Tyr residues. The Ser/Thr protein phosphatases (PPs) specifically remove phosphates from Ser and Thr; the protein tyrosine phosphatases (PTPs) are phosphotyrosine-specific; while the dual specificity PTPs can hydrolyze phosphates from both Tyr and Ser/Thr (**Table 14**) (30)(155)(201)(351)(691)(730)(763)(897).

Structure-functional considerations of kinase

Multiple sequence alignment of kinases has revealed that all protein kinases contain a highly conserved "kinase domain" which consists of 250-300 amino acid residues (289). Taken from the type α

Table 14**Phosphatase superfamily**

Class	Family	Example members
Ser/Thr protein phosphatases (PPs)	PPP	PP1 PP2A PP2B (calcineurin)
	PPM	PP2C Pyruvate dehydrogenase phosphatase
Protein tyrosine phosphatase (PTPs)	Receptor PTP (RPTP)	CD45 PTP α PTP β PTP μ
	Intracellular PTP (Non-receptor PTP)	SHP-1 (SH2-containing tyrosine phosphatase-1) SHP-2 FAP-1 (Fas-associated phosphatase-1)
	Dual specificity PTP	Cdc25 MAPKP (=MKP-1)

*References: (30)(155)(201)(351)(691)(730)(763)(897).

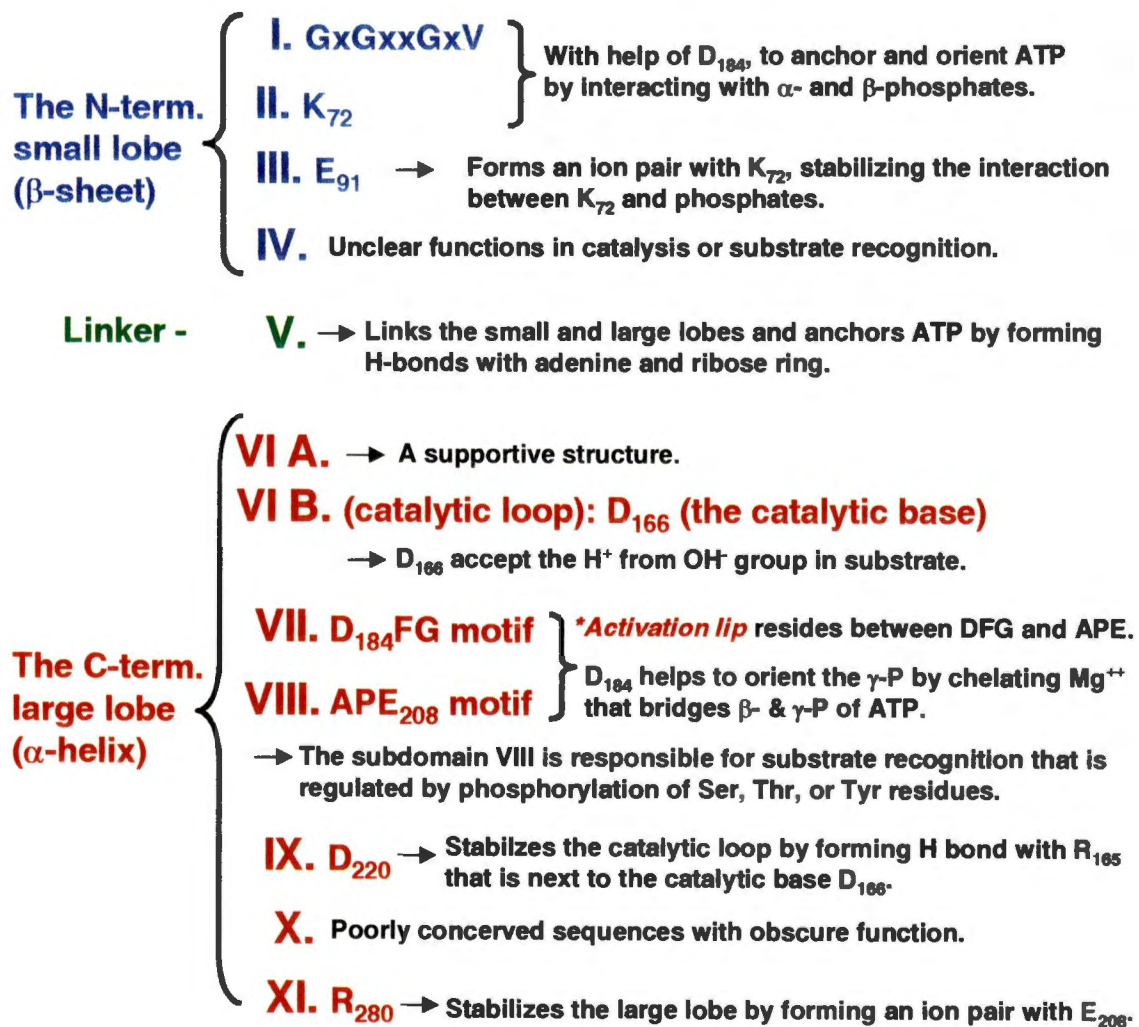
catalytic subunit of protein kinase A (PKA-C α) as an example, conserved features and essential roles of the 12 subdomain regions are summarized in (Figure 12). From the N-terminus to C-terminus of the kinase domain, there are the small lobe (subdomains I to IV) that consists of almost entirely β -sheets, the linker (subdomain V), and the large lobe (subdomains VI to XI) that is dominated by α -helix. In other parts of the kinase domain, different protein kinases exhibit variability such as additional domains, subunits, or both.

Three steps of catalysis by a kinase are: (i) binding and orientation of the ATP (or GTP) phosphate donor as a complex with a divalent cation (usually Mg⁺⁺ or Mn⁺⁺), (ii) binding and orientation of the protein substrate, and (iii) transfer of the γ -phosphate from ATP (GTP) to the acceptor hydroxyl residues (Ser, Thr, Tyr) of the substrate. Both (i) and (iii) are achieved by the highly conserved kinase domain. Taking residues of PKA-C α as an example (Figure 13): D₁₈₄ chelates Mg⁺⁺ that bridges the β - and γ -phosphates of ATP; K₇₂ and E₉₁ collaboratively orient ATP by interacting with α - and β -phosphates of ATP; and the linker further stabilizes ATP via formation of hydrogen bonds with adenine or ribose of ATP (289). Subdomain VIB is the catalytic loop that contains D₁₆₆ as the catalytic base that accepts proton from the attacking substrate hydroxyl group during a phosphotransfer mechanism (868).

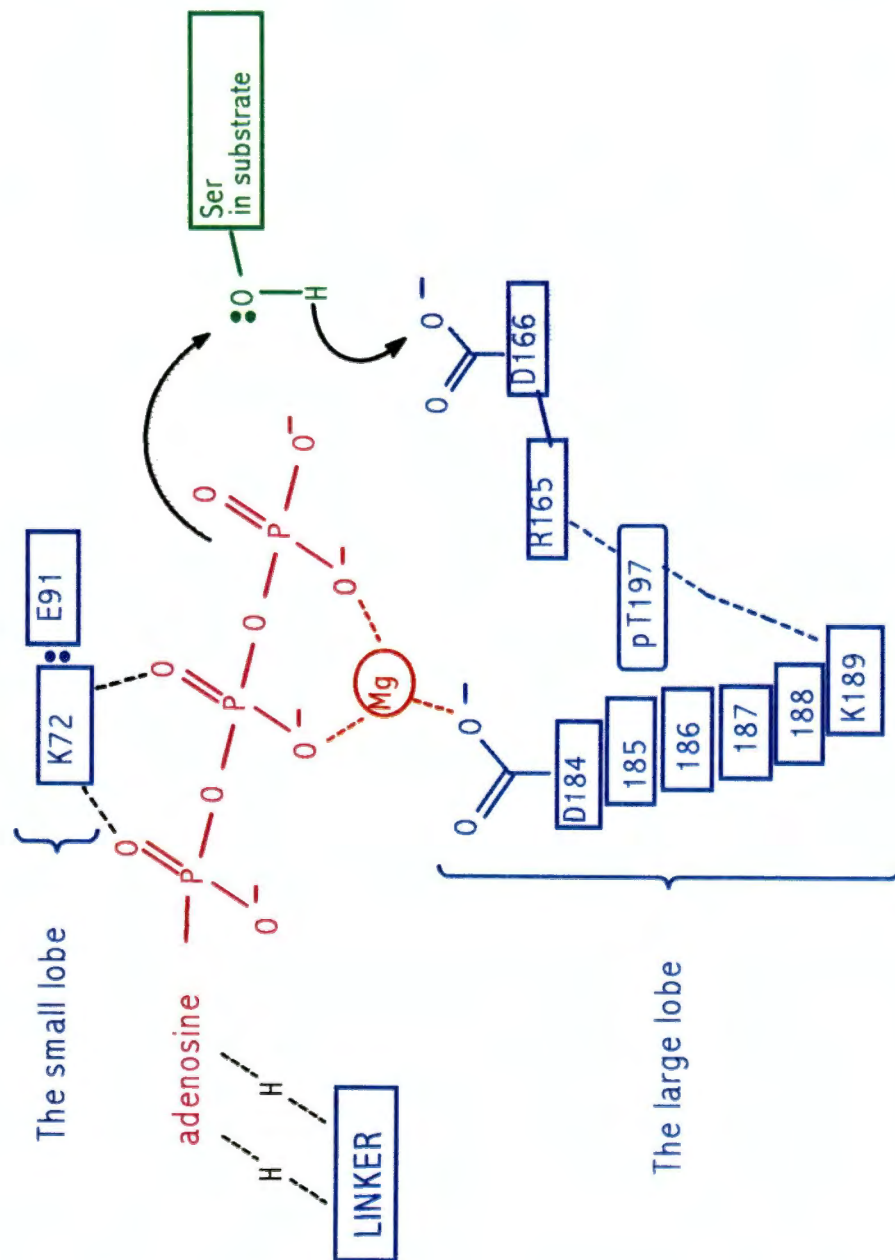
Recognition, binding and orientation of the substrate protein rely on the activation lip (segment) residing in subdomains VII and VIII. Many protein kinases may be activated by phosphorylation of residues in the activation lip. Taking PKA-C α as an example, residing in the activation lip between D₁₈₄FG and APE₂₀₈ motif is Thr₁₉₇ that has to be phosphorylated during activation of PKA (Figure 12). The phosphate oxygen of phospho-T₁₉₇ form hydrogen bonds with the charged side-chains of R₁₆₅ and K₁₈₉ as well as the hydroxyl group of T₁₉₅, hence stabilizing the subdomain VIII loop in the active conformation that permits proper orientation of the substrate peptide (289). The activation lip varies among kinases. The variability in sequences of activation lip allows the kinase to be constitutively active, to be controlled by autophosphorylation, or to be controlled by phosphorylation directed by other kinases.

General mechanisms regulating the functions of protein kinases

The activity of protein kinases may be controlled by: (i) additional subunits that may function in response to second messengers (such as the cAMP binding to the regulatory subunit of PKA); (ii) additional subunits whose expression levels vary depending on the functional state of the cell (such as cyclin and cyclin-dependent kinase); (iii) additional domains (such as SH2 and SH3 of Src) that target the



(Figure 12) Subdomains of the kinase domain in protein kinase A. Abbreviations used are: G, glycine; x, any amino acid residue; K, lysine; E, glutamic acid; D, aspartic acid; F, phenylalanine; a, alanine; P, proline; R, arginine.



(Figure 13) **Kinase-catalyzed phosphorylation.** Protein kinase A is taken as an example: kinase is in blue, **ATP is in purple**, substrate is in green, and the **Mg ion is in red**. Only some key residues are shown (D, aspartic acid; E, glutamic acid; K, lysine; R, arginine; T, threonine).

kinase to different molecules or subcellular localizations; (iv) additional domains that inhibit the kinase activity by an autoregulatory process (such as in myosin light chain kinase); or (v) phosphorylation and dephosphorylation by other kinases and phosphatases, respectively.

OVERVIEW OF MAPK PATHWAYS

Actions of various signals in the cellular signal transduction network converge at the mitogen-activated protein kinase (MAPK) cascades, which lead to and activate transcription factors in the nucleus and other effectors throughout the cell (180)(810). At least twelve MAPKs (**Table 15**) and seven MKKs (MAPKK) (**Table 16**) have been identified in the mammalian cells (459). Each MAPK is activated by its cognate MKKs through a common mechanism involving phosphorylation of Thr and Tyr residues in the regulatory triplet, Thr-X-Tyr, located in the activation lip between subdomains VII and VIII. MKKs can be activated by various MAPK kinase kinases (MAPKKKs), including Raf family members, MOS, TPL2, TGF- β -activated kinase (TAK1), MEKK family members, mixed lineage kinases (MLKs), germinal center kinases (GCKs) and apoptosis signal-regulating kinase (ASK1). Thus, multiple signals from cell receptors mainly converge at the level of MKK activation.

Comparisons among MAPK pathways

ERK (extracellular signal regulated protein kinase) was first described as a protein kinase that phosphorylates microtubule-associated proteins 1 and 2 (MAP1, MAP2) (336)(637). Likewise, JNK/SAPK was first discovered as a cycloheximide-activated MAP2 kinase (434). It was also identified as a protein kinase which copurified with and phosphorylated c-Jun (6)(316). p38 MAPK was first characterized as a protein phosphorylated upon treatment with lipopolysaccharide (286) and as a “reactivating kinase (RK)” that phosphorylates and activates phosphatase-treated MAPK-APK2 (655).

ERKs, JNK/SAPK, and p38 share 40–45% overall sequence identity. Generally speaking among MAPK pathways, the ERK pathway is often stimulated by growth and differentiation factors (459), whereas the JNK and the p38 MAPK pathways are mainly activated by stress-related stimuli, such as UV or gamma irradiation, ischemia and reperfusion, and inflammatory cytokines (**Figure 6**) (393)(394)(435)(558). Nevertheless, opposing effects of JNK and p38 have been reported: for example, p38 mediates, but JNK suppresses, the development of myocyte hypertrophy (575).

Table 15

MAPK family

Subfamily	*Phosphorylation motif	Kinase	Alternative names
ERK	T-E-Y (Thr-Glu-Tyr)	ERK1 ERK2	p44MAPK p42MAPK
JNK	T-P-Y (Thr-Pro-Tyr)	JNK1 JNK2 JNK3	SAPK γ SAPK α SAPK β
p38	T-G-Y (Thr-Gly-Tyr)	p38 α	SAPK2a, RK (reactivating kinase), CSBP1 (**CSAIDs-binding protein 1)
		p38 β	SAPK2b, CSBP2
		p38 γ	SAPK3, ERK6
		p38 δ	SAPK4
Others		ERK3 ERK4 ERK5	SAPK5, BMK1 (big MAP-kinase 1)

* Phosphorylation motif resides between the DFG (Asp-Phe-Gly) motif of kinase subdomain VII and the APE (Ala-Pro-Glu) motif of kinase subdomain VIII.

** CSAIDs: cytokine-suppressive anti-inflammatory drugs.

***References: (132)(459).

Table 16
MAPKK family

MAPK kinase	Alternative names	Substrates
MKK1	MEK1 (MAPK or ERK kinase-1)	ERK1, ERK2
MKK2	MEK2 (MAPK or ERK kinase-2)	ERK1, ERK2
MKK4	SEK1 (SAPK or ERK kinase-1), JNKK (JNK kinase)	JNK1, JNK2, JNK3, p38 α , p38 β
MKK7		JNK1, JNK2, JNK3
MKK3		p38 α , p38 β , p38 γ , p38 δ
MKK6	MEK6 (MAPK or ERK kinase-6)	p38 α , p38 β , p38 γ , p38 δ
MKK5	MEK5 (MAPK or ERK kinase-5)	ERK5

* References: (132)(459).

ERK pathways

ERK1(42 kD) and ERK2 (44 kD) are expressed ubiquitously in mammalian cells at micromolar levels (344), with some tissue type-specific variations (555). Both enzymes are activated by MKK1/2 through phosphorylation within their activation lip at Thr-Glu-Tyr (T-E-Y) in a reaction where phosphorylation of Tyr precedes that of Thr (**Table 15**)(299).

MKK1 and MKK2, both about 44 kD in mass, are activated by various upstream kinases through phosphorylation of Ser residues at the sequence Ser₂₁₈-Met-Ala-Asn-Ser₂₂₂ (numbered as in human MKK1) within the activation lip (892)(891). The upstream kinases for MKKs include: Raf family members (Raf-1, B-Raf) (836), Mos (620), Tpl-2 (678), and MEKK-1 (544). In vitro activation of MKKs through phosphorylation of Ser₂₁₈ and Ser₂₂₂ residues is followed by intramolecular autophosphorylation at Thr₂₃, Ser₂₉₈, and Tyr₃₀₀ (642).

Substrates for ERKs include various transcription factors (Elk-1, c-Jun, c-Fos family members, c-Myc, p53) and signaling proteins (MAPK-APKs, cPLA2, Sos1). Myelin basic protein (MBP) is commonly used as substrate for ERKs in *in vitro* assays (**Figure 6**).

Elk-1, also known as p62 ternary complex factor (p62^{TCF}), can be phosphorylated by ERK (369)(504), JNK (250)(819)(896), and p38 (624), regulating serum response element (SRE) transcription through its interaction with serum response factor (SRF) (256). ERK can phosphorylate c-Jun both at Ser₆₃ and Ser₇₃ within its activation domain (627) and at residues at the C-terminus of c-Jun (545) that may lead to negative regulation through inhibition of DNA binding (12)(112). ERK also phosphorylates c-Fos, Fra-1, and Fra-2 *in vitro* and *in vivo* (101)(589). In cell extracts, ERK binds to the N-terminus of c-Myc (273) and phosphorylates its Ser₆₂ (12)(143). The tight interaction between ERK and c-Myc is disrupted by phosphorylation of c-Myc, suggesting functional interactions between enzyme and substrate (459). ERK also phosphorylates the tumor suppressor gene product p53 (540).

Activated ERK may phosphorylate MAPK-activated protein kinases (MAPK-APK)-1 (207)(266)(734)(740), MAPK-APK-2 (39), and MAPK-APK-3 (493)(525). Substrates for MAPK-APK-1 include transcription factors such as cAMP-response element-binding protein (CREB), CREB-binding protein (CBP), c-Fos, Nur77, and serum response factor, suggesting that ERK may regulate gene expression indirectly through activation of MAPK-APK-1 (459). Both MAPK-APK-2 and MAPK-APK-3 phosphorylate heat shock protein 27 (hsp27), which may act as an actin capping protein (186)(525). Thus, ERK may regulate cytoskeletal assembly through MAPK-APKs. Cytosolic phospholipase A2 (cPLA2),

upon phosphorylation by ERK (469)(574), becomes activated and is translocated to the plasma membrane (177)(670). ERK also phosphorylates the Ras guanine nucleotide exchange factor, Sos1 (107)(134). Phosphorylation of Sos1 leads to destabilization of interactions among Sos1-EGFR, Sos1-Grb2, and Sos1-Shc, downregulating Ras-Raf activation (665). Thus ERK may negatively regulate its own activity through phosphorylation of Sos1.

JNK/SAPK pathways

Ten JNK isoforms encoded by three genes (*jnk1*, 2, 3) have been identified (271)(358). Stress-related stimuli do not alter expression levels of JNKs but rather activate existing JNK through dual phosphorylation of Thr and Tyr in the Thr-Pro-Tyr (T-P-Y) motif (156)(436). Two direct upstream kinases for JNKs are: MKK4 (also known as SEK1, JNKK1) that also activates p38 (157)(467)(680) and MKK7 (JNKK2) that has been shown to exclusively activate JNK (765). Activated JNK/SAPKs phosphorylate a variety of transcription factors including c-Jun, leading to transcriptional activation through interactions with c-Jun responsive DNA elements such as TPA-response element (TRE) (459). In addition to responding to extracellular stimuli, the JNK/SAPK pathway is also activated by intracellular stresses including inhibition of protein synthesis, treatment with antimetabolites, DNA damage (105)(393)(435), or microtubule dysfunction induced by chemotherapeutic agents (803).

p38 pathways

Four forms of p38 MAPK (p38 α , p38 β , p38 γ , p38 δ) have been identified (Table 15) (132)(251)(372). Mxi2, a C-terminal truncation of p38 α resulting from alternative splicing of p38 α , was discovered by its interaction with Myc and Max (882). p38 MAPKs are activated by MKK3, MKK4 (JNKK/SEK1), and MKK6 through phosphorylation of Thr and Tyr on the Thr-Gly-Tyr (T-G-Y) motif (Table 15)(Table 16).

Substrates for p38 include transcription factors (ATF2/CRE-BP2, CHOP/Gadd153, Elk-1, MEF2s) and signaling proteins (MAPK-APK-2, MAPK-APK-3, cPLA2) and MBP has been used as substrate in immunocomplex kinase assay for p38. Both p38 and JNK (54)(272) phosphorylate the bZIP transcription factor ATF2, enhancing its transcriptional activity. The C/EBP family member CHOP/Gadd153, upon phosphorylation by p38, inhibits adipocyte differentiation through heterodimerization with other C/EBP family members (806). Members of the myocyte-enhancer factor 2 (MEF2) transcription factor, such as MEF-2A (890) and MEF-2C (285), are also substrates for p38. p38 also phosphorylates Elk-1 (624), MAPK-APK-2 (187), MAPK-APK-3 (493), and cPLA2 (59)(422).

Activation of p38 is frequently associated with stress-related stimuli and apoptosis, however, conflicting data exist depending on cell types and stimuli. A pro-apoptotic role of p38 has been demonstrated in HeLa cells upon inhibition of basal ERK activity (43), neurons upon withdrawal of trophic factors (335), cultured neutrophils undergoing spontaneous apoptosis (214), cardiomyocytes (807), serum-starved fibroblast and NGF-deprived PC12 pheochromocytoma cells (432)(842), glutamate-induced apoptosis in cerebellar granule cells (400), fibroblasts treated with sodium salicylate (699), and IgM-induced apoptosis of B-lymphocytes (258). A p38-dependent reorganization of actin is further proposed to be required for membrane blebbing during apoptosis (352). On the other hand, p38 has been shown to be required for B-cell proliferation (137), protect cells from apoptosis (576)(654), or not have any effect on apoptosis (679)(804).

Scaffolding proteins maintaining pathway specificity

On the stage of MAPK signaling pathways, the players include at least twelve MAPKs (**Table 15**), seven MAPKKs /MKKs (**Table 16**), and about a dozen of MAPKKKs (Raf, MOS, MEKKs 1-5, TPL2, TAK1, ASK1, MLKs, DLK/MUK) (459). Each MAPK may have multiple, sometimes even overlapping, substrates as exemplified in **Figure 6**, further creating problems of potential cross-regulation between different sets of kinases. To achieve exquisite specificity for an extracellular stimulus reliably activating the proper target, the cells have developed some “custom-designed MAPK cascade regulatory proteins” (180), such as JNK interacting protein-1 (JIP-1) (818), MEK partner 1 (MP1) (690), and potentially MEKK-1 (180)(846).

These scaffolding proteins may form multi-enzyme complexes with a kinase in a signaling cascade, selectively enhancing activation of this pathway and excluding cross-talk with others (180). JIP-1, first characterized as a cytoplasmic inhibitor of the JNK pathway when overexpressed (165), was recently demonstrated to bind JNK, MKK7, and MLK/DLK, channeling signals through the specific set of kinases leading to JNK activation (818). MP1, which has no enzymatic activity, has been shown to scaffold only MKK1 and ERK1, facilitating ERK activation (690). The small, 13.5-kD MP1 may bind MKK1 and ERK1 through a common site and then dimerize (180). The 195-kD MEKK1 phosphorylates MKK1, MKK2, and MKK4 *in vitro*, however, it preferentially activates JNK through MKK4 over ERK activation through MKK1 and MKK2 (544)(847)(855)(880). More interestingly, although MKK4 activates both JNK and p38, MEKK1 is a poor activator of p38 *in vivo*, suggesting a specifically

regulatory role of MEKK1 for JNK (880), perhaps through complex formation with MKK4 and JNK.

SPECIFIC CONSIDERATIONS OF THE JNK/SAPK PATHWAY

Genes, isoforms, and tissue distribution

Three genes (*jnk1*, *jnk2*, *jnk3*) encode at least ten JNK/SAPK isoforms (156)(436). Four isoforms of JNK1 ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$), four isoforms of JNK2 ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$), and two isoforms of JNK3 ($\alpha 1$, $\alpha 2$) result from alternative mRNA splicing (Table 17) (271). Two types of alternative splicing of JNKs have been described (271). Firstly, all three genes can be expressed as 46-kD and 55-kD proteins due to differential processing of the 3' coding region of the corresponding mRNA. Therefore, the JNK isoforms with molecular weight of 46 kD and 55 kD detected by in-gel kinase assays (316) represent mixtures of JNK isoforms: JNK1- $\alpha 1$, JNK1- $\beta 1$, JNK2- $\alpha 1$, JNK2- $\beta 1$, and JNK3- $\alpha 1$ may migrate together in the 46-kD band; while JNK1- $\alpha 2$, JNK1- $\beta 2$, JNK2- $\alpha 2$, JNK2- $\beta 2$, and JNK3- $\alpha 2$ may cluster in the 55-kD band (271). No functional differences between these 46-kD and 55-kD isoforms have been reported (358). The second type of alternative splicing of mRNA of *jnk1* and *jnk2* (but not found in *jnk3*) have functional consequences. This type of alternative splicing involves the mutually exclusive utilization of two exons encoding the kinase subdomains IX and X, and the resulting JNK1 α vs JNK1 β (likewise, JNK2 α vs JNK2 β) exhibit different substrate specificity *in vitro* (Table 17) (271)(358). Expression of *jnk1* and *jnk2* is ubiquitous in many tissues whereas *jnk3* expression is found only in brain, heart, and testis (156)(358)(436)(856).

Regulators of JNK activation

Two direct activators of JNK (Table 16) are MKK4/JNKK/SEK1 (157)(467)(680) and MKK7 (325)(557)(765)(860).

The first characterized MAPKKK is a MEKK family member: MEKK1 was originally identified as a truncated 73-kD protein (439) and later cloned to be a full-length 195-kD protein (848). MEKK1 phosphorylates MKK1, MKK2, and MKK4 *in vitro*, however, it preferentially activates JNK through MKK4 *in vivo* (544)(847)(855)(880). MKK4 also can be activated by MEKK2 (53), MEKK4 (246), and MEKK5/MAPKKK5 (805), but not by MEKK3 (53).

Other MAPKKKs activating MKK4 and subsequently JNK include TPL-2 (678), TAK1 (556)(852), ASK1 (356), and some MLK family members (320)(321). TPL-2 was discovered as an

Table 17
Human JNK isoforms

Genes	Isoform	M. W.	Accession numbers* for nucleotide sequence	Preference for substrate binding
<i>jnk1</i>	JNK1- α 1	46 kD	L26318	c-Jun = ATF2
	JNK1- α 2	55 kD	U34822	
	JNK1- β 1	46 kD	U35004	c-Jun > ATF2
	JNK1- β 2	55 kD	U35005	
<i>jnk2</i>	JNK2- α 1	46 kD	U34821	c-Jun > ATF2
	JNK2- α 2	55 kD	L31951	
	JNK2- β 1	46 kD	U35002	c-Jun < ATF2
	JNK2- β 2	55 kD	U35003	
<i>jnk3</i>	JNK3- α 1	>46 kD	U34820	c-Jun > ATF2
	JNK3- α 2	>55 kD	U34819	

Reference: (271).

* Numbers in the EMBL GenBank and DDBJ Nucleotide Sequence database.

oncogene in rat T-cell lymphoma (609)(610). TGF- β -activating kinase 1 (TAK1) was identified as a kinase upregulated in response to TGF- β and bone morphogenic protein 4 (MBP4) (852). Apoptosis signal-regulating kinase 1 (ASK1), which shares sequence similarity to MEKKs, activates both MKK3 and MKK4, leading to activation of p38 and JNK (356). Through interactions with TRAF2 (581) and with DAXX (93), ASK1 conveys signals from various membrane receptors to the MKK4-JNK signaling cascade. Among the mixed lineage kinase (MLK) family members, MLK2 (168)(321), MLK3 (231)(633) and DLK/MUK (320)(330) activate MKK4.

Further upstream regulators that lead to JNK activation include the Rho family of small GTPases (Rac1, Rac2, Cdc42, RhoA) (136)(543)(753), p21 Ras-activated kinases (PAKs) (26)(218)(889), germinal center kinase (GCK) (618), and hematopoietic progenitor kinase 1 (HPK1) (341)(409). Rac and Cdc42 may interact with PAKs (PAK1, PAK2, PAK3) to activate the JNK pathway (26)(218)(889), whereas JNK activation by RhoA does not involve PAK (753). Instead, RhoA may regulate the JNK pathway through interaction with protein kinase N (PKN) (565). MKK4 also can be activated by some members of the GCK family, such as GCK (618) and HPK1 (341)(409).

Effects of JNK activation

Activation of the JNK pathway has been shown to regulate gene expression, stability of target proteins, and other signal transduction pathways.

Transcriptional activation

Many downstream effectors of JNK are transcription factors including c-Jun, ATF2, ATFa, and Elk-1. Activating protein-1 (AP-1) was originally identified as a DNA binding activity recognizing a DNA element (TGACTCA) found in the enhancer region of the human metallothionein IIA gene and SV40 (208)(449). AP-1 now becomes a collective term for the dimeric transcription factors composed of bZIP (basic region leucine zipper) subunits that bind to a common DNA element, the AP-1 binding site (394). The bZIP proteins (419) include members of the Jun family (c-Jun, JunB, JunD) (154), Fos family (c-Fos, FosB, Fra1, Fra2) (515)(881), ATF family (ATF2, ATF3/LRF1, B-ATF) (169)(340)(394), and many others (C/EBP, CHOP, CREB).

Jun-Fos and Jun-Jun dimers preferentially bind to the DNA sequence TCACTCA that is known as the phorbol 12-O-tetradecanoate-13-acetate-responsive element (TRE), whereas Jun-ATF and ATF-ATF bind to the DNA sequence TGACGTCA that is the cAMP-responsive element (CRE)

(276)(394)(719). Both c-Jun (6)(316) and ATF2 (54)(272)(775) are substrates for JNK, thus JNK regulates AP-1 activity. Studies using *jnk3* *-/-* mice further provide *in vivo* evidence that the JNK signaling pathway regulates AP-1 activity (358)(856). Transcriptional activation by AP-1 is further regulated through interactions with transcriptional coactivators, such as CREB-binding protein (CBP) (21)(28)(390)(433) and Jun activation domain binding protein (JAB1) (119). When phosphorylated by JNK, c-Jun gains enhanced affinity for CBP (28). JAB1 selectively interacts with c-Jun and Jun D but not with JunB, providing a potentially differential transcription activation for distinct genes (119). In summary, AP-1 regulates various biological functions through: (i) different combinations of dimerization forming different AP-1 factors, (ii) phosphoregulation of subunits of AP-1 by specific protein kinases, and (iii) further interactions between AP-1 factors and transcriptional coactivators (393)(394).

Regulation of protein stability

Direct associations between members of phosphorylation/dephosphorylation cascade of the JNK/SAPK pathway and regulation of target protein stability through ubiquitination have been recently established (222). Depending on its activation status, JNK may act oppositely to affect c-Jun stability. One unique feature of JNK is the tight binding of non-activated JNK to its substrates c-Jun, ATF2, p53, and to the nonsubstrate JunB (222). For instance, both the delta region and proline- and glutamine-rich (P-Q) regions collaborate to tether non-activated JNK to c-Jun (517). Binding of JNK, even in the form of a phosphorylation mutant on residues 183 and 185 (221), targets c-Jun to ubiquitination (767). Activated JNK, however, can stimulate c-Jun phosphorylation which prevents its ubiquitination (221)(767).

Inactive JNK also targets ubiquitination of ATF2 and JunB (223). Phosphorylation of ATF2 on Thr₆₉ and Thr₇₁, either by JNK (272) or p38 (632), prevents its ubiquitination. On the other hand, Elk1 is phosphorylated by JNK (90)(819), but JNK neither associates with nor targets Elk1 for ubiquitination (223).

Inactive JNK is able to bind to p53, targeting its ubiquitination (219)(222), whereas activated JNK can phosphorylate p53 and induce conformational changes and dissociation from JNK (5)(219)(541). Ubiquitination of p53 is also regulated by mdm2 (295)(331)(429). JNK-p53 complexes are mainly found in G₀/G₁, whereas mdm2-p53 complexes are identified in S and G₂/M phase of the cell cycle (219). A proposed hypothesis includes that: (i) in nonstressed cells, JNK targets p53 ubiquitination of G₀/G₁, whereas mdm2 targets p53 ubiquitination of S and G₂/M populations; and (ii) when cells are bombarded with a stress such as UV, activated JNK (as well as DNA-PK) phosphorylates p53 and changes its

conformation, which is no longer recognized by mdm2 and cannot be targeted for ubiquitination (222)(220).

Regulation of other signaling proteins

JNK/SAPK phosphorylates MAPK-APK-3 (MAPK-activated protein kinase-3) (493). A substrate for MAPK-APK-3 is heat shock protein 27 (hsp27), which may act as an actin-capping protein (186)(525). Thus, JNK may involve with regulation of cytoskeletal assembly.

APOPTOSIS INDUCTION AND OTHER FUNCTIONS OF JNK/SAPK

Activation of the JNK/SAPK pathway has been frequently demonstrated to be involved in the apoptotic process (**Table 18**). However, the role of JNK activation in apoptosis appear to be cell type-specific and stimulus-specific, since substantial amounts of evidence indicate no role of JNK activation in certain cases of apoptosis (**Table 19**). Furthermore, instead of induction of apoptosis, JNK activation may promote cell survival, cell proliferation, or tissue regeneration (**Table 20**).

JNK/SAPK activation associated with apoptosis

Apoptosis in neuronal cells

The most established pro-apoptotic role of JNK activation is in neuronal apoptosis (11)(85)(108)(203)(292)(311)(322)(391)(453)(481)(495)(715)(842)(856). During the ontogenic development of nervous system, more-than-needed number of cells are originally generated and many original cells die through apoptosis (283)(593). Among original cells, only those which reach out to perfectly connect with appropriate target cells can access sufficient trophic factors to survive, making the final nervous system. It is not surprising to observe that withdrawal of nerve growth factor (NGF) is a consistent stimulus for apoptosis induction in pheochromocytoma PC12 cells and other neuronal cells (**Table 18**). JNK activation is also involved in some receptor-mediated apoptosis after treatment with kainic acid (856) or dopamine (391)(495). On the other hand, acute transient JNK activation may have functions, instead of apoptosis induction, which help in dealing with some non-lethal environmental stresses, such as new environment for mice (849) (**Table 19**).

Apoptosis induced by stress-related stimuli

As the term stress-activated protein kinase (SAPK) indicates, JNK/SAPK responds to various

Table 18

Positive correlations between JNK activation and apoptosis

Cell types	Induction of apoptosis	Year	References
Ovarian cancer BR cells	Paclitaxel	1999	(800)
Cerebellar granular neuron	Suppression of PI3-K activity	1999	(715)
PC12 pheochromocytoma	Withdrawal of trophic factors	1999	(453)
Neonatal sympathetic neurons	NGF withdrawal	1998	(11)
Developing brain neurons	Transient hypoxia	1998	(108)
Bovine aortic endothelial cells	Mannitol (300 mOsm)	1998	(502)
PC12 cells	Dopamine	1998	(391)
Immature thymocytes	Initiation of negative selection	1998	(643)
Rat hippocampal neurons	Polyglutamine-huntingtin	1998	(481)
Cos-7 cells	Overexpression of G α 12 & G α 13	1998	(42)
H9C2 cardiac muscle cells	Oxidative stress (menadione, H ₂ O ₂)	1998	(770)
PC 12 cells	Manganese	1998	(322)
Breast cancer MDA-MB435	Vitamin E succinate	1998	(872)
Jurkat (T-cells)	Dominant active MEKK1	1998	(199)
Prostate cancer DU145	Radiation	1998	(65)
Bladder cancer MGH-U1	Intracellular acidification	1998	(879)
Jurkat cells	Etoposide, teniposide, UV	1998	(395)
Cervical cancer HeLa cells	H ₂ O ₂	1998	(804)
Adult mammalian brain	Ischemia, axotomy	1998	(311)
PC12 cells	Ceramide	1998	(292)
Multiple myeloma cells	IL-6 withdrawal	1998	(844)
Remote myocardium	Induced myocardial infarction	1998	(461)
Rat mesangial cells	TNF- α	1998	(270)
HL-60	Anisomycin	1998	(726)
HL-60, U937, Jurkat	Edelfosine (ET-18-OCH ₃)	1998	(229)
Jurkat cells	UV, γ -irradiation, anisomycin	1998	(198)
Jurkat cells	Fas ligation	1998	(667)
U937, 293 kidney cells	proteasome inhibitors	1998	(531)
Rat mesangial cells	TNF- α with cycloheximide or act-D	1998	(269)
Rat striatal neuron cells	Dopamine	1998	(495)
U937 leukemia cells	Bufalin	1998	(811)
Mouse keratinocyte PAM212	Cisplatin	1998	(681)
Jurkat, HeLa, 293 cells	Isothiocyanates (PMITC, PEITC)	1998	(104)
Hippocampal neurons	Kainic acid (glutamate-R agonist)	1997	(856)
Jurkat cells	Dominant active GTPase Cdc42	1997	(117)
PEER (T-cells)	Heat	1997	(560)
Jurkat cells	Fas ligation, synthetic C6-ceramide	1997	(69)
Heart, kidney	Ischemia-reperfusion	1997	(863)
Madin-Darby canine kidney	Loss of integrin-ECM contacts	1997	(84)
HeLa, 293, L929 cells	Overexpression of DAXX	1997	(859)
Cultured synovial cells	Fas ligation with anti-Fas	1997	(588)
Murine mast cells	Bruton's tyrosine kinase-ind. apopt.	1997	(399)
Developing rat brain	Injection of botenic acid	1997	(203)
Small cell lung cancer cells	UV radiation	1997	(74)
293, Jurkat, Mv1Lu cells	Overexpression of ASK1	1997	(356)
Prostate cancer cells	Androgen ablation	1997	(498)
Jurkat, 293 cells	UV, γ -radiation, anti-Fas	1996	(105)(103)
Epithelial cells	Detachment from matrix	1996	(217)
T-cells, B-cells	Fas ligation	1996	(76)
Oligodendrocytes	NGF binding to its receptor p75	1996	(85)
H9 T-cell leukemia line	Adriamycin	1996	(871)
T-lymphocytes	Fas ligation	1996	(824)(442)
U937 (monoblastic leukemia), BAE (bovine aortic EC)	Ceramide	1996	(782)
PC-12 cells	NGF withdrawal	1995	(842)

Table 19**No correlation between JNK activation and apoptosis**

Cell Types	Apoptotic stimuli or Diseases	Year	References
LNCap prostate cancer	Protease inhibitors	1998	(313)
Breast cancer MCF7 cells	Ceramide pathway in TNF-mediated apoptosis	1998	(16)
Mouse Hepa1C hepatoma cells	Benzo(a)pyrene	1998	(450)
Rat cardiac myocytes	Staurosporine	1998	(875)
Madin-Darby canine kidney (MDCK) cells	Detachment of epithelial cells (anoikis)	1997	(407)
Motor neurons	Amyotrophic lateral sclerosis (ALS)	1997	(533)
JNK activation in mouse brain	Environmental stimuli that do not induce apoptosis	1997	(849)
Multiple myeloma cells	Dexamethasone	1997	(97)
Syk-, Lyn-deficient chicken B-cell line	Osmotic stress	1997	(628)
293 (human kidney) cells	TNF-R1-mediated apoptosis	1997	(572)
Jurkat cells	Fas ligation-induced apoptosis	1997	(452)
MCF7, HeLa, 293 cells	TNF-R1-mediated apoptosis	1996	(483)

Table 20**Functions of JNK activation other than induction of apoptosis**

Cell Types	Observed functions of JNK	Year	References
Mouse fibroblasts	Protect cells from UV-induced apoptosis	1999	(825)
Bone marrow-derived mast (BMMC) cells	Rac1/JNK pathway is required for Kit ligand-induced proliferation of mast cells	1998	(760)
Adult rat hepatocytes	Required for liver regeneration after partial hepatectomy	1998	(70)
T-cells	Required for T-cell stimulation	1998	(580)
L929, KYM-1 cells	Early transient JNK activation protects cells from TNF-induced apoptosis	1998	(654)
NIH3T3 fibroblasts	Constitutive activation of JNK is required for cell transformation by the constitutively active EGFR variant typeIII	1998	(19)
BAF3 pre-B cells (IL-3-dependent)	Required for cell proliferation of BAF3 cells	1997	(720)
TRAF2 ^{-/-} cells	Deficiency of JNK activation is correlated with enhanced TNF-induced apoptosis (JNK promotes survival)	1997	(861)
T-lymphocytes	TRAF2/JNK pathway promotes survival	1997	(448)
T-lymphocytes	SEK1/JNK pathway protects T-cells from Fas- and CD3-mediated apoptosis	1997	(579)

stresses and may lead to apoptosis (65)(74)(198)(395)(531)(726). These stresses include: UV, γ -irradiation (65)(105), heat (560), oxidative stresses (H_2O_2 , menadione) (770)(804), osmotic stress (502), hypoxia (108), ischemia-reperfusion (461)(311)(863), intracellular acidification (879), and antimetabolites (anisomycin, proteasome inhibitors). Involvement of JNK in osmotic stress-induced apoptosis may be cell type-specific. For example, JNK activation was observed in the apoptosis of bovine aortic endothelial cells induced by hyperosmotic mannitol (300 mOsm) (502); whereas the osmotic stress-induced apoptosis in chicken B cell lines (DT40 cells) was noted to be independent of JNK activation (628).

Apoptosis induced by lack of survival signals

In addition to the most extensively demonstrated example of neuronal apoptosis induced by withdrawal of NGF (11)(453)(842), JNK activation is involved in apoptosis induced by withdrawal of trophic factors in many other systems. For instance, androgen ablation induces apoptosis in prostate cancer cells (498) and IL-6 withdrawal causes apoptosis of multiple myeloma cells (844). Survival signals can also be provided by contacts between integrins and extracellular matrix (ECM). Loss of contacts between integrins and ECM may result in "anoikis", a type of apoptosis in epithelial cells (84)(217). JNK activation has been implicated in these two reports (84)(217), although a conflicting report exists. Using the same Madin-Darby canine kidney (MDCK) cells as Cardone *et al* did (84), Khwaja and Downward observed that (i) dn JNKK inhibited JNK activation without protection from anoikis, and (ii) caspase inhibitors prevented anoikis without affecting JNK activation induced by detachment of MDCK cells (407).

Apoptosis induced by cancer therapies

JNK activation has been involved in apoptosis of cancer cells induced by treatments such as adriamycin (871), γ -radiation (65)(105)(198), isothiocyanates (104), cisplatin (681), bufalin (811), edelfosin (ET-18-OCH₃) (229), etoposide and teniposide (395), vitamin E succinate (872), and paclitaxel (Taxol®) (800).

Fas-mediated apoptosis

Involvement of JNK in Fas-mediated apoptosis of lymphocytes and cultured synovial cells has been indicated in several studies (69)(76)(103)(442)(588)(667)(824). However, JNK activation may be a result, rather than a cause, of Fas-mediated apoptosis (452). JNK activation following Fas ligation requires activated caspases (76)(766) and caspase-dependent cleavages of p21-activated kinase (PAK)

(667)(794) and MEKK-1 (84)(147), two regulators of the JNK pathway, have been demonstrated in Fas-mediated apoptosis. In summary, the early activation of caspases in the Fas-initiated apoptotic pathway can account for both apoptotic characteristics and JNK activation.

While JNK activation appears to be a consequence in Fas-mediated apoptosis, in other circumstances, activated MEKK-1 and/or JNK may upregulate expression of Fas ligand (FasL), resulting in apoptosis (198)(199)(311)(395)(453). Upregulation of FasL expression in apoptosis induced by DNA damaging agents was noted to require activation of AP-1 (395) and one AP-1 complex (ATF2 and c-Jun heterodimer) has been demonstrated to bind to a novel MEKK-1-regulated response element in the FasL promoter (199). Thus, MEKK-1 and the transcription factors regulated by JNK, at least partly, promote apoptosis through upregulation of FasL.

Other functions of JNK/SAPK activation (Table 20)

Depending on the types of cells and stimuli, activation of the JNK pathway may be associated with: (i) protection of cells from apoptosis (448)(579)(654)(825)(861), (ii) cell proliferation (720)(760), (iii) T-cell stimulation (580), (iv) cell transformation (19), and (v) liver regeneration after partial hepatectomy (70).

PACLITAXEL-INDUCED APOPTOSIS

DEVELOPMENT AND PRODUCTION OF PACLITAXEL (TAXOL®)

Historical developments of paclitaxel (PTX) discovery are summarized in **Table 21**. Despite the demonstrations of PTX extraction from cultured plant tissues in 1992 (204) and the total synthesis of PTX in 1994 (578) (**Table 21**), neither is yet economically practical for PTX production at a commercial level. On the other hand, the yield of PTX extracted from the Pacific yew bark is extremely low in the range of 0.014-0.017% (784). An alternative, semisynthetic production of PTX from the more abundant precursor taxanes present in yews has been developed (608).

A complex mixture of taxanes (PTX, 10-deacetyltaxol, cephalomannine, baccatin III, 10-deacetylbaccatin III, taxol C) at various levels (608) can be extracted from many species of *Taxus* plants all over the world (**Table 22**). The most valuable precursor materials for semisynthesis are the taxane

Table 21

Historical development of paclitaxel discovery

Year	Key development
1955	NCI started planning for the Cancer Chemotherapy National Service Center to facilitate discovery of new anti-cancer drugs.
1962	Specimens of the Pacific yew (<i>Taxus brevifolia</i>) were collected by a USDA team from the Gifford Pinchot National Forest of Washington state.
1966	Paclitaxel, the substance with anti-tumor activity against nasopharyngeal KB cells, was purified from the bark of <i>T. brevifolia</i> .
1971	The molecular formula for paclitaxel was determined to be $C_{47}H_{51}NO_{14}$.
1974	Intraperitoneal (i.p.) injection with paclitaxel increased life span of mice i.p. implanted with melanoma B16 cells.
1976	NCI developed the human tumor xenograft model in the nude mice for screening of anti-tumor drugs. Paclitaxel caused regression of breast cancer xenograft and growth inhibition of lung and colon cancers.
1980	Paclitaxel was demonstrated to interfere with microtubule function through binding to microtubule polymer.
1980	Paclitaxel was reported to be present in other yews: <i>T. baccata</i> and <i>T. cuspidata</i> .
1982	Investigational New Drug (IND) application of paclitaxel was filed to the Food and Drug Administration (FDA).
1984	FDA approved paclitaxel for clinical trials.
1989	The Johns Hopkins group reported that paclitaxel was effective in treatment of refractory ovarian cancer.
1991	Bristol-Myers Squibb Company won the Cooperative Research and Development Agreement (CRADA) and was responsible for increasing the production of paclitaxel from bark and for developing an alternative supply of paclitaxel.
1992	The first report of paclitaxel production from plant cell cultures of <i>T. cuspidata</i> and <i>T. canadensis</i> .
1992	Taxol® (paclitaxel) was approved for marketing for the treatment of refractory ovarian cancer.
1994	The first report of total synthesis of paclitaxel.

References: (204)(522)(578)(608)(694)(693)(809)

Table 22
Paclitaxel-producing yews

Common name	Scientific name	Geographic distribution
Pacific yew	<i>Taxus brevifolia</i>	Western parts of Canada and the United States.
Mexican yew	<i>Taxus globosa</i>	Mexico, El Salvador, Honduras, and Guatemala.
Florida yew	<i>Taxus floridana</i>	Northern Florida.
Canadian yew	<i>Taxus canadensis</i>	Canada: from Newfoundland to Manitoba; United States: from Iowa to North Carolina.
English yew	<i>Taxus baccata</i>	Europe and north Africa.
Japanese yew	<i>Taxus cuspidata</i>	Eastern Asia (China, Japan, Korea, and parts of Russia)
Himalayan yew	<i>Taxus wallichiana</i>	From eastern Afghanistan to Tibet and China.

Reference: (608)

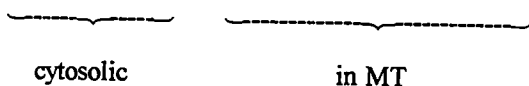
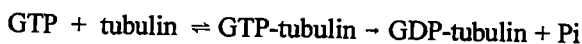
“nucleus” component of baccatin III and 10-deacetylbaccatin-III (10-DAB) (**Figure 14**). Three enzymes, C-7 xylosidase, C-10 deacetylase, and C-13 taxolase, are discovered to be valuable in enrichment of 10-DAB concentrations in the mixture of taxanes (578)(608) and the semisynthetic production of PTX is currently used to supply an increasing demand for PTX (658).

MICROTUBULE FUNCTIONS AND BINDING OF PACLITAXEL

Structure-functional considerations of microtubules

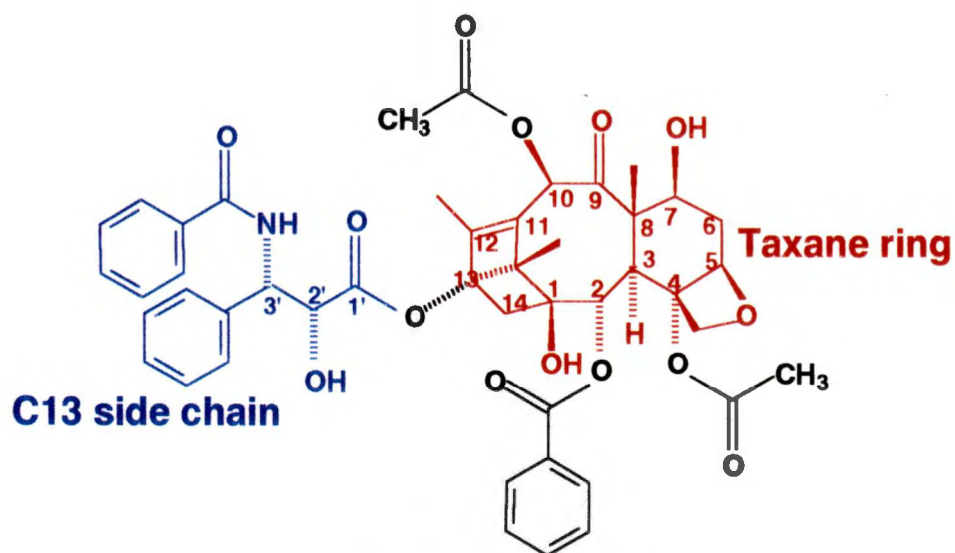
MTs are made of α - and β -tubulin heterodimers of about 110 kD. Both types of tubulin at an 1:1 ratio are arrayed head-to-tail like beads on a string, which is called a protofilament (774). Thirteen protofilaments line up side by side to form a sheet that wraps around its longitudinal axis and form the cylinder-like MT (172). MT can grow by the reversible, non-covalent addition of tubulin at both ends; however, its polarity is created by the alternate stacking of two types of tubulin. The fast-growing “plus” end is ringed by α -tubulin and the slow-growing “minus” end by β -tubulin (**Figure 15**) (812).

GTP binds to both types of tubulin: at the non-exchangeable site (N-site) of α -tubulin and at the exchangeable (E-site) of β -tubulin (159). A tubulin heterodimer can be added to a MT when both α - and β -tubulin have bound GTP. After a tubulin heterodimer is incorporated into a MT, the GTP at the E-site of β -tubulin is hydrolyzed irreversibly to GDP. The bulk of a MT therefore consists mainly of GDP-bound β -tubulin, except both its ends are still capped with GTP-bound subunits. The GTP-bound tubulin caps are believed to be critical for stability of the MT ends (**Figure 15**) (774)(485)(159). Hydrolysis of GTP removes the constraints of a simple equilibrium from MT assembly (812), as shown in the formula:

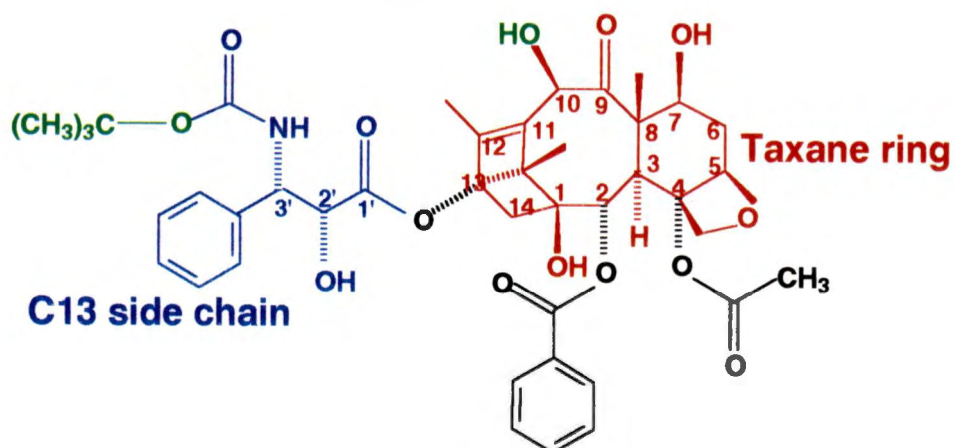


The direct coupling of MT assembly dynamics to GTP hydrolysis further provides the potential for polymerization and depolymerization, allowing the unique assembly behaviors such as “dynamic instability” and “treadmilling” (159).

“Dynamic instability” was proposed in 1984 based on an analysis of the length distributions of fixed MTs (547). According to this model, each MT persists in prolonged states of polymerization and depolymerization that interconvert infrequently, although the whole population of MTs may exhibit a bulk

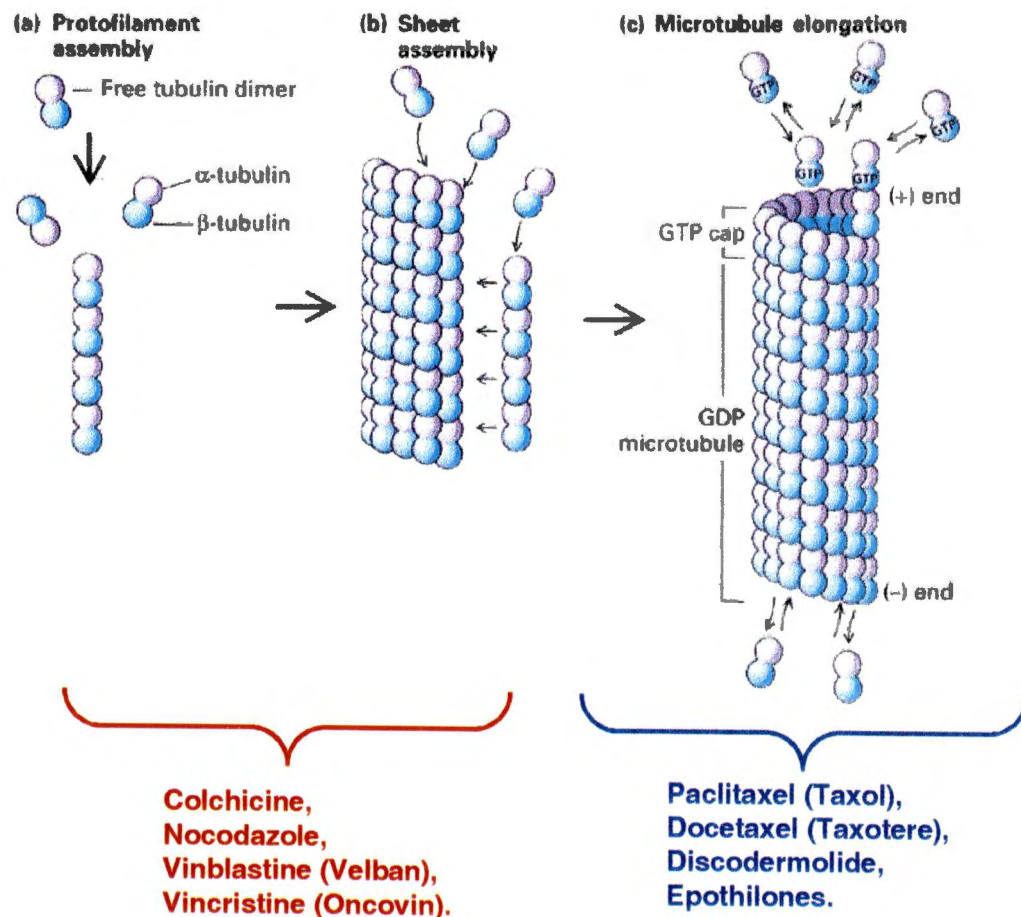


Paclitaxel (Taxol)



Docetaxel (Taxotere)

(Figure 14) Chemical structures of two taxanes. Both the **taxane ring** and the **C13 side chain** are required for anti-tumor activity of taxanes. Structures in **docetaxel** that are different from those in **paclitaxel** are labeled in **green**.



(Figure 15) Microtubule assembly is targeted by various microtubule-active agents. (a) Tubulin heterodimers form protofilament. **(b)** Thirteen protofilaments line up side by side to form a sheet that wraps into the cylinder-like microtubule. **(c)** Microtubule can elongate or shorten at both ends, however, both polymerization and depolymerization are faster at the (+) end than at the (-) end. At saturating concentrations, the microtubule-active agents that preferentially bind to tubulin are labeled in red color, whereas those only bind to microtubules are labeled in blue.

steady state. Several real-time, dark field and Normarski (differential interference contrast) video microscopy studies confirmed the existence of dynamic instability (333)(792). Increasing amount of *in vitro* and *in vivo* evidence has convinced researchers that dynamic instability is the predominant mechanism governing MT assembly (88)(244)(189)(830).

Dynamic instability is characterized by four parameters: polymerization rate, depolymerization rate, the frequency of catastrophe (the transition from polymerization to depolymerization), and the frequency of rescue (transition from depolymerization to polymerization) (159). MT polymerization is dependent on the free tubulin concentration, whereas depolymerization is independent of it. Thus, increasing the tubulin concentration increases the polymerization rate, which may result in a decreased catastrophe frequency. The relationship between polymerization rate and catastrophe frequency, however, is not supported by every study. The catastrophe frequency has been demonstrated to be independent of polymerization rate (585).

Intuitively, dynamic instability seems to require an energy source, which is likely to be GTP hydrolysis by β -tubulin. Studies using nonhydrolyzable GTP analogues, however, revealed that polymerization does not require GTP hydrolysis and that the MT lattice is more stable with a GTP analogue-bound β -tubulin than with GDP-bound subunits (411)(530). Therefore, the primary role of GTP hydrolysis is likely to destabilize the MT lattice through weakened contacts among GDP-bound subunits (80). On the other hand, soluble tubulin undergoes GTP hydrolysis only very slowly; whereas the rate of GTP hydrolysis highly increases when tubulin subunits are incorporated into MTs (141)(82). In this assembly-driven GTP hydrolysis, the formation of inter-subunit contacts during polymerization may enhance tubulins function as a GTPase activating protein (GAP), accelerating GTP hydrolysis by itself (159). Thus, tubulin can be considered as a *cis*-acting factor in the regulation of MT dynamics. At the end of a MT, the ring of newly incorporated tubulins which have not undergone hydrolysis remain stable, acting as a stabilizing cap of MT (547). The GTP-cap hypothesis has been supported by microscopic studies using nonhydrolyzable GTP analogues (81)(175).

MT dynamics are also regulated by several *trans*-acting factors that regulate the catastrophe frequency and the polymerization rate (722). Two catastrophe-promoting factors are Op18/stathmin (17)(38)(488)(764) and XKCM1 (Xenopus kinesin central motor 1) (158)(789). Op18/stathmin has been shown to bind to free tubulin and sequester it into a pool where it cannot polymerize into MTs (38). XKCM1 is a motor protein that binds to an assembled MT and alters its dynamics (789). Both

Op18/stathmin and XKCM1 may also be involved in the regulation of increased MT dynamics during mitosis (353). On the other hand, MT-associated proteins (MAPs) bind to the MT lattice and thereby promote polymerization (503). Phosphorylation of MAPs, which increases during mitosis, reduces their affinity to MTs, perhaps also accounting for increased MT dynamics during mitosis (514)(592).

MTs function both as constitutive cytoskeletal components in the interphase cell and as spindle fibers for chromosome segregation during mitosis. Dynamic instability allows MTs to move through and fill the newly formed regions of cytoplasm, facilitating recruitment of the membrane system to use MT motor proteins (746). Dynamic instability is also the mechanism for a MT to “search and capture” its target (*e.g.* duplicated chromatids) in the three-dimensional space more effectively than equilibrium polymerization (298)(329)(159).

The “treadmilling” model of MT assembly was proposed in 1978 based on the observation of continuous incorporation of tubulin into MT at a steady state when tubulin subunits are constantly added at one end with a balanced loss of subunits at the other end (506). Treadmilling of MTs in a plus-to-minus direction, *i.e.*, tubulin subunits adding to the plus end and dropping from the minus end, was visualized using dark-field microscopy in 1988 (337). Observations of treadmilling have been made both in mitotic cells (813) and in interphase cells (645)(644). By definition, the rates of both assembly and disassembly of MTs are much higher at the plus end than at the minus end; thus, how can a state of treadmilling exist? A “biased dynamic instability” mechanism has been proposed that some factors including MAPs may regulate dynamic instability in an end-specific manner, controlling the rates of assembly and disassembly, thus catastrophe is suppressed at the plus end and rescue is suppressed at the minus end (812).

The most dramatic function of MTs is to equally separate duplicated chromatids into two daughter cells during mitosis. Indeed, MT dynamics are 20- to 100-fold higher in mitotic cells than in interphase cells (380). When cells enter mitosis, the cytoskeletal MT network is dismantled and spindle MTs are oriented with their minus ends at the spindle pole and their plus ends pointing to the midplane of the cells, reaching the kinetochores of chromosomes (35). The source of force required to capture and pull chromosomes is believed to be generated by MT polymerization and depolymerization (87)(160). Nevertheless, forces and movements required for mitosis may also be provided by members of the MT-associated motor family, such as the plus-end-directed kinesins and the minus-end-directed dyneins (35) (722).

Kinetic suppression of microtubule dynamics by microtubule-active agents

Various chemical agents, mostly derived from natural products, bind to tubulin or MTs and inhibit cell division at the metaphase-anaphase transition of mitosis through interference with mitotic spindle functions (380). Many of this group of “MT-active” or “anti-mitotic” agents become valuable chemotherapeutic drugs against cancers. They may fall into one of two groups according to their preferred targets being tubulin (colchicine, vinblastine=Velban®, vincristine=Oncovin®, nocodazole) or MTs (paclitaxel=Taxol®, docetaxel=Taxotere®, discodermolide, epothilones) (**Figure 15**)(48)(380)(421)(694)(752).

Although at high concentrations, these anti-mitotic agents exhibit differential affinities to either tubulin or MTs; studies using lower concentrations revealed that both groups of agents cause a common kinetic suppression of MT dynamics (380). At high concentrations, colchicine (603), vinblastine (163) (604), and nocodazole (778) destabilize MTs; whereas at low concentrations, they suppress MT dynamics without accompanying MT depolymerization. In summary, the kinetic suppression (stabilization) of MT dynamics is a common mechanism for these MT-active agents (380).

Paclitaxel binds to microtubule at β -tubulin

Both the taxane ring and the C-13 side chain are essential for the anti-tumor activity of PTX (**Figure 14**) (809) and docetaxel (Taxotere®) (49), both derived from *Taxus*. Epothilones A and B, natural products from bacterium *Sorangium cellulosum* (56), despite no structural similarity with taxanes, also bind to MTs and induce the formation of hyperstable tubulin polymers (421). This structural differences may explain that epothilones remain cytotoxic on PTX-resistant cells which express multidrug resistance (*mdr*) gene (421).

Six α -tubulin isotypes and six β -tubulin isotypes have been identified in human cells. Expression of these isotypes differs in a tissue-, cell- and tumor-specific manner (380). Differences in the content of β -tubulin isotypes in MTs particularly affect the dynamics and drug binding of MTs (397)(437). For instance, β III and β IV isotypes are associated with PTX resistance, explaining overexpression of these isotypes in taxol-resistant ovarian cancer tissues (397) as well as in cultured leukemia, prostate cancer, and lung cancer cells (366)(397)(634).

Four regions of β -tubulin are important for binding of PTX: (i) a.a. residues 1-36 at the N-terminus and (ii) residues 217-231 at central region (27)(284), both are the binding sites for PTX; (iii) a

surface-exposed, drug-sensitive loop from residues 270 to 290, and (iv) an amphipathic helical region from residues 370-390. Exposure of the 270-290 loop to chymotrypsin cleavage is enhanced by binding of colchicine and vinblastine (672) but is suppressed by PTX (674). Unfolding of the 370-390 helical regions to chymotrypsin cleavage is enhanced by binding of colchicine (673) but is suppressed by vinblastine (672). Sequence analyses of β -tubulin in PTX-resistant ovarian cancer PTX-10 and PTX-22 cells revealed that two a.a. residues Phe₂₇₀ and Ala₃₆₄ are important modulators for PTX's interaction with tubulin, perhaps through regulations of the 270-290 loop sequence and the 370-390 amphipathic helical region (247). Single amino acid mutations of either Phe₂₇₀ or Ala₃₆₄ blocks PTX binding to MTs, resulting in PTX-resistance.

Our further understanding of structural interactions between tubulin and MT-active agents may have clinical importance. For example, combinations of anti-mitotic agents that bind to different sites on tubulin or MTs have been demonstrated to exert additive or synergistic effects against cell growth (234) (605)(616)(723).

CLINICAL USES OF PACLITAXEL

Taxanes (paclitaxel, docetaxel) may have the broadest anti-tumor spectrum among all classes of anticancer agents (658). Both agents have been demonstrated effective as the second-line treatment for patients with advanced stages of refractory cancers. Furthermore, taxanes may have greater activity in early stage diseases, making a greater impact on survival and cure of cancer patients. Current clinical trials are focusing on development of strategies using taxanes as first-line agents in cancer patients.

Paclitaxel-responsive tumor types

PTX (721) is currently approved in the United States as a second-line drug to treat: (i) ovarian cancer after the failure of previous platinum (cisplatin, carboplatin) treatment (78)(275)(522)(660) and (ii) breast cancer after failed treatment with anthracyclines (doxorubicin, epirubicin, daunorubicin, idarubicin) (328)(454)(641). PTX is also active in treatment of lung cancer (91)(192)(568), head and neck cancers (209), bladder cancer, and esophageal cancer (660)(658). On the contrary, it may not be beneficial in treatment of colorectal, renal, prostate, pancreatic, gastric, and brain cancers (658).

Since PTX has been shown to enhance the effects of ionizing radiation in murine mammary

carcinoma (535), HL60 cells (561), laryngeal carcinoma (626), its combined use with radiotherapy in treatment of locally advanced lung cancer (113)(114)(116) may be useful. However, the radiosensitizing effect of PTX appears to be cell type-specific, because it only exhibits minimal effect in cervical cancer cell lines (190).

Adverse effects of paclitaxel

Common adverse effects associated with PTX are (i) major hypersensitivity reactions (HSR), manifested by dyspnea with bronchospasm, urticaria, and hypotension (815)(663)(661), (ii) neutropenia (663)(661), (iii) neurotoxicity, including peripheral sensory loss as well as motor and autonomic dysfunctions (659), and (iv) cardiac effects such as bradycardia, myocardial ischemia, atrial arrhythmia, and ventricular tachycardia (662)(661)(248). Almost major HSR occur within the first 10 min after treatment with the first or second dose of PTX. To prevent HSR, premedication with dexamethasone, diphenhydramine, and one of histamine H₂-antagonists (cimetidine, famotidine, ranitidine) before treatment is recommended (658). The onset of neutropenia is usually during the days 8-10 after administration of PTX and typically recovers by day 15-21 (658). Other side effects include nausea, vomiting, diarrhea, and reversible hair loss (661)(660).

MECHANISMS OF PACLITAXEL-INDUCED CYTOTOXICITY

Potential advantages of using PTX in cancer therapy include: (i) killing cancer cells by PTX does not rely on a functional p53 (149)(197)(777)(829), (ii) PTX even kills cancer cells with p53 mutations more efficiently than those with wt p53 (788), and (iii) it preferentially induces G₂/M arrest and apoptosis in transformed cells but rather induce reversible G₁ arrest in non-transformed cells (768). All of these features of PTX suggest that it may differentially kill cancer cells but spare normal cells.

Apoptosis accounts for paclitaxel-induced apoptosis

Apoptosis was first shown to be a mechanism of PTX-induced cytotoxicity in human lymphoid leukemia cells in 1993 (46). That observation has been supported by numerous similar reports in other leukemia cells (462), ovarian cancer cells (297), gastric cancer cells (95), head and neck tumors (232), prostate tumors (862), adrenocortical carcinoma cells (194), and human glioma cell lines (754). A positive

correlation of cytotoxicity and apoptosis induced by PTX has been confirmed by studies *in vitro* (233) (249) and *in vivo* (542).

Status of p53 and paclitaxel-induced apoptosis

One unique feature of PTX anti-tumor actions is that they do not rely on p53-dependent pathways to initiate apoptosis in treated cancer cells (149)(197)(777)(829). The p53-independent mechanism of Taxol cytotoxicity was clinically demonstrated by the fact that response rate to the treatment with PTX and radiation in cancer patients with p53 mutant was as good as in cancer patients with wt p53 (675). More importantly, loss of normal p53 function, which is found in more than 60% of human cancers, has been shown to confer sensitization to PTX (612)(785)(788)(876).

Several explanations have been proposed for the association between p53 mutations and increased sensitivity to PTX. (i) Lack of p53-dependent G₁ arrest promotes the progression of cells to the G₂/M phase, which will become of the target of mitotic arrest (788). (ii) Upregulation of p21^{WAF1/CIP1} by p53 may facilitate exit from mitotic arrest (29). (iii) MT-associated protein 4 (MAP4), which is downregulated by activation of wt p53 (567), may play a role in MT stabilization (886). The levels of MAP4 may increase in the absence of functional p53, stabilizing MTs and sensitizing cells to PTX treatment.

Bcl-2 family members and paclitaxel-induced apoptosis

Like apoptosis initiated by other stimuli, PTX-induced apoptosis is also regulated by members of the Bcl-2 family (Figure 7). Apoptosis induced by PTX has been shown to be inhibited by overexpression of Bcl-2 (747)(346)(241) or Bcl-X_L (475), and to be enhanced by Bcl-X_s (736), Bak (717), Bad (733), or Bax (732)(731).

PTX treatment itself may be able to modulate expression of the Bcl-2 family members and may also cause post-translational modification of Bcl-2 molecules. PTX has been shown to downregulate Bcl-X_L expression or upregulate Bak, depending on the cell type (477). Upregulation of proapoptotic Bcl-2 family members by PTX has been demonstrated in Bak (376) and Bax (725). PTX has been shown to induce Bcl-2 phosphorylation, which has been proposed to promote PTX-initiated apoptosis (51)(281) (279)(652). Raf-1 has been proposed to be required for the PTX-induced Bcl-2 phosphorylation (51)(52), however, contrary data exist (355). On the other hand, recent reports demonstrate that phosphorylation of

Bcl-2 occurs only in cells blocked at the G₂/M phase after PTX treatment (279)(689), arguing that Bcl-2 phosphorylation is a mere consequence of mitotic arrest. Furthermore, since the role of Bcl-2 phosphorylation in promoting (51)(92)(99)(281)(652) or inhibiting apoptosis (334)(360) remains controversial; its role in regulating apoptosis initiated by PTX remains to be clarified.

Apoptosis following the cell cycle arrest

Mitotic arrest of PTX-treated cells has been associated with apoptosis (379)(486)(829), however, the biochemical events downstream of kinetic stabilization of MT dynamics which lead to apoptosis remain largely unclear (380). Furthermore, substantial evidence indicates that G₂/M arrest may not be the only mechanism to induce apoptosis (440)(534)(542)(553); additional phosphoregulatory pathways may be involved in inducing apoptosis (51)(281)(619)(827). Interestingly, PTX has been shown to activate ERK1/2 and this may, in turn, accelerate progression of the cell cycle, further facilitating the eventual G₂/M arrest (466).

PTX-induced apoptosis can occur either directly from mitotic arrest (829) or following aberrant mitotic exit into a G₁-like “multinucleate state” (379)(468)(829). In addition to cell type specificity (768), the fate of cells following PTX treatment depends on: (i) the concentration of PTX used (378)(465) and (ii) the duration of exposure (465). For instance, a short (1 h) exposure of HL-60 cells to a low concentration (20 nM) of PTX may induce a reversible mitotic block without apoptosis, whereas either 60 nM PTX for 1 h or 10 nM for 12 h induces apoptosis (465).

The spindle assembly checkpoint is also involved in regulation of PTX-initiated apoptosis (722). Several mammalian gene products have been identified to function as the spindle assembly checkpoint, such as *h*sMAD2 (463)(464)(722), BUB1 (750), BUB3 (722)(749) (these three are kinetochore-localized), PP2A, and MAPK (722). Protracted activation of these gene products may be involved in apoptosis followed by mitotic arrest. For instance, overexpression of AV BUB1 proteins in HeLa cells reduces the severity of nocodazole-induced apoptosis (722)(750). The mechanism by which the spindle assembly checkpoint initiates apoptosis, however, remains unclear.

Apoptosis by aberrant activation of cyclin-dependent kinases (CDKs)

PTX-initiated apoptosis has been associated with activation of p34^{cdc2} (167)(345)(354)(707), Cdc2-like kinase (689), and other CDKs (226)(529)(619). Recent studies suggest that catastrophically

activated Cdk2 may be terminal effectors in the apoptotic pathway (293)(458), further strengthening the close relationships between PTX-induced p34^{cdc2} activation and apoptosis. However, whether the activated Cdc2 is the cause (870) or the consequence (471) of mitotic arrest remains unclear. Alternatively, both Cdc2 activation and mitotic arrest may be two parallel consequences of MT dynamic suppression (167) (470).

Activated cyclin B1/Cdc2 has also been suggested to be responsible for Bcl-2 phosphorylation of mitotic cells (471). The p53-upregulated p21^{WAF1/CIP1} has been shown to associate with Cdc2 and to inhibit its activity (29)(870), thereby helping cells survive PTX-induced apoptosis.

Paclitaxel may activate the release of cytotoxic cytokines

Treatment with a high concentration (30 μ M) of PTX has been shown to stimulate local release of TNF- α , a cytokine frequently associated with apoptosis, from murine macrophage (440). Despite its unlikely clinical relevance due to the supra-pharmacological concentration of PTX required, this observation still strengthens the notion that PTX has multiple effects on the cell. PTX has also been shown to transcriptionally activate IL-8 expression in ovarian cancer cells (447) and the AP-1 as well as NF- κ B sites of the IL-8 promoter are required for activation by PTX (446).

Other mechanisms of paclitaxel-induced apoptosis

PTX has been shown to regulate apoptosis at the transcriptional level, such as upregulation of c-Mos (472), cyclin B1 (472)(470), and the pro-apoptotic Bak (477)(376) and Bax (725) and downregulation of the anti-apoptotic Bcl-X_L (477). It upregulates the gene *krox-24*, whose gene product exhibits tumor suppressor activity, and several enzymes (2'5'-oligoadenylate synthase, cyclooxygenase-2, and an I κ B kinase termed *chuk*) that is involved in regulation of apoptosis (553).

RESISTANCE AND ENHANCEMENT OF PACLITAXEL-INDUCED APOPTOSIS

Mechanisms of resistance to paclitaxel treatment

In vitro studies have demonstrated that cancer cells become resistant to PTX through a variety of mechanisms: (i) increased drug efflux resulting from upregulation of either *mdr-1* gene and the subsequent increased expression of phosphoglycoproteins (45)(346) or some other membrane transporter

(731); (ii) amino acid mutation in β -tubulin that abolishes PTX binding (247); (iii) differential expression of various tubulin isotypes (397); (iv) the presence of defective caspases, such as in prostate cancer PC-3 cells (606) and breast cancer MCF-7 cells (367); and (v) upregulation of the anti-apoptotic Bcl-2 family members such as Bcl-2 (241) and Bcl-X_L (475), and/or downregulation of the pro-apoptotic Bax (347).

Enhancement of paclitaxel-induced apoptosis by adjuvant treatments

Various adjuvant treatments have been tested for enhancement of the taxanes-triggered apoptosis in cancer cells. (i) Verapamil, a membrane channel blocker, is able to reverse PTX resistance due to overexpression of the *mdr-1* gene (45). (ii) Tamoxifen has been demonstrated to act synergistically with docetaxel against breast cancer cells (202). (iii) An inhibitor of cytoplasmic phospholipase A2, quinacrine, has been shown to exhibit a synergistic effect with PTX against some hormone-refractory prostate cancer cells (146). (iv) TNF- α has been shown to potentiate the anti-tumor effects of PTX in nude mice bearing human breast, ovarian, and liver tumors (703). (v) Farnesyl transferase inhibitors (FTI) have been shown to act synergistically with PTX to inhibit cell growth. FTI causes increased sensitivity to induction of metaphase block by MT-active agents, suggesting that a farnesylated protein may regulate the mitotic check point (551). (vi) A macrocyclic lactone protein kinase C (PKC) activator, bryostatin 1, has been observed to enhance apoptosis of leukemia cells induced by PTX (799). (vii) An inhibitor of phosphatidylinositol synthesis, inostamycin, reduces the dosage of PTX required for apoptosis induction in small cell lung cancer cells (716). (viii) Expression of the pro-apoptotic BAD has been shown to reverse PTX resistance in ovarian cancer cells, suggesting that small molecules which mimic the effects of BAD may become potential adjuvant drugs for PTX treatment (733).

PTX has been found to be a potential radiosensitizing agent that augments the efficiency of radiotherapy (761)(242). For patients with locally advanced unresectable non-small cell lung cancer, the combined treatment with PTX and radiation has demonstrated an overall response rate of 77% (115). Multiple mechanisms may be involved in the radiosensitizing effects of PTX, including G₂/M arrest of the cell cycle (761) and tumor reoxygenation (535)(536). Cells in the G₂ and M phases are known to be the most sensitive to ionizing radiation (761), explaining the radiosensitization of cancer cells by PTX. On the other hand, the greatest augmentation of the radiation response was noted to not be at the time of highest mitotic arrest but at one day after PTX treatment (535), suggesting involvement of other mechanisms such as reoxygenation of hypoxic tumor cells (536).

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PART II.

RATIONALE AND OBJECTIVES

RATIONALE

1. Paclitaxel is a promising chemotherapeutic drug against both advanced stage ovarian and breast cancers. Further understanding the mechanisms by which it kills cancer cells should help us develop more effective strategies to treat patients with these two gynecological cancers.
2. The most extensively studied target of paclitaxel is the microtubule and suppression of microtubule dynamics is believed to be the mechanism by which paclitaxel exerts its effects on the cell. For instance, through disruption of functional microtubule spindles, paclitaxel arrests mitotic cells at the transition from metaphase to anaphase.
3. In addition to serving as a mechanical device to separate duplicated chromatids in mitotic cells and as a major cytoskeletal component in interphase cells, microtubules may have other physiological functions that require such complex exercises as dynamic instability and treadmilling.
4. Due to suppression of microtubule dynamics, paclitaxel-stabilized microtubules become rigid and dysfunctional. Through its binding to microtubules, paclitaxel may initiate a unique type of intracellular stress.
5. Indeed, I have observed that paclitaxel activates JNK/SAPK, which is a recently identified stress-related kinase. However, it remains unknown: (i) how paclitaxel activates the JNK pathway, and (ii) what is/are the function(s) of paclitaxel-activated JNK.

OBJECTIVES

1. I would like to characterize the pattern of paclitaxel-induced JNK activation, such as the cell type specificity, the dose-effect relationship, and the time course.
2. I want to determine whether activation of JNK is a consequence of paclitaxel binding to microtubules or is independent of microtubular interactions.
3. The signal transduction pathway(s) utilized by the paclitaxel-treated cells leading to JNK activation need to be identified.
4. Since paclitaxel kills cells through apoptosis and JNK may be involved in apoptosis, I would like to elucidate the role of JNK activation in the paclitaxel-induced apoptosis.

PART III.

MICROTUBULE-INTERFERING AGENTS ACTIVATE C-JUN N-TERMINAL KINASE/STRESS-ACTIVATED PROTEIN KINASE (JNK/SAPK) THROUGH BOTH RAS AND APOPTOSIS SIGNAL- REGULATING KINASE (ASK1) PATHWAYS

ABSTRACT

The essential cellular functions associated with microtubules (MTs) have led to a wide use of MT-interfering agents in cancer chemotherapy with promising results. Although the most well-studied effect of MT-interfering agents is an arrest of cells at the G₂/M phase of the cell cycle, other effects may also exist. I have observed that paclitaxel (Taxol), docetaxel (Taxotere), vinblastine, vincristine, nocodazole and colchicine activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway in a variety of human cells. Activation of JNK/SAPK by MT-interfering agents is dose-dependent and time-dependent and requires interactions with MTs. Functional activation of the JNKK/SEK1-JNK/SAPK-c Jun cascade was demonstrated by cotransfection with a TPA-response element (TRE) reporter construct and dominant negative signal transducers followed by chloramphenicol acetyl-transferase (CAT) assays. MT-interfering agents also activate both Ras and apoptosis signal-regulating kinase (ASK1), and coexpression of dn Ras and dn ASK1 exerted individual and additive inhibition of JNK/SAPK activation by MT-interfering agents. These findings suggest that multiple signal transduction pathways are involved with cellular detection of microtubular disarray and subsequent activation of JNK/SAPK.

INTRODUCTION

c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), are involved in a signal transduction pathway parallel to that of mitogen-activated protein kinases (MAPKs) (15)(16)(28)(31)(38)(66). This highly conserved cascade is responsive to stress-related stimuli such as UV irradiation, ionizing radiation, ischemia and reperfusion, and inflammatory cytokines, eliciting phosphorylation and activation of JNK/SAPKs (32)(33)(37)(45)(51)(65). Activated JNK/SAPKs phosphorylate a variety of transcription factors including c-Jun, leading to transcriptional activation through interactions with c-Jun responsive DNA elements such as TPA-response element (TRE). In addition to responding to extracellular stimuli (10), the JNK/SAPK pathway is also activated by intracellular stress including inhibition of protein synthesis, treatment with antimetabolites, or DNA damage (9)(32)(37). No association has been shown, however, between MT disruption and JNK/SAPK activation.

MT-interfering agents (MIAs) utilized in the present study include paclitaxel (PTX), docetaxel, vinblastine (VBL), vincristine, nocodazole and colchicine. Through differential binding to MT polymers (PTX, docetaxel) or tubulin monomer and dimers (VBL, vincristine, nocodazole, colchicine), MIAs interfere with the dynamic process of MT assembly (63). Effects of MIAs include an arrest of cells at the G₂/M phase of the cell cycle and initiation of apoptosis (4)(25)(34)(41)(60)(61). It has been proposed, however, that G₂/M arrest may not be sufficient to induce apoptosis; other phosphoregulatory pathways may also be required (3)(22)(60). On the other hand, evidence is also accumulating to indicate that JNK/SAPK activation may regulate the cell cycle (51)(56) and apoptosis (13)(33)(55)(67).

Apoptosis signal-regulating kinase (ASK1) is a recently characterized mitogen-activated protein kinase kinase kinase (MAPK-KK) (26). Overexpression of ASK1 induced apoptosis in mink lung epithelial cells, and ASK1 was activated in cells treated with tumor necrosis factor- α (TNF- α) (26). These findings suggest that ASK1 may have a role in stress- and cytokine-induced apoptosis. Here I report that MT-interfering agents activate JNK/SAPK through signal transduction by both Ras and ASK1, indicating that multiple signal transduction pathways may be required for this type of cellular stress response. These results, for the first time, demonstrate the roles of Ras, ASK1, and JNK/SAPK in signal transduction pathways initiated by microtubular disarray.

EXPERIMENTAL PROCEDURES

CELL CULTURE

Human fibroblasts (CRL1502), breast cancer cells MCF-7 and T47D, choriocarcinoma JEG-3, and osteosarcoma SAOS-2 were obtained from ATCC (Rockville, MD). Epithelial ovarian cancer cells BR and 67R (7)(64), ovarian cancer cells 1A9 and its tubulin-mutant, PTX-resistant derived cells, PTX10 and PTX22 (2)(18), and isolation of primary trophoblast from term placentae (57) were described previously. All cell lines were cultured in DMEM/F12 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin except that primary trophoblasts were cultured in DMEM-HG (Sigma) supplemented with 20% FBS. Both PTX10 and PTX22 cells were maintained in 15 ng/ml PTX and 5 μ g/ml verapamil continuously but were cultured in drug-free medium for 5 days prior to each experiment.

CHEMICALS AND CELL TREATMENT

Unless noted, all chemicals were purchased from Sigma. Docetaxel (Taxotere) was kindly provided by S.A. Coughlin (Rhone-Poulenc Pharmaceutic Inc., Collegeville, PA), and lovastatin was a gift from W.L. Henckler (Merck and Co., Rahway, NJ). All stock solutions of MIAs were prepared with DMSO at a concentration of 10 mM except that colchicine (10 mM) was dissolved in absolute ethanol and bacterial lipopolysaccharide (2 mg/ml) was dissolved in water. Lovastatin (10 mM) was prepared with 10% ethanol (36). Cell treatments were performed in serum-containing culture medium when cells were approximately 80% confluent. As reviewed by Rowinsky (53), peak plasma concentrations of PTX in patients are 0.21 to 13.0 μM . Thus, we treated cells with 1 μM PTX in most experiments. For comparison, other MIAs were used at similar concentrations. UV irradiation was performed by exposing cells to a germicidal ultraviolet lamp (254 nm, 38W, 76 cm distance between plates and the UV lamp) in a tissue culture hood for 2 min. The UV dose was approximately 40 J/m² (20). Cells were then incubated at 37°C in 5% CO₂ for 1 h before preparing cell lysates.

IMMUNOCOMPLEX KINASE ACTIVITY ASSAYS

Anti-JNK1 or anti-MAPK (ERK 2) antibodies, purified GST-c-Jun (1-79 a.a.), and protein A-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The procedure for the immunocomplex kinase assay of JNK was modified from Derijard *et al.* (16). About 100 μg of lysate prepared with lysis buffer (20 mM Tris pH 7.4, 200 mM NaCl, 0.1 % NP-40, 1 mM phenylmethylsulfonylfluoride, 1 mM Na₃VO₄, and 10 mM NaF) was immunoprecipitated with antibody excess and protein A-agarose beads at 4°C overnight, and agarose beads were washed with lysis buffer and kinase buffer (25 mM HEPES pH 7.5, 25 mM of MgCl₂, and 25 mM β -glycerophosphate). The kinase reactions for JNK/SAPK were performed by incubating immunoprecipitated proteins with kinase mixture (1 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 μM ATP, 5 μCi ³²P- γ ATP, and 0.2 μg of GST-c-Jun in kinase buffer) at room temperature for 30 min. The procedure for the immunocomplex MAPK assay was identical to the JNK/SAPK assay except an anti-ERK 2 Ab was used for immunoprecipitation and myelin basic protein (Upstate Biotechnology, Lake Placid, NY) was the substrate. Laemmli's loading buffer was added to stop the reaction and samples were resolved on SDS-PAGE. GST-c-Jun or MBP bands on

autoradiograms were analyzed with a video densitometer Lynx4000 (Applied Imaging, Santa Clara, CA).

WESTERN BLOTTING ANALYSIS

Aliquots of cell lysates resolved on SDS-PAGE, were transferred to nitrocellulose membranes, and probed with antibodies as specified followed by second antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). After washing, proteins were detected by enhanced chemiluminescence (Pierce, Rockville, IL).

PLASMID CONSTRUCTS AND TRANSFECTION

A reporter construct for the TPA-response element, p(TRE)_{x5}-TK-CAT (12) was from Z. Culig. An expression vector for β -galactosidase (pCMV-lacZ), hemagglutinin (HA)-epitope tagged expression vectors pSR α -HA-JNK1 and pSR α -HA-ERK2 (39), and dominant-negative (dn) expression vectors pSR α -dn Ras (17N) and pSR α -dn Rac (17N) (39) were from M. Karin. An expression vector for dn c-Jun (pCMV-TAM67) was from M. Birrer (49). Dominant-negative expression vectors for JNK/SAPK (pSR α -APF) and for JNKK/SEK1 (pSR α -K116R) were from G.L. Johnson (29). Expression vectors for wild type ASK1 (pcDNA3-ASK1-HA) and dn ASK1 (pcDNA3-dn ASK1-HA) were described recently (26). Liposome-mediated transfections were performed by using LipofectAmine (GibcoBRL, Gaithersburg, MD) on MCF-7 cells, and using Tfx-50 (Promega, Madison, WI) on BR cells.

CHLORAMPHENICOL ACETYL-TRANSFERASE (CAT) ASSAY

AND STATISTICAL ANALYSES

Cells in six-well plates were cotransfected with p(TRE)_{x5}-TK-CAT (1.5 μ g/well for MCF-7 and 2.5 μ g/well for BR), 0.5 μ g/well of pCMV-lacZ, and 0.5 μ g/well of either control DNA or dn expression vectors. At 24 h after transfection, cells were treated with 1 μ M MIA for 16 h. The liquid scintillation CAT assay was modified from a standard protocol (30). After transfected cells were treated with MIAs for 16 to 22 h, cells were scraped from plates, washed with 1 ml of washing buffer (40 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl), resuspended in 100 μ l of 0.25 M Tris (pH 8.0), and subjected to three cycles

of freeze-thawing. Resultant cell lysates were heated at 60°C for 10 min to inactivate endogenous CAT inhibitors and clarified by centrifuging at 16,000 x g, 4°C, for 5 min. Each sample in the CAT assay was 108 µl in volume, containing 0.25 mg/ml of butyryl Co-A and 0.15 µCi of [¹⁴C]-chloramphenicol (ICN Biomedicals, Irvine, CA) in 0.25 M Tris pH 8.0. After incubation at 37°C for 3 h, 300 µl of Pristane/Xylene (ratio=2:1) was added into each sample, vortexed for 30 s, and centrifuged at 15,000 x g for 3 min. The organic phase was re-extracted with 100 µl of 0.25 M Tris buffer (pH 8.0). Two hundred µl of each sample was counted in scintillation cocktail (Biofluor, NEN Research, Boston, MA). Expression of β-galactosidase was measured with a kit purchased from Promega (Madison, WI). Data of CAT activities were normalized with levels of β-galactosidase. Statistical analysis of CAT assay values was performed by *ANOVA* and *Student's t-test*.

ACTIVATED RAS INTERACTION ASSAY (A.R.I.A.)

Activated Ras (Ras-GTP) is precipitated by the Ras-binding domain (RBD) of Raf-1 in a GST-RBD fusion protein immobilized to glutathione beads. Affinity-precipitated Ras is detected by Western blot with anti-Ras antibodies (14)(59). The bacterial expression vector for GST-RBD, pGEX-RBD which encodes residues 1-149 of c-Raf1 (RBD), was from D. Shalloway (59).

Preparation of GST-RBD: Transformed JM109 *E. coli* was grown in 50 ml of LB (or super broth) containing ampicillin overnight. In the next morning, 450 ml of LB was added and incubated for 5 h (or till O.D. is about 0.6-0.8). GST-RBD expression was induced by 1 mM IPTG (isopropyl-1-thio-β-D-galactoside) for 2 h. After centrifugation, bacteria pellet was rinsed once with ice-cold PBS and was frozen at -70°C until use. Bacteria pellet was resuspended in 10 ml bacterial lysis buffer (BLB: 20 mM HEPES, pH=7.5, 120 mM NaCl, 10 % glycerol, 2 mM EDTA, 10 µg/ml leupeptin and 10 µg/ml aprotinin) and lysed by sonication. After centrifuged at 15,000 rpm, supernatant of sonicated lysate was collected and to which 10% NP-40 was added to a 0.5% final concentration. GST-RBD was immobilized onto 300-500 µl of 1:1 slurry GSH agarose (Sigma) during incubation with rocking at 4°C for 30 min, purified by washing agarose 6-8 times with BLB with 0.5% NP-40. Presence of GST-RBD can be detected on Coomassie blue stained gels at about 42 kD. About 30-50 µl of these GSH agarose suspension was used for each sample lysate.

Affinity precipitation of active Ras: BR cells growing to 90% confluence in 10-cm dishes were

serum-starved (0.1% FCS) for 48 h, then treated with 1 μ M of PTX or VBL for 30 or 120 min. Cells treated with 50 ng/ml of epidermal growth factor (EGF) for 10 min were used as positive controls. Treated cells were rinsed with ice-cold PBS twice and lysed with 0.4 ml/dish of Mg-containing lysis buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium vanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin). Activated Ras was precipitated by GST-RBD in excess. After three washes with MLB, activated Ras was eluted from beads by boiling in Laemmli's loading buffer, subjected to 14% SDS-PAGE, transferred to nitrocellulose membrane, and detected using anti-Ras antibody (Santa Cruz #SC-035).

IMMUNOCOMPLEX KINASE ASSAYS OF HA-JNK1, HA-ERK2 AND ASK1-HA

Since efficiencies of transient transfection in both BR and MCF-7 cells were limited (20% and 15%, respectively), we were not able to accurately evaluate the effects of transfected expression vectors on regulation of JNK/SAPK by direct measurement of endogenous JNK/SAPK activities in the whole population of cells. Therefore, we cotransfected expression vectors for HA-JNK1 with vectors expressing its potential upstream regulators, then assayed activities of epitope-tagged HA-JNK1. Since we had determined in pilot experiments that transfected HA-JNK1 was optimally activated by 4 h-treatment with MIAs in BR cells, we treated with MIAs for 4 h before measuring HA-JNK1 activity.

BR cells in 60-mm petri dishes were cotransfected with 4 μ g of pSR α -HA-JNK1 and 2 μ g of each expression vector. Total amount of DNA per dish was brought to 8 μ g by adding control vectors (pSR α or pcDNA3). At 24 h after transfection, cells were treated with 1 μ M of MIAs, cell lysates were prepared and subjected to immunocomplex kinase assay using excess anti-HA monoclonal antibody, clone 12CA5 (Boehringer-Mannheim, Indianapolis, IN), to immunoprecipitate expressed HA-JNK1. The same amounts of lysates were probed with either anti-HA or anti-JNK1 monoclonal antibodies (PharMingen, San Diego, CA). Since coexpression of some dominant negative upstream regulators inhibited HA-JNK1 expression, HA-JNK1 activities were normalized by the levels of HA-JNK1 measured in Western blot (39). Identical procedures were performed for HA-ERK2 except that MBP was the substrate and an anti-ERK antibody (Santa Cruz) was used in Western blots.

When pcDNA3-ASK1-HA was cotransfected with other expression vectors into cells, expressed ASK1-HA was immunoprecipitated with excess anti-HA antibody, extensively washed, and subjected to

identical kinase reaction as that in JNK/SAPK assay. Equivalent increase in autophosphorylation of ASK1 was observed in parallel to increased phosphorylation of ATF2 in the coupled-kinase assay for ASK1 using GST-MKK6, GST-p38 and ATF2 as sequential substrates. Therefore, in the present study, activities of ASK1 were measured by levels of *in vitro* autophosphorylation in ASK1-HA bands that migrated in SDS-PAGE at approximately 160-kD.

RESULTS

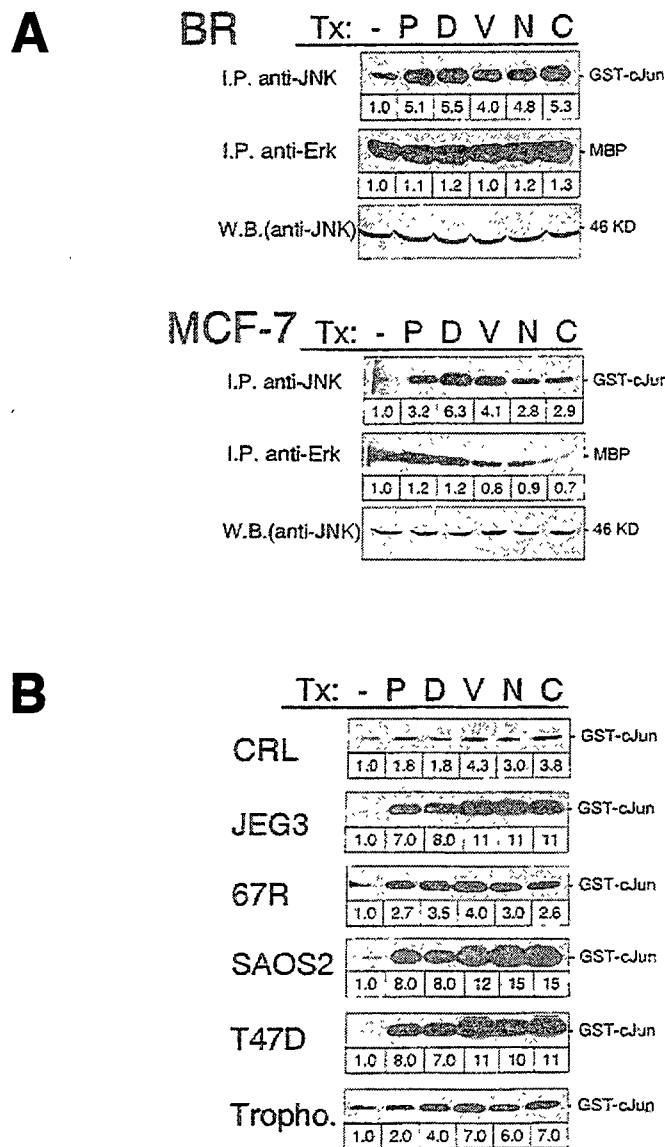
MICROTUBULE-INTERFERING AGENTS (MIAs) ACTIVATE JNK/SAPK IN A VARIETY OF HUMAN CELLS

Activities of JNK/SAPK and MAPK in MIA-treated cells were measured by immunocomplex kinase assays using GST-c-Jun and myelin basic protein (MBP) as substrates, respectively. Treatment with 1 μ M PTX, docetaxel, VBL, nocodazole, or colchicine for 2 h activated JNK/SAPK in BR ovarian cancer cells and in MCF-7 breast cancer cells. Activation of JNK/SAPK was not accompanied by alterations in JNK/SAPK protein levels as measured by Western blotting of whole cell extracts (**Figure 1A**). In contrast, treatment with MIAs did not significantly activate MAPK/ERK activities. Since BR cells had higher basal MAPK activities which could obscure modest MAPK activation by MIAs, HA-ERK2 activity was measured following 1 μ M PTX or VBL, but only a less than 2 fold activation was detected.

To test whether activation of JNK/SAPK by MIA is a general response, we measured JNK/SAPK activities in other cell lines and primary cells. MIAs activated JNK/SAPK in all tested cell types (**Figure 1B**); the varying magnitude (1.8 to 15 fold) in different cell types suggests sensitivity to MIAs may be cell type-specific. It is noteworthy that MIAs activated JNK/SAPK in both proliferating cancer cell lines and non-proliferating trophoblasts (57), suggesting that activation is independent of cell cycle progression.

MIA-INDUCED ACTIVATION OF JNK/SAPK IS DOSE-DEPENDENT AND TIME-DEPENDENT

In both BR and MCF-7 cells, MIAs activation of JNK/SAPK was dose-dependent over a range of 0.01 to 10 μ M (**Table 1**). MIAs activated JNK/SAPK within 30 min of treatment and the JNK/SAPK



(Figure 1) Microtubule-interfering agents activate JNK/SAPK in a variety of human cells.

A, ovarian cancer BR cells and breast cancer MCF-7 cells were treated with 1 μ M paclitaxel (*P*), docetaxel (*D*), vinblastine (*V*), nocodazole (*N*), colchicine (*C*), or DMSO (-) alone for 2 h before JNK/SAPK and ERK2 activities were analyzed by immuno-complex kinase assays. GST-c-Jun and MBP were used as substrates for JNK and ERK2, respectively. The kinase levels in the whole cell lysates were analyzed by Western blot (*W.B.*). *B*, human fibroblast (*CRL*), choriocarcinoma (*JEG3*), ovarian cancer cells (*67R*), osteosarcoma (*SAOS2*), breast cancer cells (*T47D*), and primary trophoblast (*Tropho.*) were treated with 1 μ M MIA (*P*, *D*, *V*, *N*, and *C*) or DMSO (-) for 2 h before JNK/SAPK activities were analyzed. Numbers under the corresponding bands indicate the -fold activation of kinase activity as based on video densitometry.

TABLE I

Dose-dependent activation of JNK/SAPK in BR and MCF-7 cells by microtubule-interfering agents

Cells were treated with MIAs at designated concentrations for 2 h. JNK/SAPK activities were assayed by immunocomplex kinase assays and quantitated by analyzing autoradiograms with a video densitometer. Fold activation shown represent the mean $\pm$ S.E.M. from 2 or 3 independent experiments as indicated in parentheses.

a. BR cells

Treatment	(no. of exp.)	0.01 μ M	0.1 μ M	1 μ M	10 μ M
Paclitaxel	(3)	1.4 $\pm$ 0.3	3.2 $\pm$ 0.9	6.7 $\pm$ 0.7	7.8 $\pm$ 1.1
Docetaxel	(2)	1.5 $\pm$ 0.5	3.9 $\pm$ 1.9	4.4 $\pm$ 1.6	15.0 $\pm$ 4.9
Vinblastine	(3)	2.6 $\pm$ 1.1	2.6 $\pm$ 0.8	3.4 $\pm$ 0.8	5.2 $\pm$ 0.5
Vincristine	(2)	1.7 $\pm$ 0.6	2.7 $\pm$ 1.3	3.4 $\pm$ 1.6	5.5 $\pm$ 0.6
Nocodazole	(3)	2.3 $\pm$ 1.0	5.0 $\pm$ 1.7	6.9 $\pm$ 1.2	10.9 $\pm$ 1.4
Colchicine	(3)	1.2 $\pm$ 0.1	2.7 $\pm$ 0.6	3.9 $\pm$ 1.0	6.6 $\pm$ 1.0

b. MCF-7 cells

Treatment	(no. of exp.)	0.01 μ M	0.1 μ M	1 μ M	10 μ M
Paclitaxel	(2)	1.3 $\pm$ 0.2	3.0 $\pm$ 1.0	3.4 $\pm$ 0.4	5.7 $\pm$ 2.3
Docetaxel	(2)	1.6 $\pm$ 0.5	5.2 $\pm$ 0.8	10.3 $\pm$ 4.2	12.0 $\pm$ 6.0
Vinblastine	(2)	2.0 $\pm$ 0.1	2.2 $\pm$ 0.2	4.0 $\pm$ 0.8	3.5 $\pm$ 0.3
Vincristine	(2)	1.8 $\pm$ 0.2	2.2 $\pm$ 0.2	3.3 $\pm$ 0.3	4.5 $\pm$ 0.5
Nocodazole	(2)	1.5 $\pm$ 0.3	2.7 $\pm$ 0.3	3.5 $\pm$ 1.5	5.4 $\pm$ 0.6
Colchicine	(2)	1.8 $\pm$ 0.2	1.8 $\pm$ 0.2	2.3 $\pm$ 1.3	4.6 $\pm$ 0.4

response peaked between 2 to 8 h declining to basal levels by 12 h (**Figure 2**). BR cells (**Figure 2A**) appeared to respond more rapidly than MCF-7 cells (**Figure 2B**), suggesting cell type-specific differences.

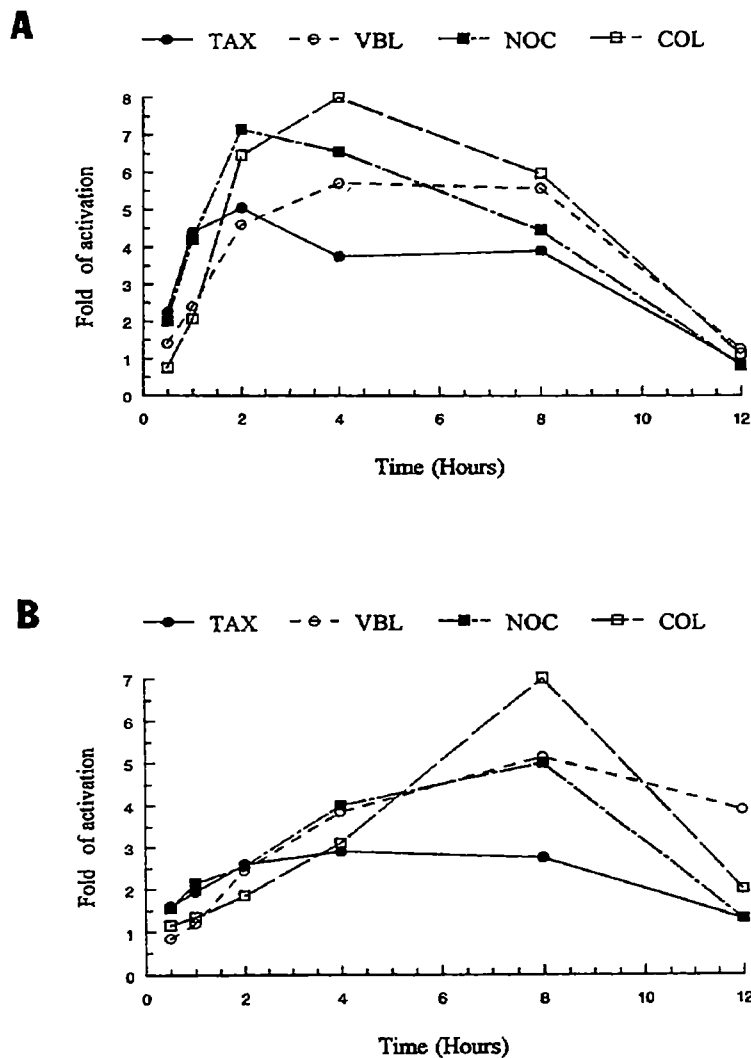
INTERACTIONS WITH MICROTUBULES ARE REQUIRED FOR ACTIVATION OF JNK/SAPK BY MIAS

To elucidate whether interactions between MIAs and tubulin/ MTs are required for activation of JNK/SAPK, we measured JNK/SAPK activities in BR and MCF-7 cells treated with an inactive precursor of PTX, 10-deacetylbaccatin III, or an inactive form of colchicine, β -lumicolchicine. Either agent at concentrations up to 10 μ M failed to activate JNK/SAPK (**Figure 3A**). Since PTX exerts lipopolysaccharide (LPS)-like effects (58) and activates JNK/SAPK in macrophages and monocytes (24), we assayed JNK/SAPK activities in BR and MCF-7 cells treated with purified bacterial LPS. No significant activation of JNK/SAPK was observed in BR cells, while in MCF-7 cells, JNK/SAPK activities fell 10-70 % below basal activities in two independent experiments (**Figure 3A**). These data do not suggest a role for LPS-like activity of PTX in JNK/SAPK activation in cancer cells.

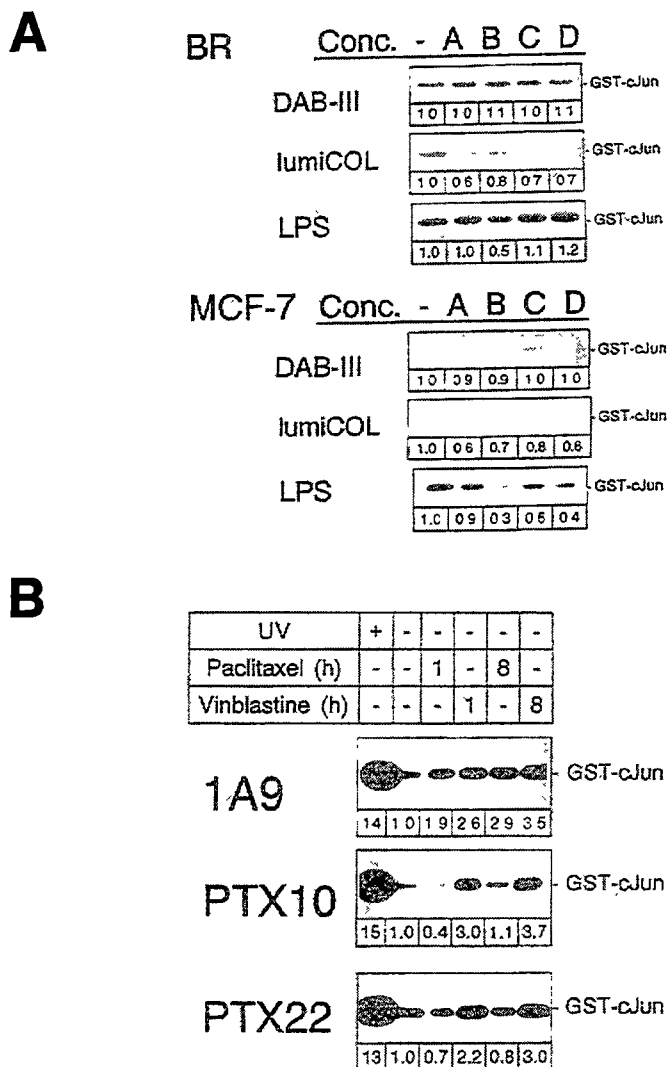
To further confirm the requirement of microtubular interactions with MIAs in the activation of JNK/SAPK, we compared JNK/SAPK activation by PTX in two PTX-resistant cell lines, PTX10 and PTX22 (which express mutant β -tubulins), with that in parental 1A9 cells (2). Both PTX and VBL activated JNK/SAPK in parental 1A9 cells but only VBL was able to activate JNK/SAPK in the PTX-resistant cell lines (**Figure 3B**). Furthermore, UV irradiation activated JNK/SAPK equally in all three cell lines, demonstrating a functional JNK/SAPK signaling cascade in these cells and, indicating the lack of JNK/SAPK activation in PTX-treated PTX10 and PTX22 cells is a result of the failure of PTX to bind tubulin (18)(48).

THE MIA-ACTIVATED, JNKK/SEK1-JNK/SAPK SIGNALING CASCADE ACTIVATES TRANSCRIPTION THROUGH C-JUN

To identify downstream effectors of MIA-activated JNK/SAPK, we transfected BR and MCF-7 cells with a p(TRE)x5-TK-CAT reporter construct for 24 h, treated with MIAs for 16 h, then evaluated CAT activity as a measure of AP-1 transcription factor activity. **Figure 4A** shows statistically significant

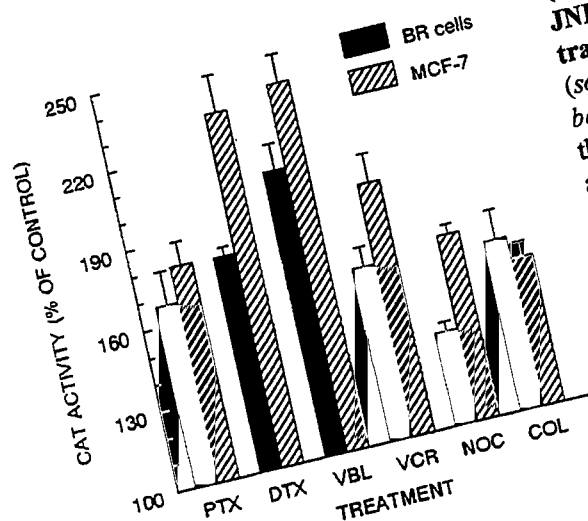


(Figure 2) Time course of JNK/SAPK activation in BR and MCF-7 cells by microtubule-interfering agents. BR cells (*panel A*) and MCF-7 cells (*panel B*) were treated with 1 μ M paclitaxel (●, *PTX*), vinblastine (○, *VBL*), nocodazole (■, *NOC*), or colchicine (□, *COL*) for 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h, then JNK/SAPK activities were analyzed by immunocomplex kinase assays. The fold activation of JNK/SAPK was calculated by comparison to basal JNK/SAPK activities. Data shown represent the means from two independent experiments. Average S.E. for each time point were about 24% and 22% of mean values of BR and MCF-7, respectively (S.E. bars not shown for clear presentation of all points).

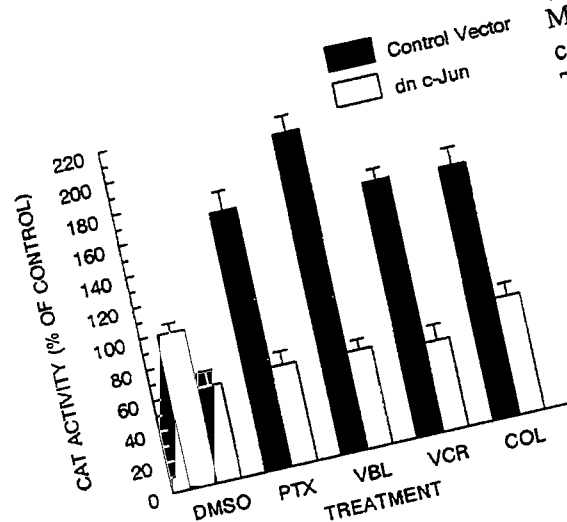


(Figure 3) Interactions with microtubules are required for activation of JNK/SAPK by MIAs. *A*, ovarian cancer BR cells and breast cancer MCV-7 cells were treated for 2 h with DMSO (-), 10-deacetylbaecatin-III (*DAB-III*), β -lumiicolchicine (*lumiCOL*), or purified bacterial LPS at concentrations of 0.01, 0.1, 1, or 10 μ M (*DAB-III* and *lumiCOL*) or μ g/ml (*LPS*) (concentrations A-D, respectively), then JNK/SAPK activities were measured by immunocomplex kinase assay using GST-c-Jun as substrate. *B*, paclitaxel-sensitive ovarian cancer 1A9 cells and the paclitaxel-resistant derivatives PTX10 and PTX22 cells were treated with 40 J/m² UV, 1 μ M paclitaxel or vinblastine for 1 or 8 h, after which JNK activities were measured. The numbers under the corresponding bands indicate -fold activation of JNK as determined by densitometry.

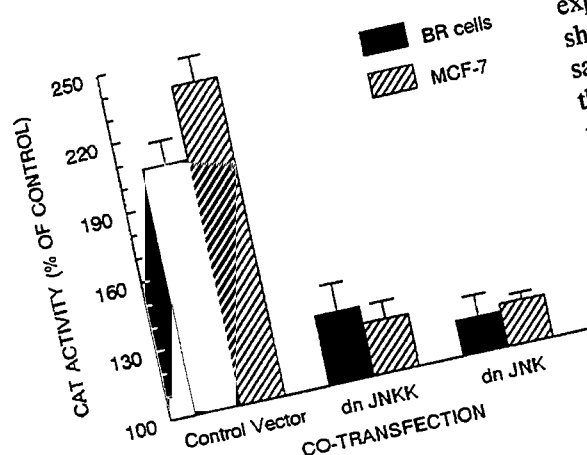
A



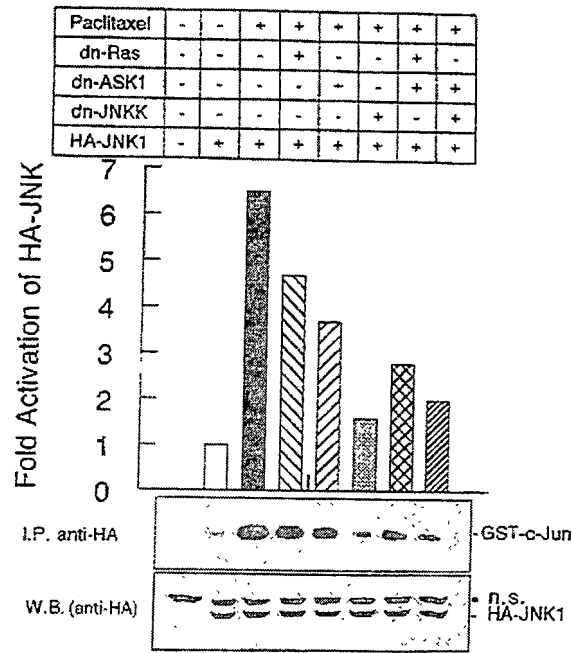
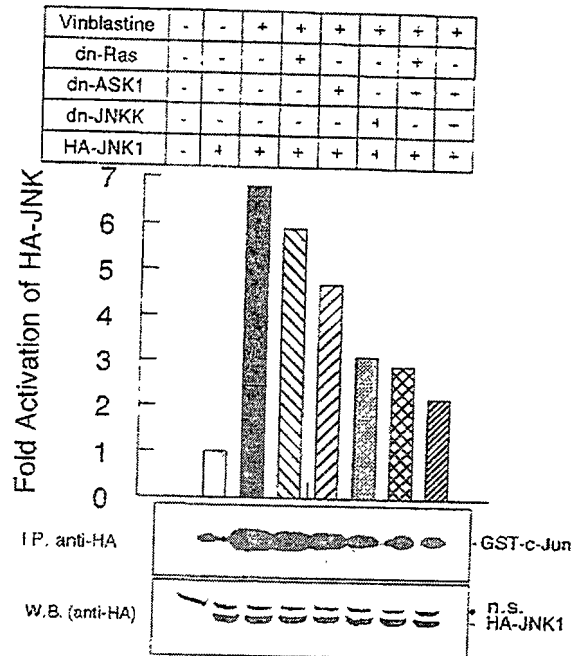
B



C



(Figure 4) The MIA-activates JNK signaling cascade activates JNK transcription through c-Jun. **A**, BR cells (solid bars) and MCF-7 cells (shaded bars) were transiently cotransfected with the p(TRE)x5-TK-CAT reporter construct and pCMV-lacZ using liposomes. Twenty-four hours after transfection, cells were treated with 1 μ M paclitaxel (PTX), docetaxel (DTX), vinblastine (VBL), vincristine (VCR), nocodazole (NOC), or colchicine (COL) for 16 h. CAT activity was measured by liquid scintillation counting and normalized by levels of β -galactosidase. The data given are percent activation of CAT activities compared with DMSO-treated controls (100 ± 6 % for BR and 100 ± 5 % for MCF-7, not shown in the graph). **B**, BR cells were cotransfected with p(TRE)x5-TK-CAT and control vector (gray bars) or dn c-Jun expression vector, pCMV-TAM67 (empty bars), then treated with 1 μ M microtubule-active agents or DMSO vehicle control as indicated. **C**, activation of transiently transfected p(TRE)x5-TK-CAT by vinblastine was measured in BR cells that were cotransfected with control pSR α vectors (Control Vector), dn JNK/SEK1 expression vector (dn JNK), or dn JNK/SAPK expression vector (dn JNK). Data of BR cells shown are the means $\pm$ S.E. of four independent duplicate samples in two independent experiments. Data of MCF-7 cells shown are the means $\pm$ S.E. of triplicate samples in a representative experiment that was repeated twice with comparable results.

A**B**

(Figure 6) Activation of transiently transfected HA-JNK1 by paclitaxel or vinblastine was inhibited by coexpression of dn Ras, dn ASK1, or dn JNKK/SEK1. BR cells in 60-mm dishes were transiently transfected with 4 μ g of pSR α -HA-JNK1 and 2 μ g of each dominant negative expression vector (or combinations as indicated) for 24 h, then treated with 1 μ M paclitaxel (**A**) or vinblastine (**B**) for 4 h. HA-JNK1 activities were measured by immunocomplex kinase assay using an anti-HA antibody (12CA5) to immunoprecipitate HA-JNK1 from cell lysates and GST-c-Jun as substrate. Activities of HA-JNK1 were normalized to levels of HA-JNK1 protein. A nonspecific band recognized by the 12CA5 antibody is labeled with an *asterisk* (*, *n.s.*). Autoradiograms and Western blot shown are from a representative experiment, which was repeated three times with comparable results.

activation, although neither as efficiently as dn JNKK/SEK1. However, coexpression of dn Ras and dn ASK1 exerted additive inhibition on HA-JNK1 activation by either PTX or VBL. In contrast, coexpression of dn ASK1 and dn JNKK/SEK1 did not inhibit HA-JNK1 activity more than dn JNKK/SEK1 alone. These results suggest that ASK1 and JNKK/SEK1 are in the same signal transduction pathway, in agreement with studies indicating JNKK/SEK1 is a downstream effector of ASK1 (26).

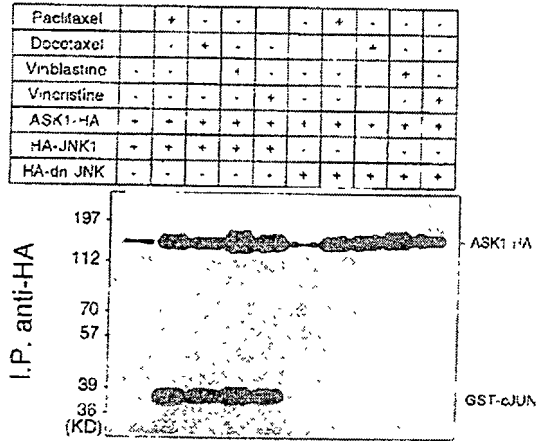
Treatment with MIAs also activated transfected ASK1-HA, as shown by autophosphorylation of ASK1-HA and activation of cotransfected HA-JNK1 (**Figure 7A**). Similarly, in cells transfected with ASK1-HA alone, treatment with MIAs induced comparable levels of autophosphorylation of ASK1-HA without phosphorylation of GST-c-Jun, which was present in the kinase reaction mixture. Furthermore, coexpressed HA-dn JNK did not phosphorylate GST-c-Jun and did not interfere with autophosphorylation of ASK1-HA (**Figure 7A**). Taken together, these results suggest that: (i) ASK1-HA does not itself phosphorylate GST-c-Jun, and (ii) the co-immunoprecipitated HA-JNK1 is unlikely to phosphorylate ASK1-HA.

Overexpression of ASK1-HA enhanced PTX-activation of HA-JNK1 16 fold over control levels, comparable to the 15-fold activation induced by UV irradiation (**Figure 7B**). The augmentation of PTX-induced HA-JNK1 activation by ASK1-HA was inhibited by coexpression of dn Ras, dn Rac, or dn JNKK/SEK1. As shown in **Figure 7B**, a 1:1 ratio of the expression vectors for dn JNKK/SEK1 and wt ASK1 did not completely inhibit the ASK1-enhanced activation of HA-JNK1. However, increasing levels of dn JNKK/SEK1 completely blocked the enhanced HA-JNK1 activation by overexpressed ASK1 without decreasing the levels of MIA-induced activation of ASK1-HA (**Figure 7C**). In agreement with the partial inhibition of HA-JNK1 by dn Ras or dn Rac (**Figure 6**), overexpression of either dn Ras or dn Rac partially inhibited ASK1-augmented activation of HA-JNK1 (**Figure 7C**).

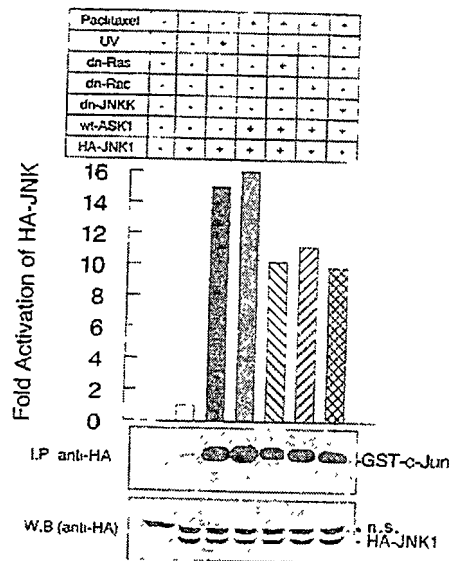
DISCUSSION

MTs serve as an intracellular scaffold and their unique polymerization dynamics are critical for many cellular functions (42)(54)(63). It is conceivable that cytoskeletal dysfunction, manifested as either a disrupted MT network or a stabilized, "rigid" MT cytoskeleton, could bear intracellular stress. In the present study we report that disruption of the equilibrium between tubulin monomer/dimers and MT polymers with MT stabilizing (PTX, docetaxel) or destabilizing (VBL, vincristine, nocodazole,

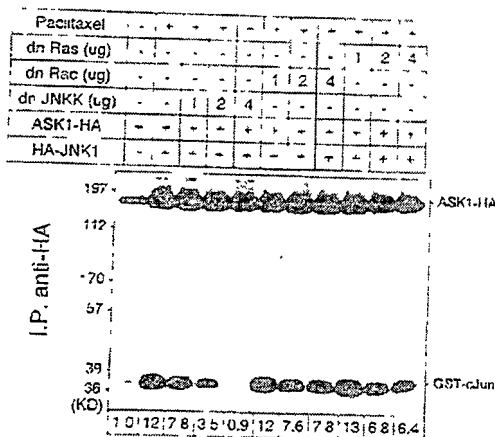
A



B



C



(Figure 7) MIAs activate the ASK1-JNKK-JNK signaling cascade. *A*, BR cells in 60-mm dishes were transiently cotransfected with 4 μ g of pcDNA3-ASK1-HA and 4 μ g of either pSR α -HA-dn JNK for 24 h, then treated with 1 μ M paclitaxel, docetaxel, vinblastine, or vincristine for 4 h. ASK1-HA and HA-JNK1 or HA-dn JNK were co-immunoprecipitated by anti-HA antibody and used in a kinase reaction containing GST-c-Jun. Activities of ASK1-HA and HA-JNK1 were measured by levels of autophosphorylation of ASK1-HA and phosphorylation of GST-c-Jun, respectively. *B*, BR cells were cotransfected with 4 μ g of HA-JNK1, 2 μ g of ASK1-HA, and 2 μ g of dominant negative expression vectors as indicated, then treated with 1 μ M paclitaxel for 4 h. For comparison, BR cells that were cotransfected with 4 μ g of HA-JNK1 and 4 μ g of control vectors were treated with UV irradiation. Activities of HA-JNK1 measured by immunocomplex kinase assay were normalized to levels of HA-JNK1. A non-specific band recognized by the 12CA5 Ab is labeled with an asterisk (*, *n.s.*). *C*, BR cells were cotransfected for 24 h with 3 μ g of HA-JNK1, 1 μ g of ASK1-HA, and increasing amounts (1, 2, and 4 μ g) of each dominant negative expression vector as indicated. Transfected cells were then treated with 1 μ M paclitaxel for 4 h and assayed for activities of ASK1-HA and HA-JNK1. The numbers under the corresponding bands of GST-c-Jun in the autoradiogram indicate the -fold activation of HA-JNK1 as based on video densitometry. Data shown are from a representative experiment, which was repeated twice with comparable results.

colchicine) agents activated the stress-activated protein kinase (JNK/SAPK) signaling cascade. In both BR and MCF-7 cells, JNK/SAPK remained activated for up to 8 h after treatment with MIAs (**Figure 2**). Since induction of JNK/SAPK in T-cell activation and apoptosis can occur in a transient or persistent pattern, respectively (9), the sustained activation of JNK/SAPK following MIA treatment may reflect the apoptosis-inducing nature of these drugs.

For the MIAs used in this study, no membrane-associated receptor or target has been identified (58)(63). Our data demonstrate the requirement of binding to tubulin and/or MTs for MIA activation of JNK/SAPK, because inactive structural derivatives of some MIAs (10-deacetylbaccatin III and β -lumicolchicine) which do not bind tubulin/MTs did not activate JNK/SAPK (**Figure 3A**); and activation did not occur in the PTX-resistant cell lines, PTX10 (F270V) and PTX22 (A364T), which have single amino acid mutation in β -tubulin which abolish binding of PTX to MT and account for PTX resistance (18)(48). The notion that microtubular interactions are required for MIA-activated JNK/SAPK is further strengthened by the observation that while both PTX and VBL activated JNK/SAPK in the parental 1A9 cells, only VBL, but not PTX, activated JNK/SAPK in the PTX resistant PTX10 and PTX22 cell lines (**Figure 3B**).

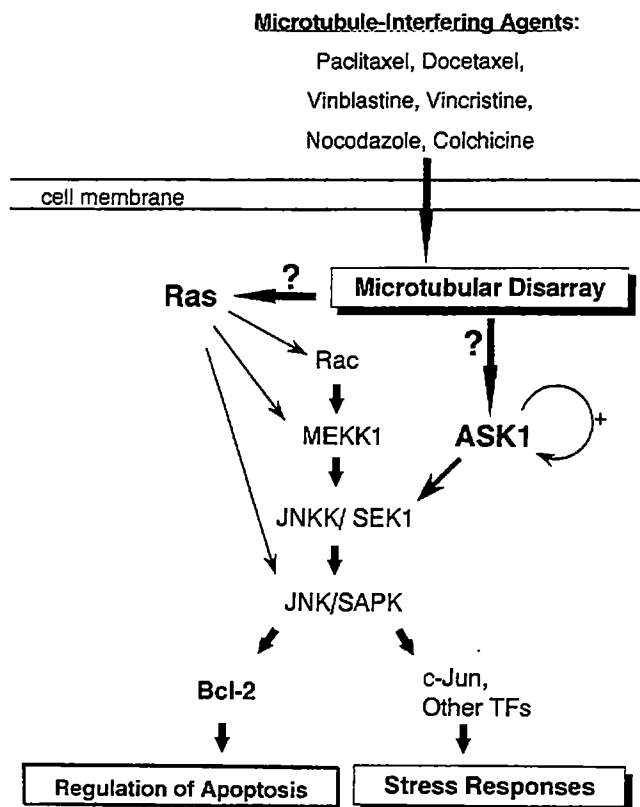
We have verified that the JNKK/SEK1-JNK/SAPK-c Jun signaling cascade was activated by MIAs with the following evidence. Firstly, MIA treatment activated transcription from a TRE-CAT reporter construct and this activation was inhibited by coexpressed dn c-Jun (**Figures 4A & 4B**), indicating c-Jun was a downstream effector responsive to treatment with MIAs. Secondly, coexpression of dn JNK/SAPK or dn JNKK/SEK1 inhibited MIA-induced TRE reporter activity (**Figure 4C**). These results suggest that MIA-activated JNK/SAPK may regulate transcription by activation of c-Jun and formation of functional c-Jun/c-Fos heterodimers (AP-1).

Multiple signal transduction pathways are required for activation of the JNK/SAPK cascade when a cell is perturbed by physical stress (52) and activation of the JNK/SAPK pathway by environmental stress can occur via Ras-dependent or Ras-independent pathways (35). Farnesylation and geranylgeranylation, the major posttranslational modifications of Ras and Rac, respectively, are essential for membrane anchoring and physiological functions (23). Our observations that lovastatin at concentrations which block farnesylation and geranylgeranylation (17) was unable to completely block JNK/SAPK activation by MIAs suggest involvement of other pathways, independent of the Ras-Rac cascade.

Indeed, we demonstrated a requirement for both Ras and ASK1 signaling for full activation of JNK/SAPK by MIAs. Firstly, treatment with PTX or VBL activated Ras (**Figure 5B**). Secondly, dn Ras and dn ASK1 exerted individual and additive inhibition of HA-JNK1 activation by MIAs (**Figure 6**). Thirdly, MIAs activated ASK1-HA with corresponding activation of HA-JNK1 (**Figure 7A**). Lastly, overexpression of ASK1-HA augmented MIA-induced activation of HA-JNK1 (**Figure 7B**) and this augmentation could be completely blocked by high levels of dn JNKK/SEK1, but not by dn Ras or dn Rac (**Figure 7C**). Collectively, these data suggest that both Ras and ASK1 are involved in optimal activation of JNK/SAPK after microtubular disruption and that both may regulate JNK/SAPK activity through the same downstream transducer, JNKK/SEK1.

Unlike treatment with EGF which activates Ras with an amplified activation of MAPK, treatment with MIAs induced a more sustained activation of Ras (**Figure 5B**) with negligible activation of MAPK (**Figure 1A**). Explanations for this discrepancy might be twofold. First, the effects of MIAs on the cell cycle might dissociate the sequential activation in the Ras-Raf1-MEK-MAPK cascade. This is supported by a report that, during progression of the cell cycle, there is a temporal dissociation between Ras and MAPK activation, suggesting Ras may target alternate effector pathways (59). Second, in addition to activation of Ras and ASK1, microtubular disarray might also activate phosphatase(s) which may attenuate MAPK activation. Several phosphatases which might target MAPK have been identified (5)(8)(19)(47)(46).

Based on these results, we propose that activation of the stress-related JNK/SAPK pathway may be a cellular response to intracellular stress caused by interference with MT dynamics (**Figure 8**). In this model, MT-interfering agents enter the cell and disrupt the dynamics of MT assembly. Through a yet-to-be defined mechanism, microtubular disarray activates both Ras and ASK1. Activated Ras may activate the JNK/SAPK through activation of Rac (43)(50), activation of MEKK1 (44), or through direct activation of JNK/SAPK by formation of the Ras-JNK complex (1). On the other hand, the signal from activated ASK1 may involve autophosphorylation followed by sequential activation of JNKK/SEK1 and JNK/SAPK. JNK/SAPK in turn activates downstream effectors, including c-Jun and other transcription factors, mediating cellular responses to this stress. Furthermore, disruption of MT integrity results in phosphorylation of an anti-apoptosis regulator, Bcl-2 (2)(21), and Bcl-2 can be phosphorylated by JNK/SAPK in the presence of Rac1 (40). Since the protective effects of Bcl-2 may be regulated by its phosphorylation status (27), these studies collectively suggest a potential role of activated JNK/SAPK in



(Figure 8) Interaction of signal transduction pathways activated by microtubule-interfering agents. Intracellular stress caused by microtubule-interfering agents activates both Ras and ASK1 signaling cascades, resulting in activation of JNK/SAPK. Activated JNK/SAPK in turn activates transcription factors, including AP-1, to mediate cellular responses to the stress. Activated JNK/SAPK may also regulate apoptosis through phosphorylation of Bcl-2.

apoptotic regulation of cancer cells after chemotherapy with MIAs.

It is intriguing that MIAs with stabilizing or destabilizing effects on MTs elicit similar activation of JNK/SAPK. These observations suggest a surveillance mechanism exists which signals the functional integrity of MTs to nuclear transcription factors. Interestingly, tubulin itself exhibits GTPase activity and acts as a nucleotide-binding protein (6), implying that tubulin may function in a fashion similar to Cdc42/Rac in the JNK/SAPK signal transduction pathway (11)(37). The mechanism(s) by which microtubular disarray activates both Ras and ASK1 remains to be elucidated.

APPENDIX

Additional experiments and results, which were not included in the paper (62), are described in this section.

CELL CYCLE ARREST

To investigate the role of mitotic arrest in PTX-initiated apoptosis, one experimental approach is to study PTX actions in G₁-arrested cells. If mitotic arrest is the sole mechanism for PTX-induced apoptosis, inhibition of cell cycle progression into the G₂/M phase by blocking cells at the G₁ phase should protect these cells from PTX-initiated apoptosis. Therefore, I have tried several methods in attempt to block ovarian cancer cells at the G₁ phase.

In the first set of experiments, ovarian cancer BR or BG-1 cells growing on 6-well plates to 50% confluence were subjected to serum starvation (0% FBS) for 16 to 48 h, followed by treatments with or without 1 μ M PTX, and analyzed for cell cycle profiles with flow cytometry. In the second set of experiments, cells were treated with 40 μ M lovastatin for 16 to 48 h followed by PTX treatment and analyzed for the cell cycle profiles.

Either serum starvation or lovastatin treatment, at best arrested 80% of BR cells at G₁ phase, (*i.e.*, at least 20% cells were still progressing through the cell cycle). Thus, after treatment with PTX, most of serum-starved cells or lovastatin-treated cells still progressed to and were blocked at the G₂/M phase, with cell cycle profiles similar to those in cells that were not serum starved nor lovastatin treated. Since both of these treatments induced cell death of BG-1 cells, they are not suitable for this purpose

either.

My third approach was the use of adenoviral vectors for CDK inhibitors (CKIs), p16 and p27. Ovarian cancer BR and BG cells were cultured with DMEM/F12 medium supplemented with 5% NBS in 6-well plates to 50% confluence. After removal of medium, each of adenoviral vectors (control, p16, p27), which was adjusted to m.o.i. = 50, in 0.5 ml of 5% NBS-DMEM/F12 was added into each well. After incubation at 37°C for 1 h, 1.5 ml of 5% NBS-DMEM/F12 was added into each well and cells were cultured for 24 h. Adenovirus-infected cells were treated with 1 μ M PTX or DMSO (vehicle control) for 16 h, trypsinized, and analyzed for the cell cycle profile with flow cytometry.

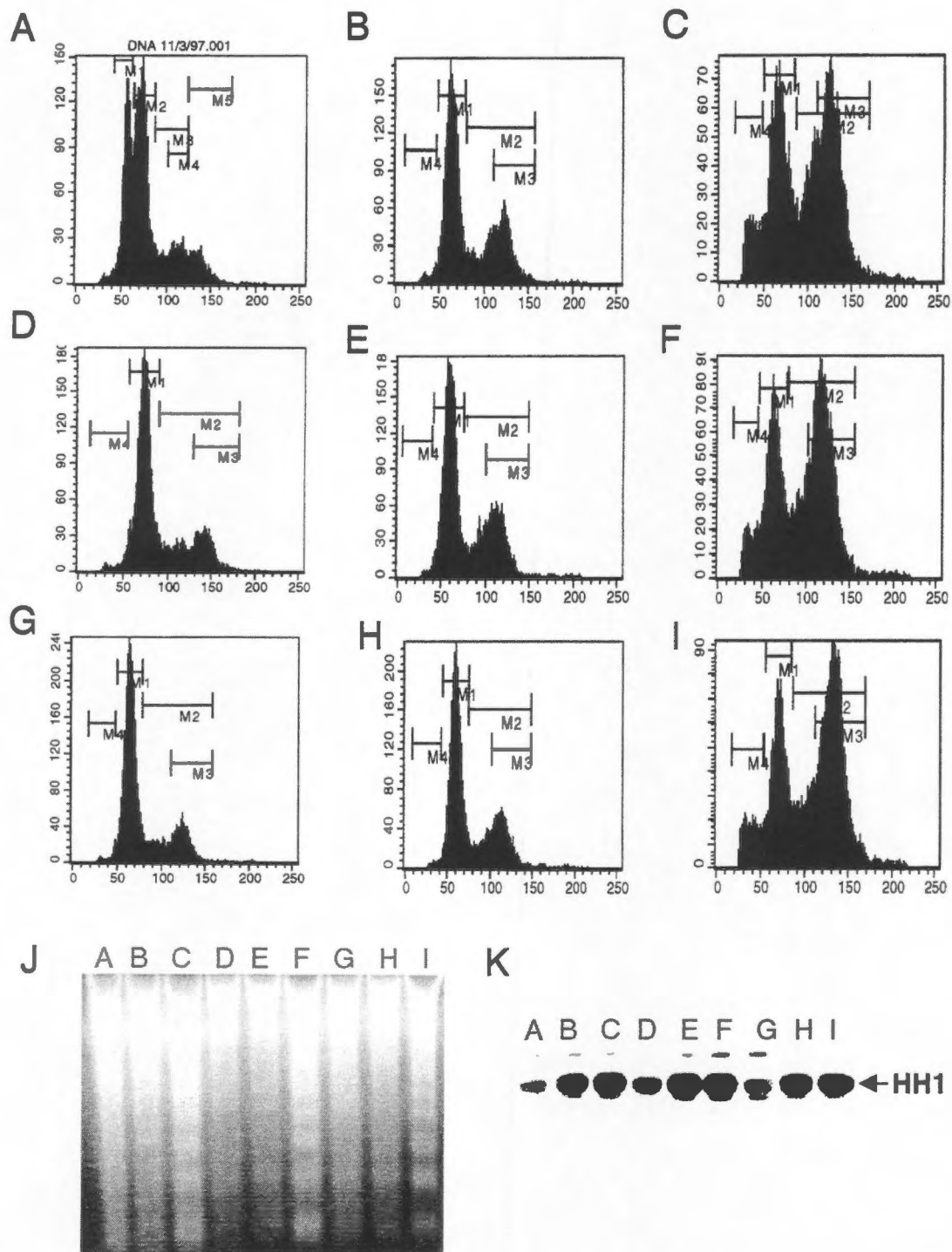
In BR cells, G₁ arrest as results of these adenoviral CKI infections could not be maintained after PTX treatment for either 6 h or 22 h (**Figure 9A-I**). Adenoviral CKI infections did not suppress DNA fragmentation or Cdc2 activation by PTX treatment (**Figure 9J & K**). Therefore, these adenoviral vectors are not suitable for this purpose in BR cells.

On the other hand, both adenovirus p16 (**Figure 10C**) and p27 (**Figure 10E**) infected BG-1 cells exhibited obvious G₁ arrest, when compared with control adenovirus-infected cells (**Figure 10A**). More importantly, after PTX treatment for 16 h, p16-arrested BG-1 cells largely remained in the G₁ phase (**Figure 10D**); and to a lesser extent, p27-arrested BG-1 cells also exhibited a predominant G₁ profiles (**Figure 10F**), when compared with control adenovirus-infected cells (**Figure 10B**).

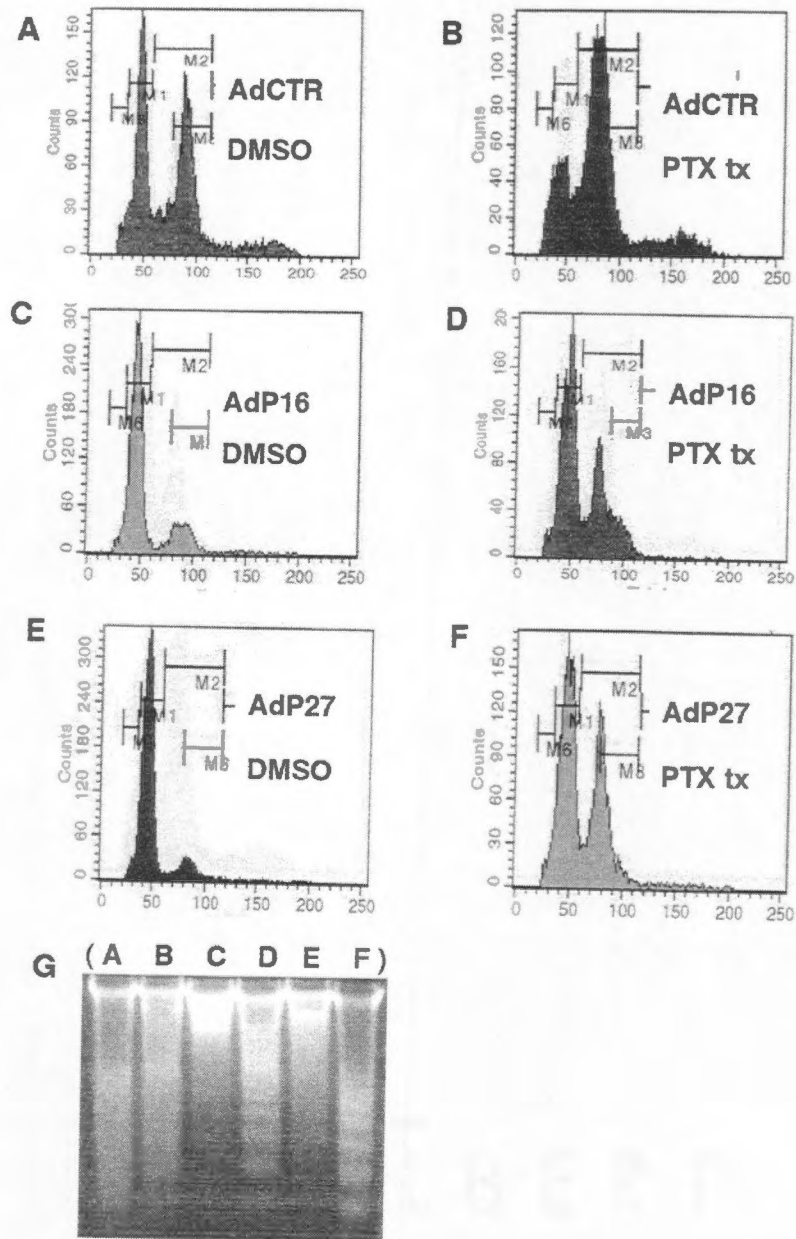
A set of parallel experiment were carried out in 60-mm dishes under the identical condition for detection of the ladder pattern of DNA fragmentation. Surprisingly, sustained G₁ arrest (**Figure 10D & F**) by p16 or p27 did not protect BG-1 cells from DNA fragmentation induced by PTX (**Figure 10G**). These results suggest that additional factor(s), other than mitotic arrest, is(are) involved in the PTX-initiated apoptotic process.

VALIDATION OF THE IMMUNOCOMPLEX JNK ASSAY

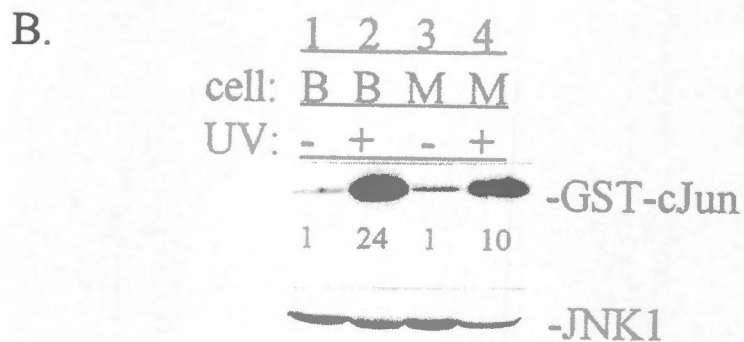
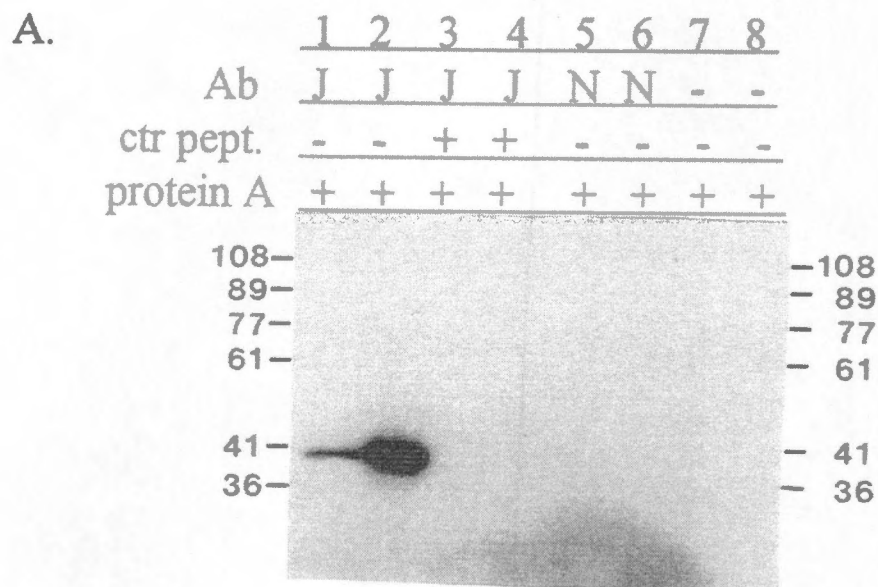
To validate the immunocomplex kinase assay for JNK, I immunoprecipitated lysates of control or UV-irradiated cells either with an affinity-purified, rabbit polyclonal Ab recognizing human JNK1 (a.a. 368-384) or with a comparable amount of non-immune rabbit IgG. To verify the specificity of the anti-JNK Ab, I used a 20-fold excess of a control peptide corresponding to the immunizing epitope of human JNK1 to block the anti-JNK Ab. **Figure 11A** shows that the anti-JNK Ab (lanes 1 and 2), but not the



(Figure 9) BR cells infected with adenoviral-p16 or -p27. *A-C*, AdControl; *D-F*, Adp16; *G-I*, Adp27. (*A, D, G*), no PTX; (*B, E, H*), PTX 6 h; (*C, F, I*), PTX 22 h. *J*, Ladder pattern of DNA fragmentation. *K*, Immunocomplex Cdc2 assay.



(Figure 10) Cell cycle profiles and paclitaxel-induced apoptosis of adenoviral p16- and p27-treated cells. Ovarian cancer BG1 cells were cultured with DMEM/F12 medium supplemented with 5% calf serum (CS) in 6-well plates to 50% confluence. After removal of medium, each of adenoviral vectors (control, p16, p27), which was adjusted to m.o.i. = 50, in 0.5 ml of 5% CS-DMEM/F12 was added into each well. After incubation at 37°C for 1 h, 1.5 ml of 5% CS-DMEM/F12 was added into each well and cells were cultured for 24 h. Adenovirus-infected BG cells were treated with 1 μ M paclitaxel or DMSO control for 16 h, and analyzed for the cell cycle profiles with flow cytometry (panels A-F). A set of parallel experiments were carried out in 60-mm dishes under the identical condition for assays on apoptotic DNA fragmentation (panel G).



(Figure 11) Validation of the immunocomplex JNK assay.

Abbreviations used are: *J*, anti-JNK Ab; *N*, non-immune rabbit IgG; *ctr pept.*, control peptide; *B*, BR cell lysates; *M*, MCF-7 cell lysates.

control rabbit IgG (lanes 5 and 6), efficiently immunoprecipitated JNK from lysates. Excess amounts of the control peptide specifically blocked JNK immunoprecipitation by this Ab (lanes 3 and 4). Non-specific binding of JNK to protein A agarose beads alone was not detected (lanes 7 and 8). **Figure 11B** compares an autoradiogram of the immunocomplex JNK assay to Western blots of the same amount of whole cell lysate from cells with and without UV irradiation. UV irradiation at dose 40 J/m² (described above) activated JNK 24 fold in BR cells and 10 fold in MCF-7 cells (upper panel) without detectably increasing JNK protein levels (lower panel). Recognition of a 46 kD band, consistent with the mobility of JNK1, validated the anti-JNK Ab used in this study.

P38 KINASE ASSAY

The procedure of p38 assay was identical to JNK assay except that anti-p38 antibody (Santa Cruz #SC-535) was used for immunoprecipitation and GST-ATF2 (Santa Cruz #SC-4114) was used as substrate. Activation of p38 by PTX treatment was not observed. However, the results were inconclusive because of the anti-p38 antibody did not recognize bands at 38 kD in Western blot analyses. The use of more reliable anti-p38 antibodies should be able to verify these data.

IN-GEL KINASE ASSAY

In an attempt to identify upstream regulator of JNK in PTX-treated cells, I have tried in-gel kinase assay using GST-JNK as substrate mixed in the agarose gel. The protocol for in-gel kinase assay was provided by Dr. J. Merryman.

Preparation of GST-JNK: The procedure of GST-JNK production in JM109 *E. coli* is identical to that of GST-RBD (described in the ARIA section). Bound GST-JNK on glutathione agarose was eluted twice by adding small volume (100-200 μ l) of eluting buffer (20 mM reduced glutathione, 50 mM Tris, pH=8.0) and spinning in compact reaction columns (CRC, Cat. #13927, United States Biochemical Corp., Cleveland, OH), which were inserted with CRC filters (pore size: 10 μ M, USB #13924). Eluents were pooled.

In-gel kinase assay:

1. Cell lysates were resolved in an 8% GST-JNK-containing polyacrylamide minigel (0.4 M Tris,

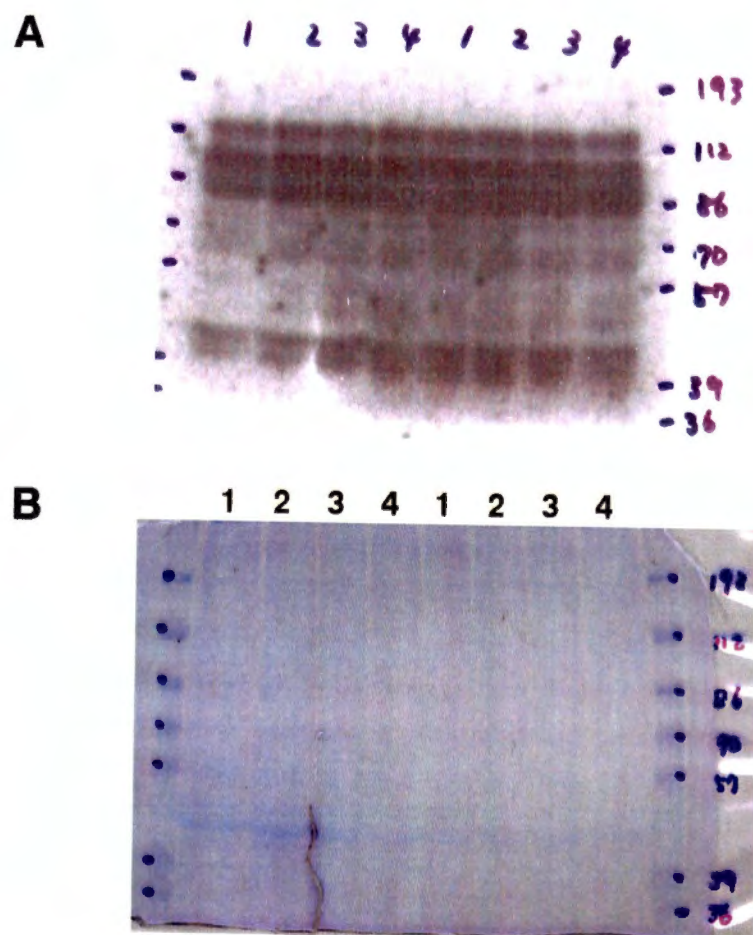
pH=8.8, 0.17% SDS, GST-JNK 200µg/ml) at constant voltage (50-75 V).

2. The gel was fixed in 50 mM Tris (pH=8.0) containing 20% isopropanol on an orbital shaker for 2 h at RT, with four changes in solution.
3. The gel was washed with 50 mM Tris (pH=8.0) containing 5 mM 2-mercaptoethanol for 2 h at RT, with four changes in solution.
4. Proteins were denatured by incubating the gel in 6 M guanidine hydrochloride for 2 h at RT, and renatured by 10 washes of 20 min each in 50 mM Tris (pH=8.0) containing 0.04% Tween-40 and 5 mM 2-mercaptoethanol.
5. The gel was pre-incubated in 40 mM HEPES (pH=8.0) containing 2 mM 2-mercaptoethanol and 10 mM MgCl₂ for 1h.
6. The gel was incubated in 6 ml kinase reaction buffer (40 mM HEPES, pH=8.0, 0.5 mM EGTA, 40 µM ATP, 10 mM MgCl₂, 25 µCi γ-³²P ATP) on an orbital shaker at RT.
7. The gel was washed with 5% TCA/1% sodium pyrophosphate for several hours until background radiation levels were decreased to that of an unused area of the gel.
8. The gel was stained in Coomassie blue, destained, and dried onto blotting paper.
9. The gel can be exposed to phosphoimaging cassette for 4 h and scanned at high resolution (optional).
10. Dried gel was exposed to X-ray film with enhanced screen at -70°C for 5-7 days.

Results: Compared with control lysates, no different pattern of bands were detected in UV-, PTX-, or VBL-treated cell lysates in my in-gel kinase assay (**Figure 12**). In addition to potential technical errors, the cause for the unsatisfactory assay was likely to be an insufficient GST-JNK amount added to the gel. Furthermore, autophosphorylation of preparations of GST-JNKK from the pGEX-JNKK, which I obtained from Anning Lin (University of Alabama, Birmingham), was detected in the following coupled kinase assays. The autophosphorylation of GST-JNKK makes it unsuitable as substrate in in-gel kinase assay.

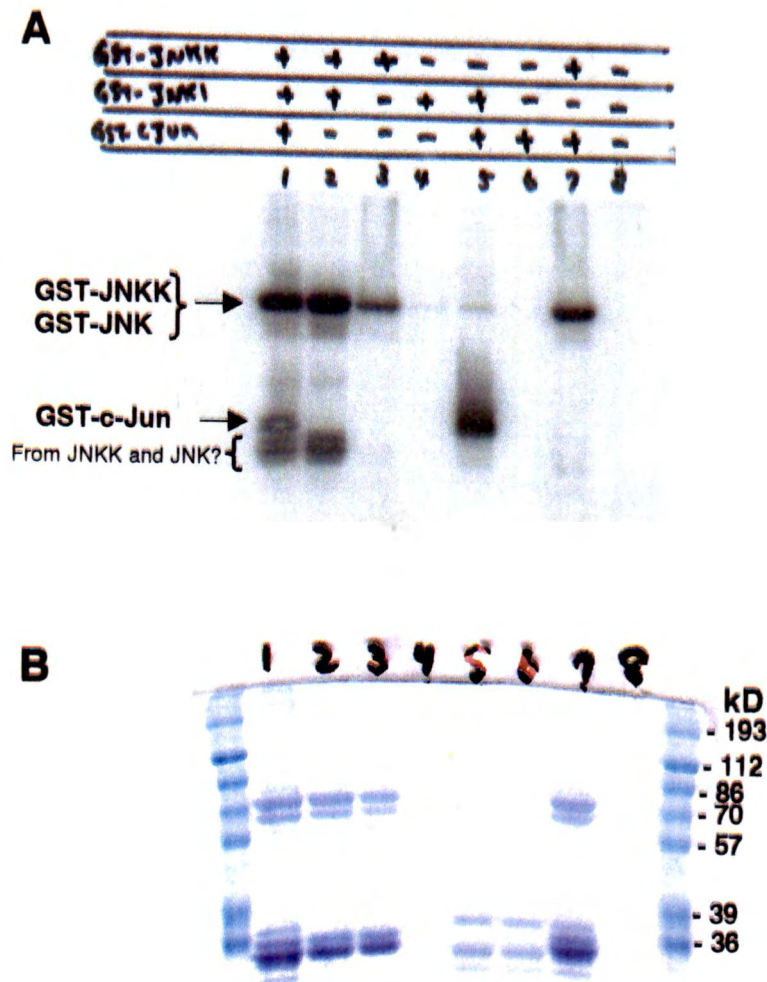
COUPLED KINASE ASSAYS

Preparation of GST-JNKK, GST-JNK, and GST-c-Jun from bacterial expression vectors: pGEX-JNKK, pGEX-JNK (both from Dr. Anning Lin), and pGEX-c-Jun (from Dr. Joyce Merryman),



(Figure 12) In-gel kinase assay using GST-JNK as substrate. MCF-7 cells were treated with control (lane 1), UV (lane 2), PTX (lane 3), or VBL (lane 4). For each lane, 100 μ g of total cell lysates were loaded and duplicated samples were loaded into this gel. **A**, An autoradiograph was derived from exposure of the dried gel between cellulose membranes to X-ray film for 5 days. **B**, The same gel was stained with Coomassie blue before drying.

respectively, are described above. Equal amount of GST-protein-bound GSH agarose beads were mixed, washed with ice-cold PBS three times and with kinase buffer once. Kinase reactions were performed in 20 μ l kinase buffer in the presence of radioactive ATP, as described in the Immunocomplex kinase assay. Alternatively, and perhaps a better way, is to use equal amount of purified GST-proteins for these coupled kinase assays. In the experiment shown in **Figure 13**, GST-JNKK exhibited autophosphorylation (lanes 3 and 7). In that experiment, SDS-PAGE did not sufficiently separated GST-JNK from GST-JNKK, therefore, both resulted in broad phosphorylated bands in lanes 1 and 2.



(Figure 13) Coupled kinase assays. Ten μ l each of suspended GST-protein-bound GSH agarose beads were mixed as designated in **A**, washed three times with ice-cold PBS and once with kinase buffer. Kinase reactions were done in 20 μ l kinase buffer in the presence of radioactive ATP, as described in the immunocomplex kinase assay. **A**, An autoradiograph was derived from 15 h exposure of the dried gel to X-ray film. **B**, A parallel set of mixed GST-proteins were resolved by SDS-PAGE and stained with Coomassie blue. *Note* the amount of proteins in *lane 4* of **B** was not accurate due to a spillage during loading.

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PART IV.

MICROTUBULE DYSFUNCTION INDUCED BY PACLITAXEL INITIATES
APOPTOSIS THROUGH BOTH C-JUN N-TERMINAL KINASE (JNK)-
DEPENDENT AND -INDEPENDENT PATHWAYS
IN OVARIAN CANCER CELLS

ABSTRACT

The antineoplastic agent paclitaxel (Taxol®), a microtubule (MT) stabilizing agent, is known to arrest cells at the G₂/M phase of the cell cycle and induce apoptosis. We and others have recently demonstrated that paclitaxel (PTX) also activates the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signal transduction pathway in various human cell types, however, no clear role has been established for JNK/SAPK in PTX-induced apoptosis. To further examine the role of JNK/SAPK signaling cascades in apoptosis resulting from microtubular dysfunction induced by PTX, I have coexpressed dominant negative (dn) mutants of signaling proteins of the JNK/SAPK pathway (Ras, ASK1, Rac, JNKK, JNK) in human ovarian cancer cells with a selectable marker to analyze the apoptotic characteristics of cells expressing dn vectors following exposure to PTX. Expression of these dn signaling proteins had no effect on Bcl-2 phosphorylation, yet inhibited apoptotic changes induced by PTX up to 16 h after treatment. Coexpression of these dn signaling proteins had no protective effect after 48 h of PTX treatment. These data indicate that: (i) activated JNK/SAPK acts upstream of membrane changes and caspase-3 activation in PTX-initiated apoptotic pathways, independently of cell cycle stage, (ii) activated JNK/SAPK is not responsible for PTX-induced phosphorylation of Bcl-2, and (iii) apoptosis resulting from MT damage may comprise multiple mechanisms, including a JNK/SAPK-dependent early phase and a JNK/SAPK-independent late phase.

INTRODUCTION

Paclitaxel (Taxol®) is an antineoplastic agent specifically targeting MTs (26)(46) and extensive studies indicate that PTX arrests cells at the G₂/M phase of the cell cycle (34). While mitotic arrest of PTX-treated cells has been observed to initiate apoptosis (33)(43)(77), the biochemical events downstream of kinetic stabilization of MT dynamics which lead to apoptosis remain largely unclear (34). Furthermore, substantial evidence indicates that the G₂/M arrest of the cell cycle may not be the only mechanism to induce apoptosis (39)(49)(50)(53); additional phosphoregulatory pathways may be involved in inducing apoptosis (6)(21)(76).

We and others have recently demonstrated that PTX activates the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathways in a variety of human cells through microtubular

interactions (1)(73). The JNK/SAPK signaling pathways respond to various stress-related stimuli and are involved in initiation of apoptosis in many cell types (19)(30)(35)(38)(36). Whether JNK/SAPK activation is required for PTX-induced apoptosis has remained unclear, however; it is known that PTX induces phosphorylation of Bcl-2 (6)(21) and that Bcl-2 can be phosphorylated by activated JNK/SAPK (45).

The purpose of this study was to examine whether inhibition of the JNK/SAPK signaling pathway protects cells from PTX-induced apoptosis and/or abrogates PTX-induced phosphorylation of Bcl-2. These results demonstrate that expression of dn ASK1 (apoptosis signal-regulating kinase 1), dn Rac, dn JNKK, or dn JNK, while exerting no effects on phosphorylation of Bcl-2, inhibits apoptosis induced by PTX treatment up to 16 h. The present study clearly indicates that activation of the JNK/SAPK signaling cascade promotes early phases of PTX-induced apoptosis, independently of cell cycle stage or Bcl-2 phosphorylation.

EXPERIMENTAL PROCEDURES

CELL CULTURE, TRANSFECTION, AND PACLITAXEL TREATMENT

Ovarian carcinoma cells BR (9)(75) were cultured in DMEM/F12 (Sigma, St. Louis, MO) supplemented with 10% NBS, penicillin, and streptomycin and incubated at 37°C in 5% CO₂. Control vectors pCMV-lacZ and pSR α empty vector as well as expression vectors for wild type (wt) JNK1 (pSR α -JNK), dominant-negative (dn) Ras (pSR α -dn Ras), and dn Rac (pSR α -dn Rac) (42) were from Z.-G. Liu and M. Karin (University of California at San Diego). A Myc-epitope tagged expression vector for wt Rac, pEXV3-Rac, was from A. Hall (University College London, UK)(22). Dominant-negative expression vectors for JNK/SAPK (pSR α -APF) and JNKK/SEK1 (pSR α -K116R) were from G.L. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO)(32). Expression vectors for wt and dn ASK1 (pcDNA3-ASK1-HA, pcDNA3-dn ASK1-HA) were described previously (12)(29)(55)(63). In addition to pSR α and pcDNA3 (Invitrogen, Carlsbad, CA) empty vectors, expression vectors used for control purposes include: pCMV-CD20 that was from E. Harlow (Massachusetts General Hospital, Boston)(69) and pcDNA3-kinase dead p70^{S6kinase}(K100R) that was generated by PCR-mutagenesis from the p70^{S6kinase} cDNA, which was originally from J. Avruch (Massachusetts General Hospital). Liposome-mediated transfections of BR cells using Tfx-50 (Promega, Madison, WI) were performed as described in

Part III (73). A CMV promoter-driven enhanced green fluorescent protein construct, pEGFP (Clontech, Palo Alto, CA), was cotransfected as a selectable marker for transfected cells. Stock solutions of PTX, actinomycin-D, and cisplatin (all from Sigma) were prepared with DMSO at concentrations of 10 mM, 1 mM, and 50 mM, respectively. In this study, 1 μ M PTX was used to treat cultured cells.

ANNEXIN-V BINDING, FLOW CYTOMETRIC ANALYSES AND SORTING

Twenty-four h after cotransfection of BR cells with pEGFP and wt or dn expression vectors, 10^5 trypsinized cells were incubated at room temperature for 15 min with 5 μ l phycoerythrin (PE)-conjugated annexin-V (AV) (Pharmingen, San Diego, CA) and 0.125 μ g/ml of 7-amino actinomycin D (7-AAD) (Sigma) in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) and analyzed by flow cytometry with a FACStar^{Plus} (Becton Dickinson, San Jose, CA). Fluorochromes such as green fluorescent protein expressed by pEGFP, PE-annexin V, and 7-AAD were excited by laser tuned to 488 nm and emissions were detected at 507 nm, 575 nm, and 650 nm, respectively. Data of 10,000 cells from each sample were analyzed with the CellQuest3 software (Becton Dickinson). To compare other apoptotic characteristics in cells with or without expression of dn vectors, the green fluorescent protein positive and negative cells were sorted using a FACStar^{Plus} flow cytometer and analyzed separately.

WESTERN BLOT ANALYSES

Aliquots of cell lysate containing equal protein mass were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-HA epitope (Boehringer Mannheim, Indianapolis, ID), anti-Myc (Calbiochem, San Diego, CA), anti-JNK (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP (Upstate Biotechnology, Lake Placid, NY), or anti-Bcl-2 (DAKO, Carpinteria, CA) antibodies followed by relevant second antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). After washing, proteins were detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL) as previously described (73).

IMMUNOCOMPLEX JNK ASSAY

Activity of the JNK/SAPK was measured as described in **Part III** (73). Endogenous JNKs were immunoprecipitated from cell lysate using an anti-JNK Ab conjugated to agarose beads (Santa Cruz Biotechnology) and the JNK activity was assayed by levels of incorporated [γ - 32 P]ATP into its substrate GST-cJun (amino acids 1-79) (Santa Cruz).

CELL CYCLE ANALYSES

Green cells expressing control vector or dn expression vectors were isolated by flow cytometric sorting, fixed with 70% ethanol, treated with 0.1 mg/ml RNase (Sigma), stained with 20 μ g/ml propidium iodide (PI), and analyzed with flow cytometry. PI-stained DNA content of each cell was used as the parameter of cell cycle profile.

CASPASE-3 ASSAY

We used the colorimetric substrate Ac-DEVD-pNA (Calbiochem) for caspase-3 assays in a procedure modified from the manufacturer's protocol. Briefly, aliquots of sonicated cell lysate were prepared in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA), incubated with 200 μ M Ac-DEVD-pNA in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol) in 96-well plates at 37°C for 24 h. Absorbance of the cleaved product was measured at 405 nm in a BioKinetic EL340 microplate Reader (Bio-Tek Instruments, Winooski, VT).

DNA FRAGMENTATION ASSAYS

Identification of the ladder pattern of DNA fragmentation was done according to a previously reported method (59). Treated cells from 6-well plates, including both floaters and attached cells, were collected by scraping in their culture media, rinsed once with ice-cold PBS, and spun at 1000 $\times$ g for 5 min. Cell pellets were resuspended in 0.5 ml digestion buffer (10 mM EDTA, 50 mM Tris pH=8, 0.5 % sodium lauryl sarcosine, 0.5 mg/ml proteinase K) and incubated at 55°C for 3 h. RNase A (final concentration at 0.1 mg/ml) was added and incubated for another 1 h. After extraction with an equal

volume of phenol-chloroform twice and by chloroform once, sodium acetate (0.3 M final concentration) and 2 to 2.5 volumes of pure ethanol were added into the aqueous phase of solution, and the resultant mixture was kept at -70°C for 20 min. DNA was precipitated by spinning at 14,000 rpm, 4°C for 10 min and dried with a Speed Vac (Savant Inc.) for 10 min. Dry DNA pellets were resuspended with 20-50 µl TE buffer (10 mM Tris pH=7.4, 1 mM EDTA) at 4°C overnight. Ten µg per lane DNA was loaded to 1.6 % agarose gel containing a minimal amount of ethidium bromide, resolved with 110 V electrical current, and visualized with UV.

To quantify fragmented DNA in apoptotic cells, transfected cells isolated by flow cytometry were treated with PTX or vehicle (DMSO) and levels of fragmented DNA resulting from apoptosis were measured with a Cell Death ELISA kit according to the manufacturer's protocol (Boehringer Mannheim).

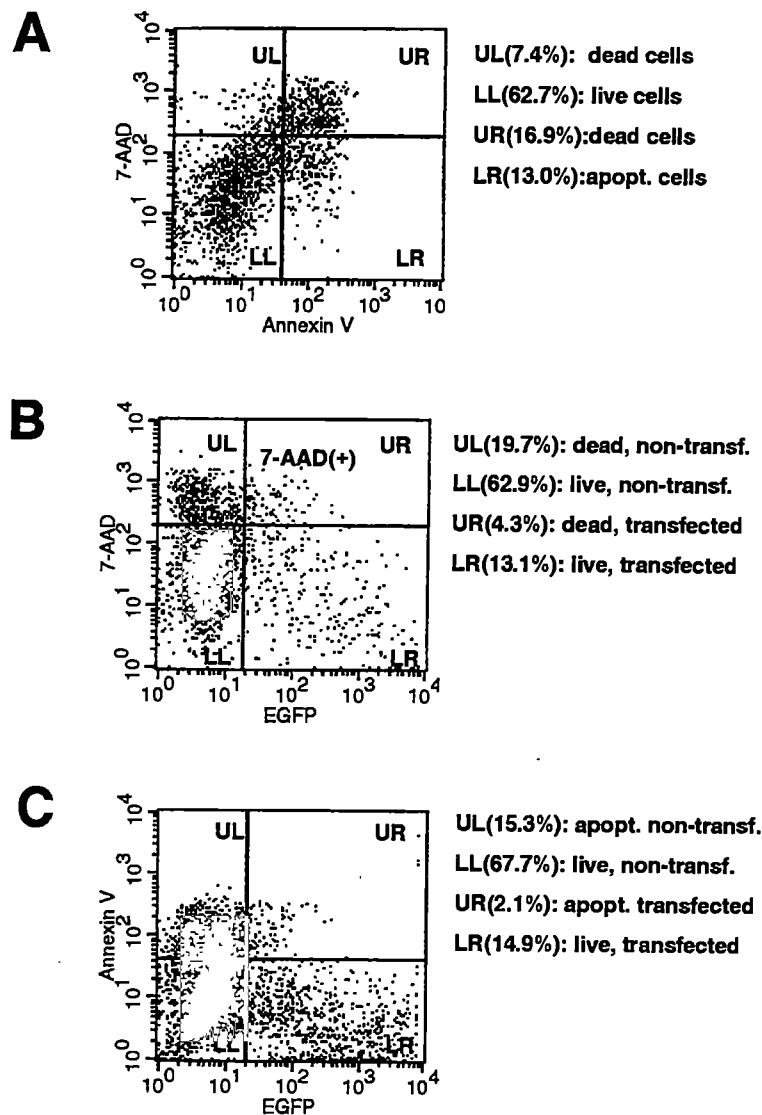
STATISTICS

Analysis of variance (ANOVA) and Scheffe test for *post-hoc* comparisons were used for statistical analyses with the STATISTICA software (Statsoft Inc., Tulsa, OK). The *p*-values equal to or greater than 0.05 are considered to be not significant.

RESULTS

EXPRESSION OF DN ASK1, DN RAC, DN JNKK, OR DN JNK TRANSIENTLY ALLEVIATES CYTOTOXICITY INDUCED BY PACLITAXEL TREATMENT FOR 16 H.

To determine whether inhibition of the JNK/SAPK signaling cascade abolishes apoptosis in cells treated with PTX, we analyzed PTX-induced apoptosis among ovarian cancer BR cells transfected with dn Ras, dn ASK1, dn Rac, dn JNKK, or dn JNK, along with pEGFP to allow selection of transfected cells. The efficacies of these dn expression vectors for inhibition of the JNK/SAPK have been demonstrated previously (73). Following PTX treatment for the indicated times, transfected cells were stained with both PE-conjugated AV (binding to both apoptotic cells and dead cells) and the viability dye 7-AAD (staining dead cells but not early apoptotic cells) to differentiate early apoptotic cells (AV positive / 7-AAD negative) from dead cells (7-AAD positive)(Figure 1).



(Figure 1) Three-color analyses for apoptosis and cell death among transfected cells by flow cytometry. Ovarian cancer BR cells were cotransfected with pEGFP and an expression vector for dn Ras for 24 h, treated with 1 μ M paclitaxel for 16 h, stained PE-conjugated annexin-V and 7-AAD, and analyzed by flow cytometry. *A*, early apoptotic cells (annexin-V positive/7-AAD negative) in LR region were differentiated from dead cells (7-AAD positive) in UR region. The UL and LL regions contain dead cells (7-AAD positive) and live cells (annexin-V positive) and live cells (annexin-V negative/7-AAD negative), respectively. *B* and *C*, to analyze early apoptotic cells among transfected cells, we first excluded dead cells (7-AAD positive) from the whole population shown in *B* and only analyzed live cells by profiles of EGFP and PE-conjugated annexin-V. Percent of early apoptotic cells among transfected (green) cells were calculated by: $100\% \times UR/(UR + RL)$ shown in *C*.

Percentages of early apoptotic cells among transfected cells treated with PTX for 16 h or 48 h are summarized in **Table I**. Data presented as the (% in PTX-treated cells / % in DMSO-treated cells) ratios among cells expressing different dn signaling proteins are compared in **Figure 2**. In transfected cells without PTX treatment, expression of dn vectors decreased the percentage of early apoptotic cells. These decreases in basal levels of apoptosis were specific for expression of these dn expression vectors because expression of irrelevant genes in pCMV-lacZ, pcDNA3-kinase dead-p70^{S6kinase}, or pCMV-CD20 did not decrease basal apoptosis (data not shown). Treatment with PTX for 16 h significantly increased apoptosis in both control vector-transfected and dn Ras-transfected cells ($p < 0.05$), but the induction of apoptosis by PTX was decreased to statistically insignificant levels (*n.s.*) in cells expressing dn ASK1, dn Rac, dn JNKK, or dn JNK (**Table I & Figure 2A**). In contrast to 16-h treatment with PTX, 48 h of treatment significantly ($p < 0.05$) increased early apoptotic cells in all transfected cells irrespective of expression of dn vector types (**Table I & Figure 2B**).

To verify that expression of dn signaling proteins efficiently inhibited endogenous JNK/SAPK activity, we cotransfected BR cells with pEGFP and dn expression vectors, isolated transfected cells by flow cytometry using expression of pEGFP (green fluorescence) as a selectable marker, treated with PTX, and analyzed by immunocomplex JNK assay. We isolated only live (7-AAD negative) green cells expressing transfected vectors (**Figure 3A**) and verified expression of dn signaling proteins by Western blot analyses (**Figures 3B & 3C**).

The inability of dn vectors to protect cells from apoptosis following 48-h treatment with PTX did not result from decreased expression of dn signaling proteins. We have confirmed substantial expression of EGFP by fluorescent microscopy (data not shown) and expression of dn signaling proteins by Western blot analyses up to 48 h of PTX treatment (**Figures 3B & 3C**). At 48h of treatment, protein levels of dn JNK or dn ASK1 in DMSO-treated cells remained as high as those at earlier time points; whereas those in PTX-treated cells slightly decreased, perhaps resulting from a general protein degradation during apoptosis (**Figures 3B & 3C**). Despite of its slight decrease in later time points of PTX treatment, expression of these dn signaling proteins efficiently suppressed PTX-induced JNK activation through all the time points (**Figures 3B & 3C**).

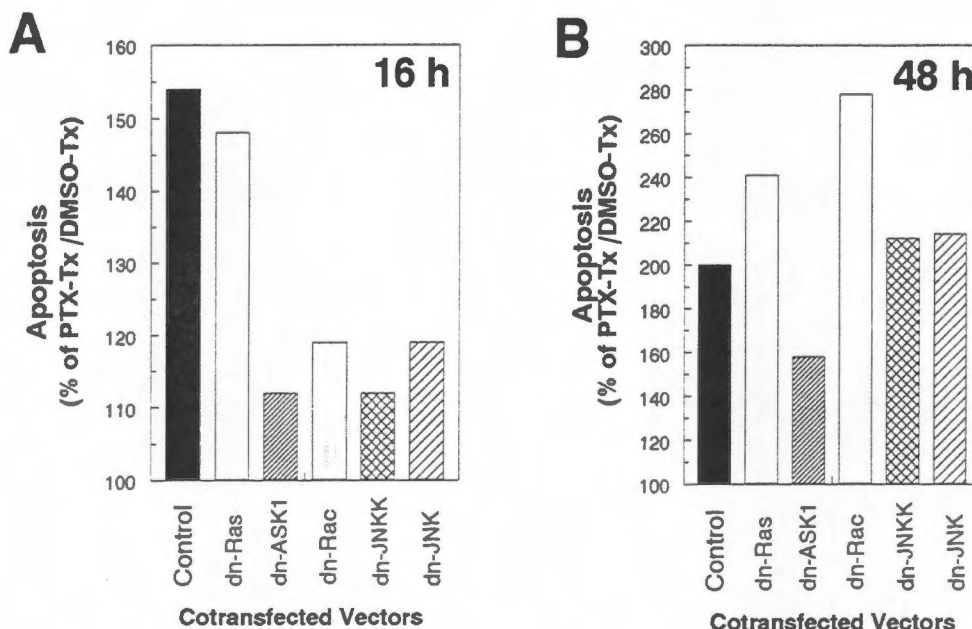
Collectively, these results suggest that expression of dn ASK1, dn Rac, dn JNKK, or dn JNK transiently protects ovarian cancer cells from PTX-induced apoptosis up to 16 h. When treated with PTX for a longer time, such as 48 h, cells may undergo apoptosis through additional, JNK/SAPK-independent

TABLE I

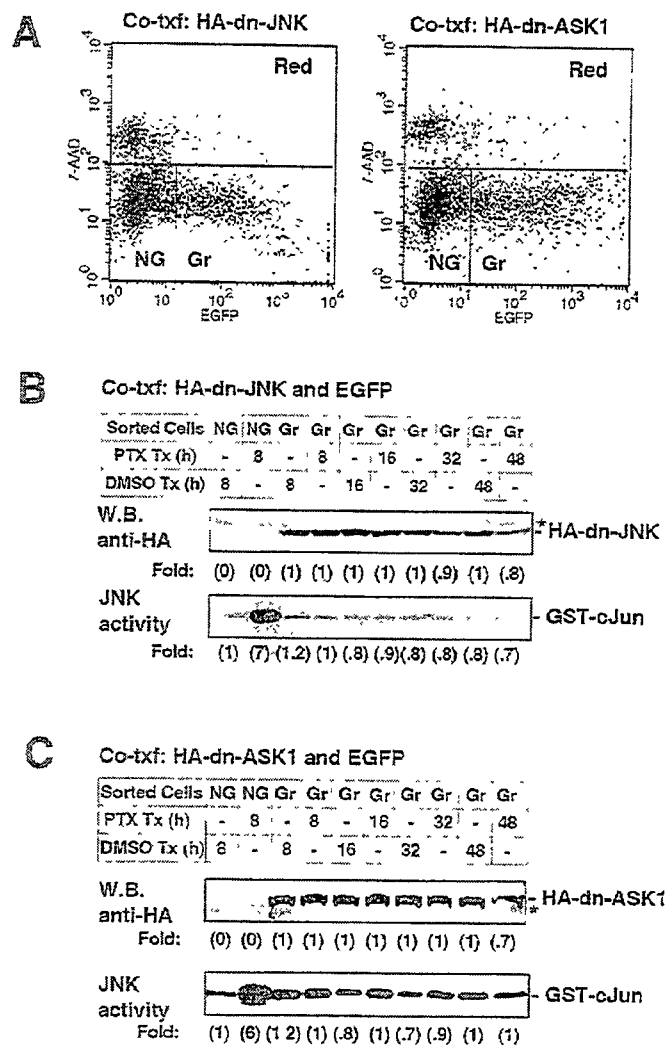
Apoptotic effects of paclitaxel treatment on transfected cells

Human ovarian cancer BR cells were cotransfected with pEGFP and empty (Control) or dominant negative (dn) expression vectors for 24 h, treated with 1 μ M paclitaxel for 16 h or 48 h, and stained with phycoerythrin-conjugated annexin-V and 7-amino actinomycin D (7-AAD) followed by flow cytometric analyses. Percentages of early apoptotic cells (annexin-V positive / 7-AAD negative) among green transfected populations, which were set as 100%, are shown as the mean $\pm$ S.E. (n = 6) of duplicates from three independent experiments. Statistical data in the right column are comparisons between DMSO- and paclitaxel-treatment (horizontal comparisons). The symbols (*, ‡) indicate significant differences ($p < 0.05$) between cells expressing dn-vectors and cells expressing control vector (*italicized values*) within each treatment group (vertical comparisons).

Tx time	Expressed Vectors	DMSO	Paclitaxel	Statistics
16 h	Control	11.5 $\pm$ 0.3	17.7 $\pm$ 0.2	$p < 0.05$
	dn Ras	6.5 $\pm$ 0.2*	9.6 $\pm$ 0.8‡	$p < 0.05$
	dn ASK1	8.5 $\pm$ 0.5	9.5 $\pm$ 0.4‡	<i>n.s.</i>
	dn Rac	5.0 $\pm$ 0.4*	5.9 $\pm$ 0.3‡	<i>n.s.</i>
	dn JNKK	8.1 $\pm$ 0.3	9.5 $\pm$ 1.1‡	<i>n.s.</i>
	dn JNK	7.3 $\pm$ 0.5*	8.7 $\pm$ 1.0‡	<i>n.s.</i>
48 h	Control	16.5 $\pm$ 1.5	33.1 $\pm$ 1.3	$p < 0.05$
	dn Ras	13.9 $\pm$ 0.8	33.8 $\pm$ 0.7	$p < 0.05$
	dn ASK1	17.8 $\pm$ 1.0	28.1 $\pm$ 1.2	$p < 0.05$
	dn Rac	13.6 $\pm$ 0.9	37.8 $\pm$ 1.4	$p < 0.05$
	dn JNKK	14.9 $\pm$ 0.6	31.6 $\pm$ 0.8	$p < 0.05$
	dn JNK	14.3 $\pm$ 1.1	32.1 $\pm$ 1.3	$p < 0.05$



(Figure 2) Expression of dn-ASK1, -Rac, -JNKK, or -JNK significantly inhibits apoptosis induced by paclitaxel treatment for 16 h. Apoptosis among transfected BR cells were analyzed by staining profiles of PE-conjugated annexin-V and 7-AAD. *A*, Comparisons of paclitaxel (PTX)-induced apoptosis among cells expressing control vector or dn-vectors after paclitaxel treatment for 16 h. *B*, Comparisons of apoptosis among cells expressing different vectors after 48-h treatment with paclitaxel. Bars in figures are calculated from the data in Table I using the formula: $100\% \times (\% \text{ in paclitaxel-treated cells} / \% \text{ in DMSO-treated cells})$.



(Figure 3) Isolation of transfected cells coexpressing green fluorescent protein and verification of expression of dn-signaling proteins in these cells during the period of treatment up to 48 h. *A*, Twenty-four h after cotransfection with pEGFP and dn-expression vectors, transfected cells were isolated by flow cytometric sorting using expression of green fluorescent protein as a selectable marker. Examples of isolating cells transfected with dn-JNK and dn-ASK1 are shown. Three regions, Gr (green), NG (Non-green), and Red (7-AAD positive) were selected to represent live transfected cells (coexpressing pEGFP), live non-transfected cells (EGFP negative), and dead cells, respectively. *B* & *C*, Western blot analyses and immunocomplex kinase assays were used to measure transfected protein levels and endogenous JNK activity, respectively. Expression of HA-dn-JNK or dn-ASK1-HA was not detected in non-green cells (NG) but was substantial in green transfected cells (Gr) and those protein levels remained largely intact up to 48 h of treatment. Efficient suppression of endogenous JNK activity by expression of dn-JNK or dn-ASK1 were demonstrated by the kinase assay using GST-cJun as substrate for JNK. Data shown are from a representative experiment, which was repeated twice with comparable results. Nonspecific bands also recognized by the 12CA5 antibody are labeled with asterisks (*).

mechanisms.

EXPRESSION OF DN SIGNALING PROTEINS OF THE JNK/SAPK PATHWAY DOES NOT ALTER CELL CYCLE PROFILES OF PACLITAXEL-TREATED CELLS.

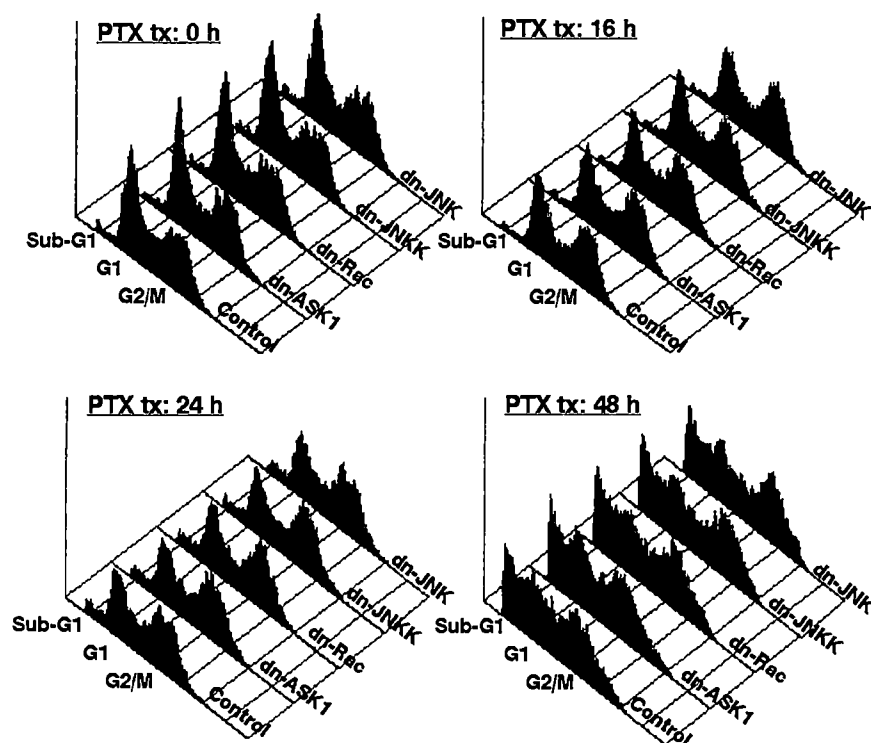
To investigate whether these dn signaling proteins inhibited PTX-induced apoptosis through regulation on cell cycle progression, we analyzed cell cycle profiles of cells expressing dn signaling proteins at 16, 24, and 48 h of PTX treatment. Compared with cells expressing control vector, expression of dn ASK1, dn Rac, dn JNKK, or dn JNK did not change cell cycle profiles, nor interfere with mitotic arrest of cells after PTX treatment (**Figure 4**).

APOPTOTIC CHARACTERISTICS IN PACLITAXEL-TREATED CELLS ARE ABROGATED BY BLOCKAGE OF THE JNK/SAPK PATHWAYS.

To examine the time course of inhibition of apoptosis by dn signaling proteins, we treated isolated cells expressing control vector or dn signaling proteins with PTX and quantified DNA fragmentation with ELISA. Expression of dn ASK1, dn Rac, dn JNKK, or dn JNK significantly inhibited PTX-induced DNA fragmentation up to 16 h, whereas the inhibition declined at 24 h and completely disappeared at 36 h of treatment (**Figure 5A**). In transfected cells treated with PTX for 16 h, expression of dn ASK1, dn Rac, dn JNKK, or dn JNK significantly inhibited caspase-3 activation by PTX (**Figure 5B**) and suppressed PTX-induced PARP cleavage (**Figure 5C**). These results are consistent with data given above for AV binding (**Table I and Figure 2**), again indicating that inhibition of the JNK/SAPK signaling pathways transiently protects ovarian cancer cells from PTX-induced apoptosis.

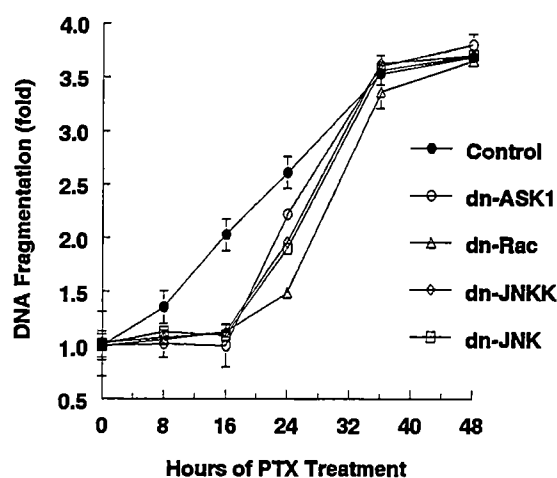
OVEREXPRESSION OF WT ASK1, WT RAC, OR WT JNK PROMOTES APOPTOSIS.

Our data would predict that overexpression of wt signaling proteins of the JNK/SAPK pathways may promote PTX-induced apoptosis. Flow cytometric analyses on AV binding confirmed that overexpression of wt ASK1, wt Rac, or wt JNK significantly ($p < 0.05$) promoted apoptosis in both DMSO-treated and 16 h-PTX treated BR cells (**Figure 6A**). Consistent with flow cytometric data,

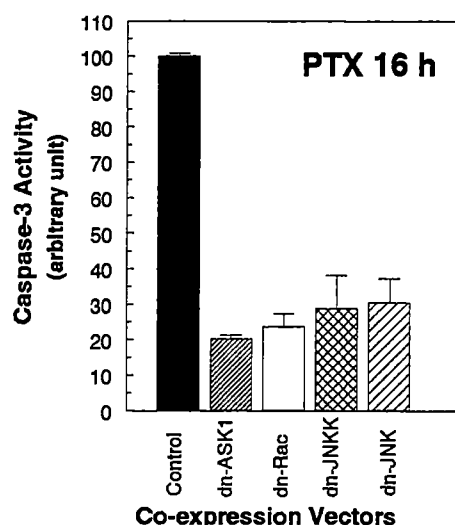


(Figure 4) Expression of dn-signaling proteins does not alter the cell cycle profiles nor prevent the G2/M arrest of paclitaxel-treated cells. Twenty-four h after transfection, cells expressing control or dn-signaling proteins were isolated by flow cytometric sorting, treated with paclitaxel for 16, 24, or 48 h, and analyzed by flow cytometry using propidium iodide-stained DNA content of each cell as parameter for the cell cycle profiles.

A



B

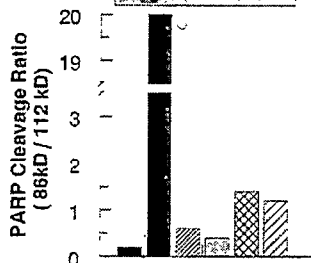


C

PTX 16 h

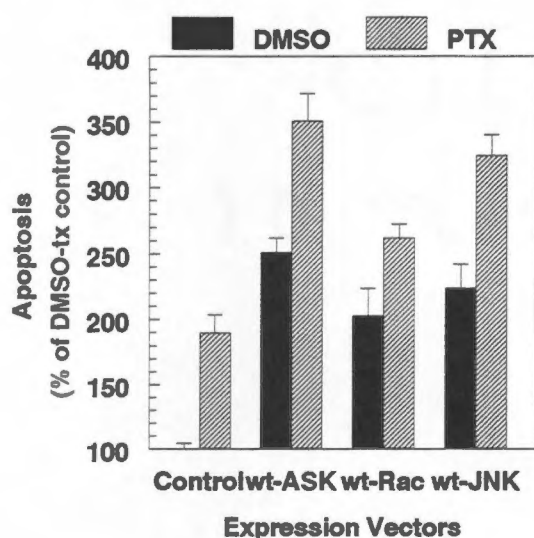
PTX Tx	-	+	+	+	+	+
Control	+	+	-	-	-	-
dn-ASK1	-	-	+	-	-	-
dn-Rac	-	-	-	+	-	-
dn-JNKK	-	-	-	-	+	-
dn-JNK	-	-	-	-	-	+

W. B. anti-PARP

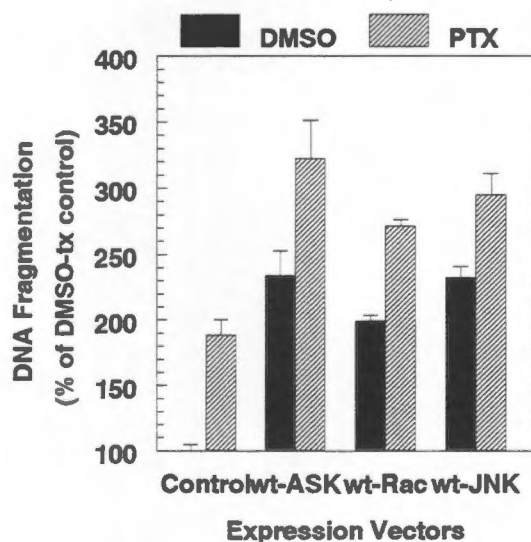


(Figure 5) Expression of dn-ASK1, -Rac, -JNKK, or -JNK transiently inhibits paclitaxel-induced DNA fragmentation, caspase-3 activation, and cleavage of PARP. Twenty-four h after transfection, sorted cells expressing different vectors were treated with paclitaxel and apoptotic features were analyzed. *A*, Time course of paclitaxel-induced DNA fragmentation measured by the Cell Death Detection ELISA. Data shown are mean $\pm$ S.E. ($n = 6$) of triplicates from two independent experiments. *B*, After treated with paclitaxel for 16 h, caspase-3 activity in cells expressing control vector or dn-vectors was measured using Ac-DEVD-pNA as a colorimetric substrate. Levels of caspase-3 activity are calculated by subtracting the mean of OD405 readings of DMSO-treated, control-transfected cells from each reading of paclitaxel-treated cells, then the resultant levels of paclitaxel-treated, control-transfected cells are set as 100 arbitrary units. Data shown are mean $\pm$ S.E. ($n = 6$) of triplicates from two independent experiments. *C*, After treated with paclitaxel (PTX) for 16 h, cleavage of PARP among transfected cells was analyzed by Western blot. The ratios between 112-kD (intact) and 86-kD (cleaved) species of PARP were compared in the bar graph. Data shown are from a representative experiment, which was repeated three times with comparable results. A nonspecific band also recognized by this anti-PARP antibody is labeled by an asterisk (*).

A

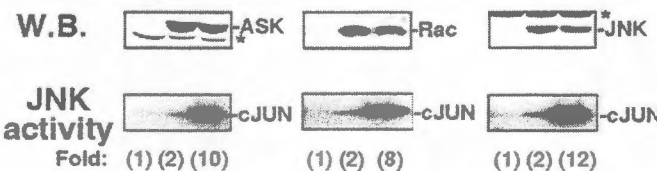


B



C

PTX 3 h	-	-	+	-	-	+	-	-	+
wt-ASK	-	+	+	-	-	-	-	-	-
wt-Rac	-	-	-	-	+	+	-	-	-
wt-JNK	-	-	-	-	-	-	-	+	+



(Figure 6) Overexpression of wt-ASK1, wt-Rac or wt-JNK1 promotes apoptosis. *A*, BR cells were cotransfected with pEGFP and expression vectors for control, wt-ASK1, wt-Rac, or wt-JNK1 for 24 h, treated with DMSO (solid bars) or 1 μ M paclitaxel (hatched bars) for 16 h, stained with PE-conjugated annexin-V and 7-AAD, and analyzed by flow cytometry as described in Fig. 1. *B*, BR cells overexpressing wt-ASK1, wt-Rac, or wt-JNK1 were isolated by flow cytometric sorting, treated with DMSO (solid bars) or 1 μ M paclitaxel (hatched bars) for 16 h, and DNA fragmentation was quantified by ELISA. OD405 readings of control-transfected cells treated with DMSO are set as 100 arbitrary unit. Data shown are mean $\pm$ S.E. ($n=8$ or 4) from several independent experiments. *C*, Isolated BR cells expressing control vector, HA-wt-ASK1, HA-wt-JNK1, or Myc-wt-Rac were treated with DMSO or 1 μ M paclitaxel (PTX) for 3 h and expression of these epitope-tagged, wt-signaling proteins were confirmed by Western blot analyses with an anti-HA (12CA5) antibody or an anti-Myc (9E10) antibody. Endogenous JNK activities in these lysates were measured by immunocomplex kinase assay using an anti-JNK antibody for immunoprecipitation and GST-c-Jun as substrate. Non-specific bands also recognized by the 12CA5 antibody are labeled by asterisks (*).

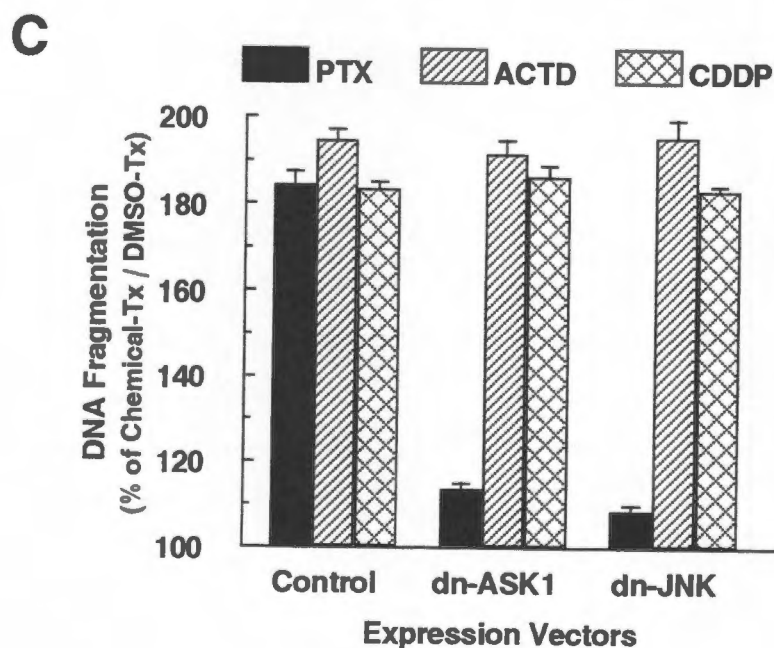
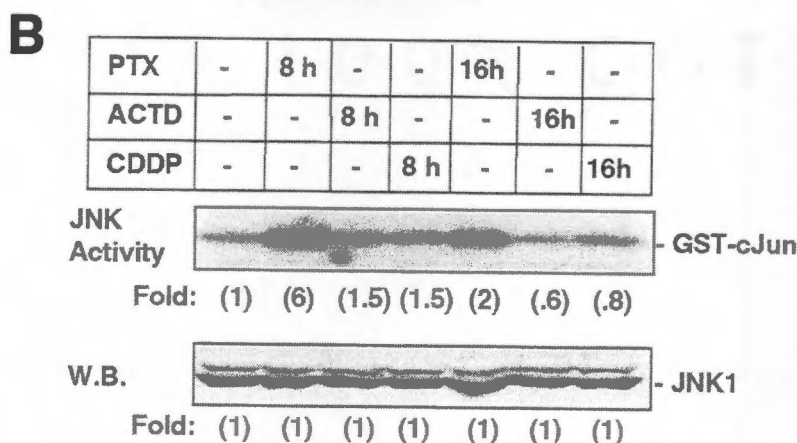
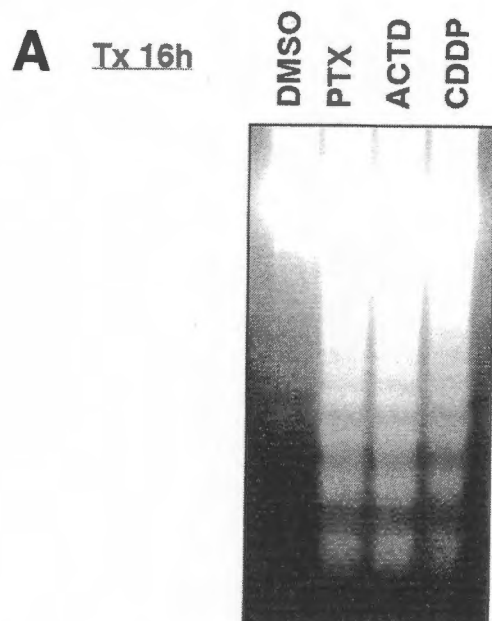
overexpression of wt ASK1, wt Rac, or wt JNK also significantly ($p < 0.05$) increased basal and PTX-induced DNA fragmentation (**Figure 6B**) and the increases in apoptosis measured by two independent methods are very similar. Western blot analyses with an anti-HA epitope antibody (12CA5) and an anti-Myc epitope antibody (9E10) confirmed expression of HA-wt ASK1, HA-wt JNK, and Myc-wt Rac (**Figure 6C**, upper panel) and immunocomplex kinase assays verified the augmentation of PTX-induced JNK activation by overexpressed signaling proteins (**Figure 6C**, lower panel). The increases in the apoptosis induced by PTX above those due to overexpression of wt signaling proteins were the same as that in PTX-treated, control-transfected cells (**Figures 6A & 6B**). Therefore, the effects of PTX and overexpression of wt signaling proteins appear to be additive. However, among cells overexpressing these wt signaling proteins, the 8-12 fold JNK activation induced by PTX (**Figure 6C**) was not proportional to the 2.5-3.5 fold increase of apoptosis (**Figures 6A & 6B**), suggesting that activation of JNK is not linearly related to apoptosis.

**SUPPRESSION OF JNK/SAPK ACTIVATION SPECIFICALLY INHIBITS
APOPTOSIS INDUCED BY 16 H-PACLITAXEL TREATMENT BUT
NOT THAT INDUCED BY ACTINOMYCIN-D OR CISPLATIN.**

To examine whether the JNK/SAPK signaling cascade plays a specific role in apoptosis resulting from microtubular dysfunction, we compared JNK activations and cytotoxicities in ovarian cancer cells treated with PTX or treated with two DNA-targeting agents, actinomycin-D (ACTD) and cisplatin (CDDP). Treatment with 5 μ M actinomycin-D or 50 μ M cisplatin for 16 h induced comparable ladder pattern of DNA fragmentation with that induced by PTX treatment (**Figure 7A**), whereas only PTX significantly activated JNK (**Figure 7B**). Moreover, expression of dn ASK1 or dn JNK only inhibited DNA fragmentation induced by 16 h-PTX treatment but not that induced by actinomycin-D or cisplatin (**Figure 7C**).

**SUPPRESSION OF THE JNK/SAPK SIGNALING CASCADE DOES NOT INHIBIT
PHOSPHORYLATION OF BCL-2 INDUCED BY PACLITAXEL.**

To determine whether PTX-activated JNK/SAPK is required for phosphorylation of Bcl-2 in



(Figure 7) Expression of dn-ASK1 or dn-JNK specifically inhibits DNA fragmentation induced by paclitaxel but not that induced by actinomycin-D or cisplatin.

A, Treatment with 1 μ M paclitaxel (PTX), 5 μ M actinomycin-D (ACTD), or 50 μ M cisplatin (CDDP) for 16 h induced the ladder pattern of DNA fragmentation resolved in 1.6 % agarose gel. B, BR cells were treated with paclitaxel, actinomycin-D, or cisplatin for 8 or 16 h, and JNK activities and JNK protein levels were measured by immunocomplex kinase assay and Western blot analysis, respectively. Relative intensities of bands are shown as fold (shown in parentheses) of DMSO-treated control. C, Twenty-four h after transfection, sorted cells expressing control vector, dn-ASK1, or dn-JNK were treated with paclitaxel (solid bars), actinomycin-D (hatched bars), or cisplatin (checked bars) for 16 h. DNA fragmentation were quantified by ELISA. Data shown are mean $\pm$ S.E. (n=3) from a representative experiment.

PTX-treated cells, we cotransfected BR cells with pEGFP and dn expression vectors and treated cells with 1 μ M PTX for 16 h. Following treatment, green fluorescent positive (transfected) and negative (non-transfected) cells were analyzed separately for phosphorylation of Bcl-2. Phosphorylated Bcl-2 bands exhibiting slower mobility on Western blot were identified from both transfected and non-transfected cells, irrespective of which dn vectors were overexpressed (**Figure 8A**). To confirm these results by an alternative approach, we cotransfected cells with pEGFP and dn vectors, isolated transfected cells by flow cytometric sorting, then treated control- or dn vector-expressing cells with PTX or DMSO for 16 h and analyzed the status of Bcl-2 phosphorylation. Consistent with previous experiments, treatment with PTX induced phosphorylation of Bcl-2 in all transfected cells, including those expressing control vector or vectors for dn ASK1, dn Rac, dn JNKK, or dn JNK (**Figure 8B**). Collectively, these results indicated that inhibition of the JNK/SAPK signaling cascade did not influence PTX-induced phosphorylation of Bcl-2.

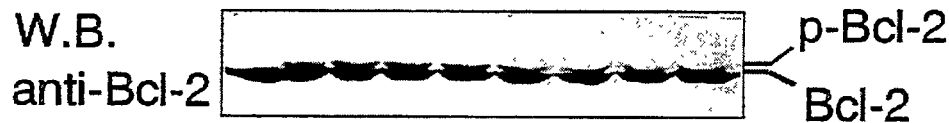
DISCUSSION

Activation of the JNK/SAPK signaling pathways has been mechanistically implicated in regulation of apoptosis (19)(30)(35)(36)(38), however, the roles of JNK/SAPK in promoting (14)(48)(78) or preventing apoptosis (54)(58) differ, depending on both cell type and apoptosis-triggering stimuli (30)(35). Furthermore, in addition to apoptosis, JNK/SAPK activation may be involved in proliferation (7)(67) and oncogenic transformation (60). On the other hand, apoptosis itself can be considered as a form of stress, hence, JNK/SAPK activation may be a stress response secondary to apoptosis, rather than a primary mediator in apoptotic pathways (30). We have previously demonstrated that in BR ovarian cancer cells treated with PTX, activation of JNK/SAPK reaches a peak at 2 h when apoptosis is still minimal (73), suggesting that JNK/SAPK activation is not a secondary response to PTX-induced apoptosis.

One of the early changes in apoptotic cell membranes is externalization of phosphatidylserine, which exerts high affinity to AV. Therefore, increased binding of AV is a sensitive indicator for apoptosis (44). Apoptotic cells are also characterized by increased activities of caspases, cleavage of 112-kD poly ADP-ribose polymerase (PARP) into a 86-kD species, and DNA fragmentation (15)(70). We have previously demonstrated that PTX induces characteristic apoptotic morphology (9)(72) and DNA fragmentation assayed by both gel electrophoresis and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method in ovarian cancer cells (72). By analyzing these

A

dn-ASK1	-	NG	-	-	-	Gr	-	-	-
dn-Rac	-	-	NG	-	-	-	Gr	-	-
dn-JNKK	-	-	-	NG	-	-	-	Gr	-
dn-JNK	-	-	-	-	NG	-	-	-	Gr
PTX16h	-	+	+	+	+	+	+	+	+

**B**

dn-ASK1	-	-	+	+	-	-	-	-	-
dn-Rac	-	-	-	-	+	+	-	-	-
dn-JNKK	-	-	-	-	-	-	+	+	-
dn-JNK	-	-	-	-	-	-	-	+	+
PTX16h	-	+	-	+	-	+	-	+	-



(Figure 8) Blockage of the JNK/SAPK signaling cascade does not interfere with paclitaxel-induced phosphorylation of Bcl-2. *A*, BR cells were cotransfected with pEGFP and dn-expression vectors for 24 h, treated with paclitaxel for 16 h, then transfected (green, *Gr*) and non-transfected (non-green, *NG*) populations were separately isolated by flow cytometric sorting. By Western blot analyses, phosphorylated Bcl-2 (*p-Bcl-2*) was identified as an additional band exhibiting slower mobility on SDS-PAGE when it was compared with Bcl-2 in DMSO-treated control. *B*, Cotransfected BR cells with pEGFP and different dn-expression vectors were isolated by flow cytometric sorting, treated with either paclitaxel (*PTX*) or DMSO for 16 h, and analyzed for the status of Bcl-2 phosphorylation by Western blots. Data shown are from a representative experiment, which was repeated twice with comparable results.

biochemical apoptotic characteristics in PTX-treated cells overexpressing wt or dn signaling proteins of the JNK/SAPK pathways, our results herein demonstrate an apoptosis-promoting role for JNK/SAPK in ovarian cancer cells treated with PTX (Figures 2, 5, & 6). Since suppression of the JNK/SAPK signaling cascade only protects cells from PTX-induced apoptosis but not from apoptosis induced by DNA targeting agents, actinomycin-D or cisplatin (Figure 7), the present study further suggests an apoptosis-promoting role of the JNK/SAPK cascade specifically in apoptotic process resulting from microtubular dysfunction.

JNK/SAPK has been shown to be involved in activation of caspases that are required for execution of the apoptotic process (8)(10)(14)(62)(65). The role of JNK/SAPK in activation of caspases is, however, not straightforward. JNK/SAPK could be either upstream (14)(65) or downstream of (8)(10)(62) caspase activation, depending on cell type and apoptosis-initiating agents. Since PTX has been shown to activate caspases (28)(66), we analyzed the role of JNK/SAPK in caspase-3 activation of PTX-treated cells. Our data show that inhibition of the JNK/SAPK cascade prevented PTX-induced caspase-3 activation, PARP cleavage, and DNA fragmentation (Figure 5), indicating that JNK/SAPK is upstream of caspase-3 activation in PTX-initiated apoptosis. These and other studies, where anticancer agents were used to induce apoptosis (14)(65), suggest that JNK/SAPK acts upstream of caspase activation in chemotherapy-initiated apoptosis.

Intriguingly, while expression of dn Ras decreased basal levels of apoptosis (Table I), it did not significantly abrogate PTX-induced apoptosis (Table I & Figure 2). Although we previously reported that both Ras and ASK1 are required for optimal activation of JNK/SAPK by PTX (73), results of the present study failed to clarify a pro- or anti-apoptosis role for Ras in the apoptotic process initiated by PTX. In addition to activation of MAPK and JNK/SAPK pathways, Ras may also provide a survival signal that is mediated by the phosphoinositide 3'-OH kinase (PI 3-K)-dependent activation of the protein kinase B/Akt (24). Furthermore, inhibition of Ras activity induces apoptosis (3) and exerts synergistic inhibition of cell growth with PTX (52). These studies further collaborate the complex and multifunctional roles of Ras in both cell growth and apoptosis.

In contrast to Ras, it is clear that the related small G-protein, Rac, has a critical role in PTX-induced apoptosis at 16 h, probably via activation of JNK/SAPK. Previous studies have demonstrated that Rac activate the JNK/SAPK pathway (2)(16)(51)(56)(74) and our studies clearly indicate that MT damage activates Rac (Figure 6C) and that Rac is at least partially responsible for JNK activation and the resulted apoptosis (Figures 2, 5, & 6). It is interesting that among the upstream signaling proteins employed in

this study, the wt ASK1 appeared to have the highest apoptosis-promoting effect (**Figure 6**). Possible explanations for these observations include that ASK1, when overexpressed, may also activate other apoptosis-related kinase such as p38 (12)(29)(55).

Since expression of dn signaling proteins remained largely intact at 48 h after DMSO treatment (**Figure 3**), the disappearance of protection from basal apoptosis can not be explained by a decrease of dn signaling proteins. Forty-eight hours of treatment with DMSO (vehicle control) at a 0.1% final concentration did not activate JNK/SAPK (73) or initiate apoptosis. However, liposome-mediated transfection with control DNA into BR cells was observed to exert mild cytotoxicity and caspase-3 activation, hence it is possible that transfection by itself may initiate an apoptotic process that eventually overrides the transient protection by dn signaling proteins of the JNK/SAPK pathway. Nevertheless, treatment with PTX for 48 h caused a further significant increase in apoptosis above the relatively high basal levels (**Figures 2B & 5A, Table I**), suggesting coexistence of multiple apoptotic pathways. This suggestion is supported by the ability of dn signaling proteins to inhibit apoptosis in both control and PTX-treated cells at 16 h- but not 48 h-treatment. Therefore, the pathways leading to apoptosis after the 16 h- versus 48 h-treatment appear to be fundamentally different.

Multiple mechanisms have been suggested to be involved in PTX-induced apoptosis, such as: abortive mitosis after PTX-induced G₂/M block (33)(43)(77), activation of p34^{cdc2} (17)(27)(66) and other CDKs (18)(47), activation and local release of an apoptosis-inducing cytokine (39), and induction of transcription regulators and enzymes that modulate apoptosis (53). Whereas overexpression of wt ASK1, wt Rac, or wt JNK promotes apoptosis in BR cells (**Figure 6**), expression of dn ASK1, dn Rac, dn JNKK, or dn JNK only transiently inhibits PTX-induced apoptosis (**Table I & Figure 2**). Protection from PTX-induced DNA fragmentation by expression of these dn signaling proteins disappeared when cells were treated for 24 to 48 h (**Figure 5A**). These results are in agreement with data described in **Part III** that PTX-induces JNK/SAPK activation in BR cells is transient with a peak at 2-4 h and declines afterward (73). Since suppression of the JNK/SAPK signaling cascade does not alter cell cycle profiles nor interfere with the PTX-induced mitotic arrest that peaks at 24 h of PTX treatment (**Figure 4**), G₂/M block of the cell cycle may mainly account for the later phase of PTX-induced apoptosis. Our results further suggest that the late phase of PTX-induced apoptosis is independent of JNK/SAPK activity. Several recent studies have suggested that catastrophic activity of CDKs may be a terminal effector in apoptotic pathway (23)(41).

Results in this study do not support the hypothesis that activated JNK/SAPK is required for phosphorylation of Bcl-2 in PTX-treated cells (45). Bcl-2 is known to protect cells from apoptosis (37) and PTX has been shown to induce both phosphorylation of Bcl-2 and apoptosis (6)(21)(20)(61). However, the roles of Bcl-2 phosphorylation in promoting (6)(11)(13)(21)(61) or inhibiting apoptosis (25)(31) remain controversial.

Some reports suggest that Bcl-2 may act upstream of JNK/SAPK (40)(57). In BR cells, however, JNK/SAPK activation peaked at 2 h (73), while Bcl-2 phosphorylation and the G₂/M arrest of the cell cycle required 12 to 16 h of PTX treatment. These temporal differences suggest that phosphorylated Bcl-2 is unlikely to act upstream of JNK/SAPK. The observations that inhibition of JNK/SAPK did not interfere with PTX-induced G₂/M arrest and Bcl-2 phosphorylation (**Figures 4 & 8**) suggest that PTX-activated JNK/SAPK is independent of Bcl-2 phosphorylation occurring in PTX-treated cells. Recent reports further demonstrate that phosphorylation of Bcl-2 occurs only in cells blocked at G₂/M phase after PTX treatment (20)(64). Therefore, JNK/SAPK activation and Bcl-2 phosphorylation may reside in distinct, independent pathways. Instead of the JNK/SAPK cascade, PKA activation has been suggested to account for Bcl-2 phosphorylation in cells with MT damages (68). The role(s) of Bcl-2 phosphorylation in regulating apoptosis in general and in PTX-induced apoptosis in particular apparently requires further clarification.

Our results, for the first time, identify a Bcl-2 phosphorylation-independent role of JNK/SAPK in promoting PTX-induced apoptosis and demonstrate that multiple mechanisms are involved in apoptosis resulting from MT damage, including a JNK/SAPK-dependent early phase and a JNK/SAPK-independent late phase.

APPENDIX

Several experiments and results that were not included in the paper (71) are described in this section.

FLUORESCENT MICROSCOPY

To analyze early apoptosis and cell death, BR cells growing to 50 % confluence on chambered coverglass (Lab-Tek, Nunc Inc, Naperville, IL) were treated with 1 μ M PTX for 8 h, stained with FITC-

conjugated AV (Pharmingen) and PI for 10 min according to the manufacturer's protocol, and observed at the 200X magnification under the fluorescent microscope (Olympus, Japan). To detect apoptosis in transfected cells, BR cells were transfected with pEGFP, treated with PTX or DMSO control for 8 h, stained with PE-conjugated AV (Pharmingen) for 10 min, and examined with fluorescent microscopy. Results are shown in **Part I, Figure 2**.

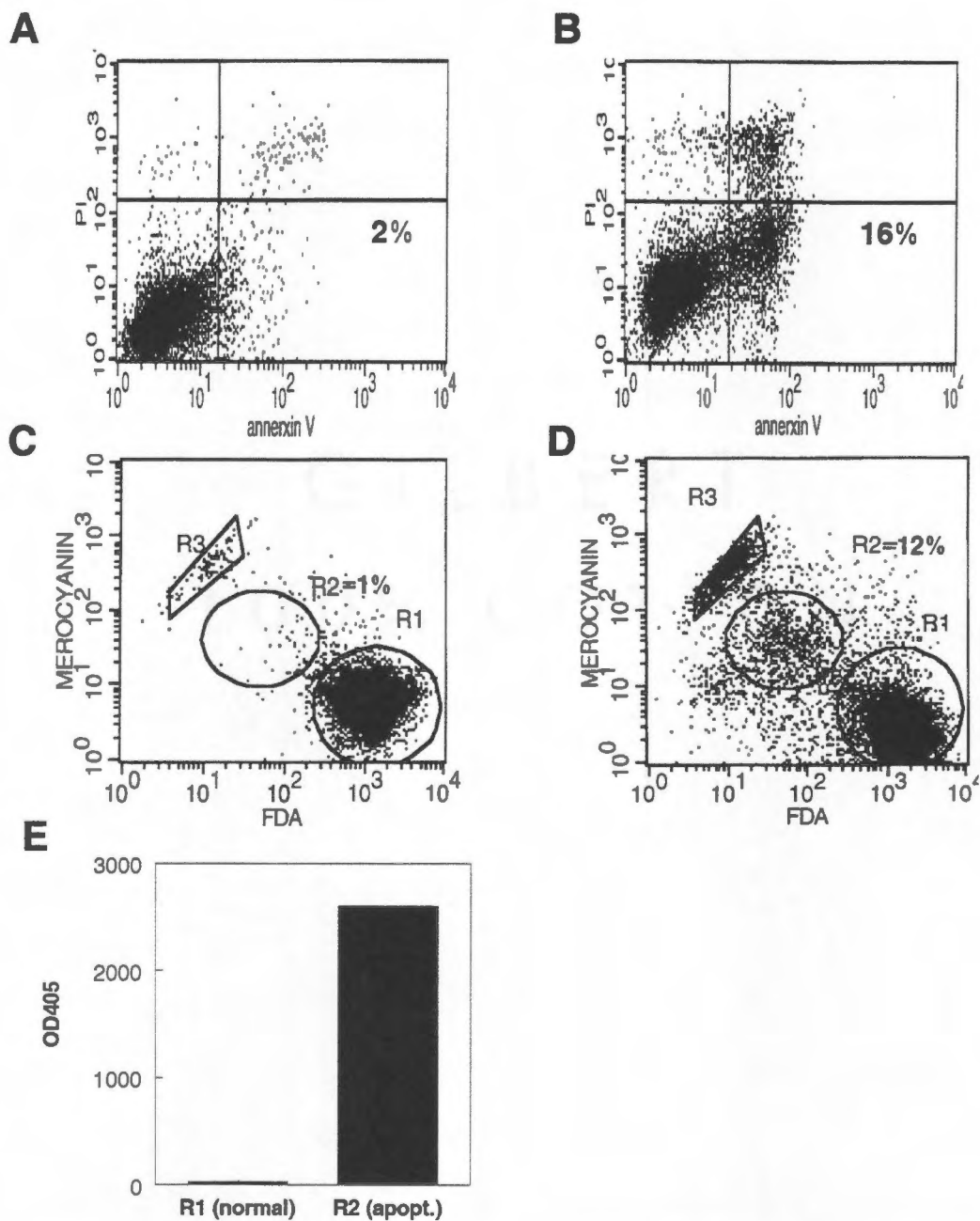
FLOW CYTOMETRIC SORTING USING OTHER MARKERS

In an attempt to isolate apoptotic cells by flow cytometric sorting, I have used either FITC-conjugated AV/PI or FDA/MC540 (both from Sigma) as markers for apoptotic cells. Both methods were able to differentiate apoptotic cell populations from dead or normal cells (**Figure 9**). However, FITC-conjugated AV is not economically practical as an apoptotic marker for sorting because of its high cost.

The procedures for the combined uses of FDA and MC540 are described here. PTX -treated cells were trypsinized, resuspended in DMEM/F12 with 10% NBS, incubated with both 0.02 ng/ml FDA and 1 μ M MC540 at 37°C for 30 min, then cells were put in ice before flow cytometric analyses and/or sorting were performed. Three populations were identified: R1 (high FDA/low MC540), R2 (intermediate FDA/intermediate MC540), and R3 (low FDA/high MC540) represented normal, apoptotic, and dead cells, respectively (**Figures 9C & D**). Cells in R1 and R2 regions were separately collected and the apoptotic characteristics were verified by a DNA fragmentation ELISA (**Figure 9E**). The combined use of FDA/MC540 is an economical and specific (**Figure 9E**) method to isolate a large number of apoptotic cells, from which we can study biochemical changes, and even discover novel factors, specifically associated with apoptosis.

CELL CYCLE ANALYSES OF SORTED APOPTOTIC CELLS

At different time intervals of 1 μ M PTX treatment, apoptotic BR cells were isolated from the total PTX-treated cells by flow cytometric sorting according to their FDA/MC540 profiles as described in **Figure 9**. Some left-over total PTX-treated cells were used as the non-sorted control. Both isolated apoptotic cells and total cells (non-sorted) were fixed with 70% ethanol overnight, and analyzed for cell cycle profiles by flow cytometry.



(Figure 9) Apoptotic analyses and sorting. Apoptosis in BR cells treated with DMSO control (*A, C*) or paclitaxel (*B, D*) were analyzed by staining profiles of AV/PI (*A, B*) or FDA/MC (*C, D*). *D*, Regions R1 and R2 which represent normal and apoptotic cell populations, respectively, were collected separately and analyzed with a DNA fragmentation ELISA (*E*).

Patterns of cell cycle profiles derived from PI staining in apoptotic cells were shifted to the left side (empty areas in **Figure 10**), when compared with that of non-sorted cells (filled areas in **Figure 10**), indicating a lower DNA content in these apoptotic cells. One possible explanation for the observation is that PTX treatment changes cell cycle profiles of apoptotic cells into mainly sub-G₁ and S-phases, which represent apoptotic bodies and cells with aberrantly activated CDKs, respectively. Further biochemical analyses on CDK activities and levels of cell cycle-related proteins should be able to verify this hypothesis. If so, these data would support a role of CDK in execution of apoptosis.

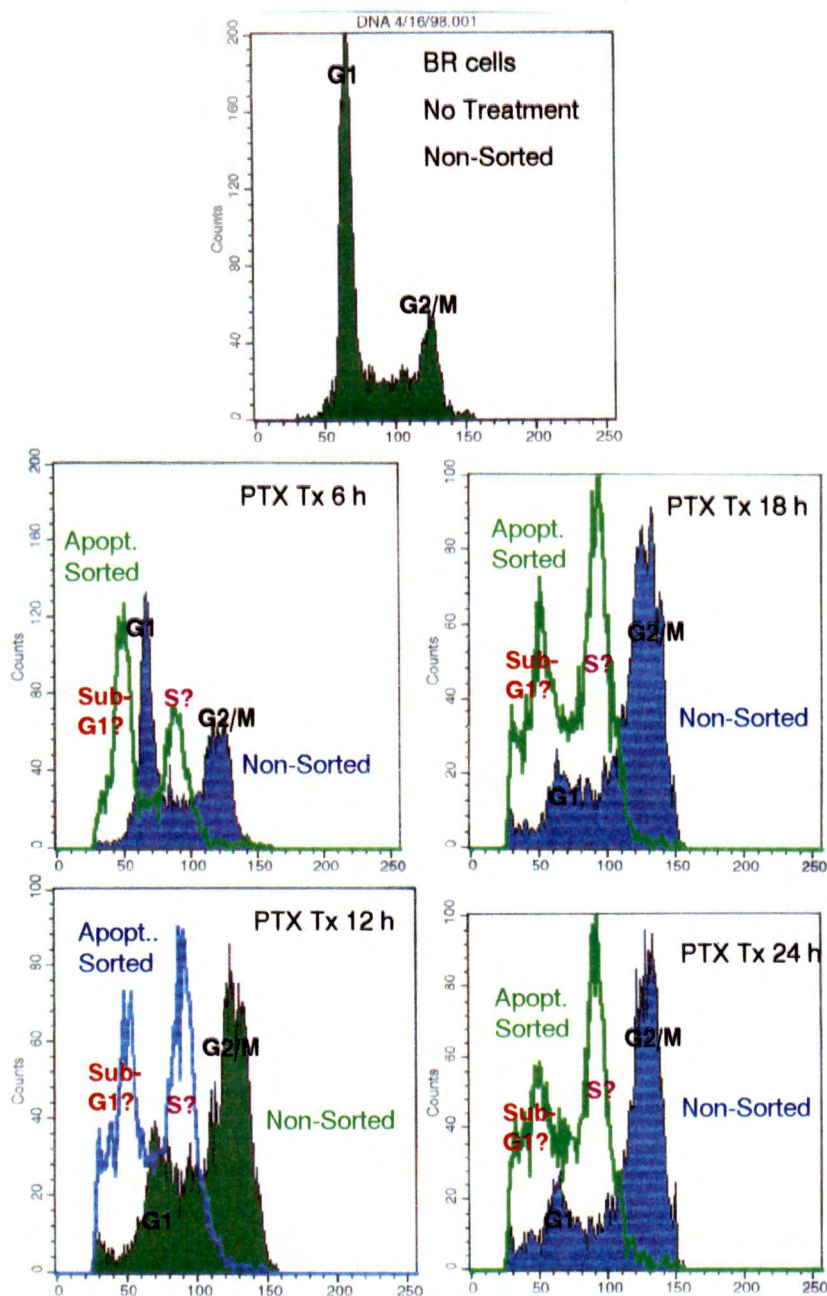
An alternative explanation for this observation is that chromatin becomes more compact in apoptotic cells, restricting the access of PI to bind DNA and resulting in decreased intensities of PI staining (the Y axis of these figures).

SAPONIN PERMEABILIZATION AND CELL CYCLE ANALYSES

In an attempt to isolate PTX-treated cells at different cell cycle phases for further analyses on their kinase activities and various protein levels, I tried to permeabilize live cells using 0.1% saponin (Sigma) in trypsinized cell suspensions, then stained DNA with propidium iodide and analyzed cell cycle profiles. Treatment with saponin sufficiently permeabilized the cell membrane and allowed entry of propidium iodide, resulting in a satisfactory cell cycle analysis and sorting. However, protein quantitation on cell lysates prepared from those sorted cells indicated that most cytoplasmic proteins leaked from saponin-treated cells. Therefore, this method is not suitable for isolating cells according to their cell cycle profiles for further biochemical analyses. Centrifugal elutriation appears to be a better choice for this purpose.

CENTRIFUGAL ELUTRIATION

Centrifugal elutriation was performed according to a previously reported method (4)(5). Ten T150 flask of ovarian cancer BR cells growing to 80% confluence (total $\sim 4.3 \times 10^8$ cells) were trypsinized and resuspended with 30 ml of 5% NBS-DMEM/F12 and kept in ice before centrifugal elutriation was done with a Beckman elutriation centrifuge (model J2-21) equipped with the JE-6B elutriator rotor. A speed-constant (rotor speed set to 2,000 rpm) centrifugal elutriation was performed with the initial flow rate of



(Figure 10) Cell cycle profiles of paclitaxel-induced apoptotic cells. After treatment with paclitaxel for designated hours, apoptotic cells (*Apopt. Sorted*), which were cells in R2 region as described in Figure 9, were isolated with flow cytometric sorting using FDA/MC540 profiles as selectable markers, fixed with 70% ethanol, and analyzed for cell cycle profiles. *Non-sorted* cells were mixed populations of cells in regions R1, R2, and R3 as described in Figure 9.

normal saline set to 10 ml/min which was gradually increased to 45 ml/min. The total of 12 fractions (40 ml per fraction) of elutriated cell suspension were collected. Cells from each fraction were collected by centrifuge at 600 x g for 10 min, resuspended with 1 ml 5% NBS-DMEM/F12, kept in ice before analyzed for the cell cycle profiles and Western blot analyses.

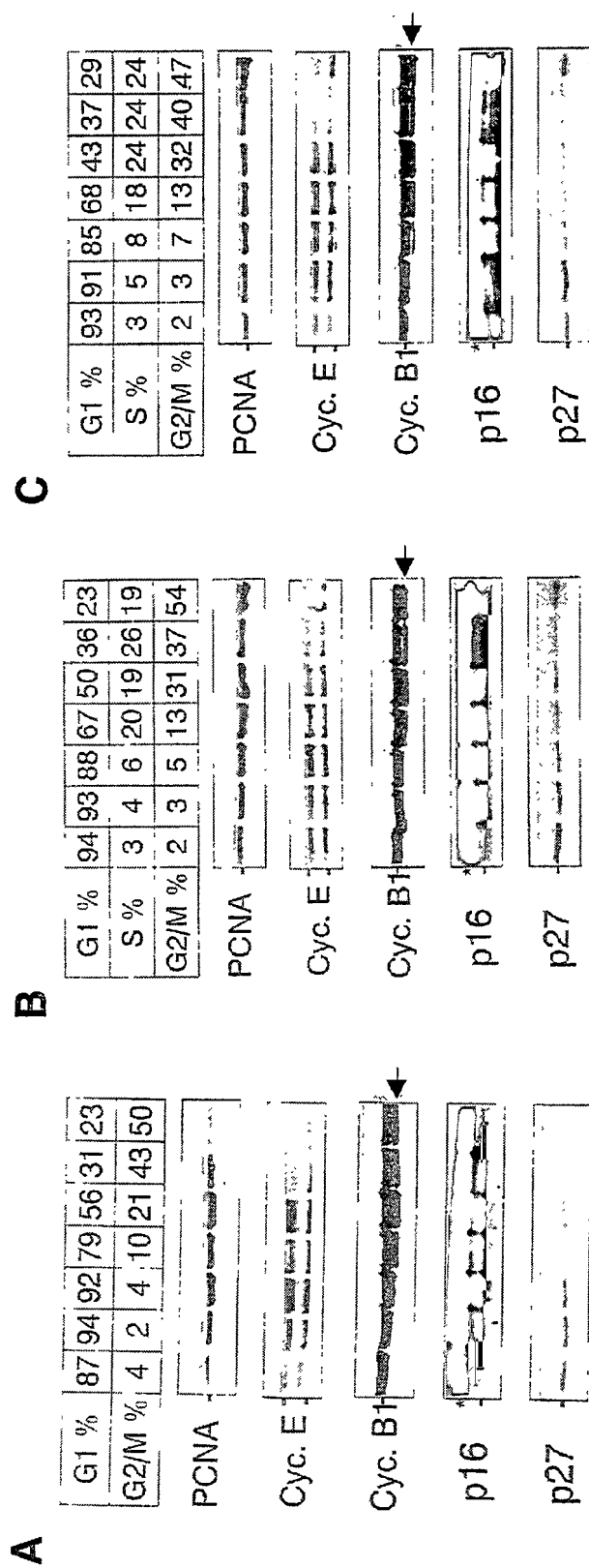
Results of three centrifugal elutriations (**Figure 11**) demonstrate that centrifugal elutriation is a practical method to separate a large number of cells (in the 10^8 range) by the different sizes of cells through the cell cycle. Correlations between cell cycle profiles and cell cycle-related proteins (PCNA, cyclins B1 and E, two CKIs, p16 and p27) also confirm a sufficient fractionation of cells at different phases of the cell cycle by this method.

In addition to the advantage of harvesting a large number of cells in less than 1 h, separation of G₁ from G₂/M phase cells by centrifugal elutriation does not rely on arrest of the cell cycle by chemical treatments or metabolic manipulations, thereby avoiding undesired effects potentially caused by these additional treatments. Nevertheless, since it separates cells by sizes and densities, and cycling cells from one cell line to another may change sizes at different ratios, the rotor speed and the change of flow rates have to be optimized for separation of each cell line.

GST-FUSION PROTEIN PULL DOWN ASSAYS

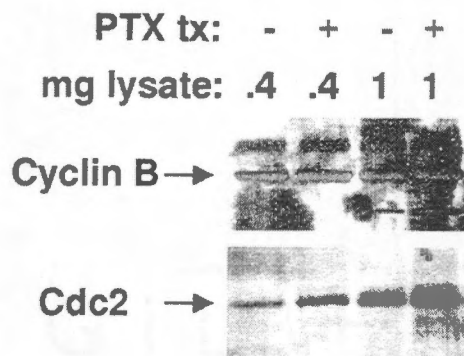
Preparations for purified GST-JNK or GST-JNKK were identical for that for GST-RBD as described in the **Part III, ARIA** section.

Ovarian cancer BR cells were treated with 1 μ M PTX or DMSO control for 16 h and cell lysates were prepared with magnesium-containing lysis buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM MgCl₂, 1 mM EDTA, 1 mM sodium vanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin). Equal amount of cell lysates (400 and 1,000 μ g in parallel experiments) were incubated with 30 μ l of GSH beads bound by excess GST-JNK or GST-JNKK on a rocker at 4°C for 1 h. After three washes with MLB, GSH beads with proteins interacting with JNK or JNKK were spun down, boiled in Laemmli's loading buffer, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and detected by anti-Cdc2 (Santa Cruz #54) or anti-cyclin B1 (Santa Cruz #SC-245) Abs in Western blot. Results shown in **Figure 12** suggest an *in vitro* complex formation among GST-JNK and cyclin B1 and Cdc2.



(Figure 11) Western blot analyses for cell cycle-related proteins in cells fractionated by centrifugal elutriation.

Three sets of fractions of paclitaxel-treated BR cells (A-C), which were separated by sizes through centrifugal elutriation, were analyzed for cell cycle profiles and cell cycle-related proteins. Specific bands recognized by anti-cyclin B1 Ab in Western blot analyses are labeled with arrows. Non-specific bands also recognized by anti-p16 Ab are marked with asterisks. Abbreviations: *PCNA*, proliferating cell nuclear antigen; *Cyc.*, cyclin.



(Figure 12) GST-protein pull down experiments. Ovarian cancer BR cells were treated with 1 uM PTX or DMSO control for 16h and cell lysates were prepared with magnesium-containing lysis buffer. Cell lysates (0.4 and 1 mg in parallel experiments) were incubated with GST-JNK bound to GSH agarose at 4°C for 1 h. After three washes, proteins interacting with GST-JNK thus bound to agarose beads were analyzed with Western blot using anti-Cdc2 and anti-cyclin B Ab (Santa Cruz).

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PART V.

GENERAL DISCUSSION

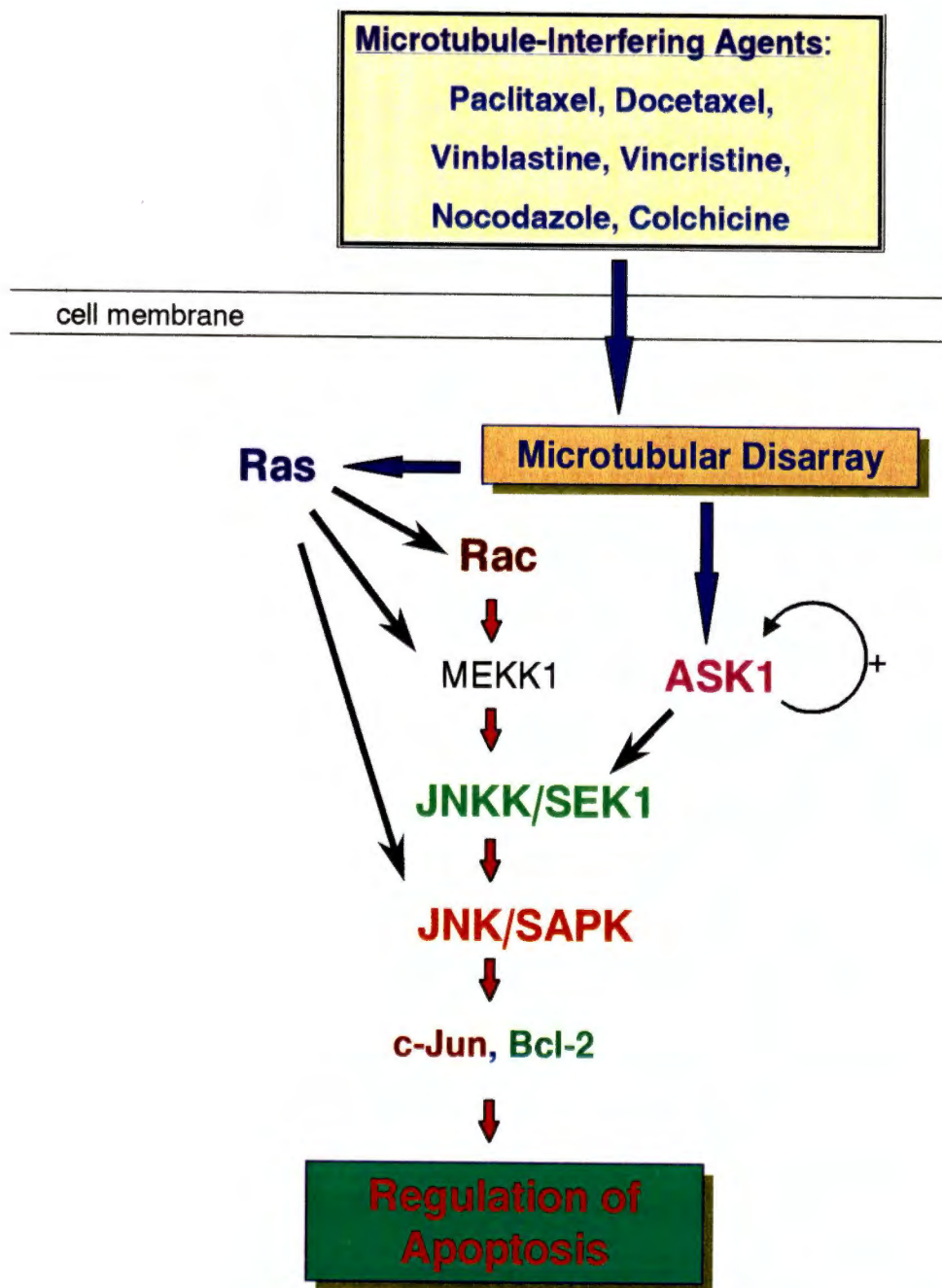
Paclitaxel (Taxol®) is a very useful chemotherapeutic agent in the treatment of ovarian cancer and breast cancer. It has become clear that paclitaxel (PTX)-initiated apoptosis accounts for its anti-tumor effect. Although many studies have associated mitotic arrest of PTX-treated cells with its cytotoxicity against cancer cells (41)(60)(103), the biochemical events downstream of kinetic stabilization of microtubule (MT) dynamics which lead to apoptosis of cancer cells remain largely unclear (42).

Besides the inhibition of mitotic spindle formation, PTX has been demonstrated to have multiple functions, such as: (i) upregulation of aromatase and steroidogenesis (11), (ii) activation of p34^{cdc2} (19)(36)(38)(78), Cdc2-like kinase (77), and other CDKs (73), (iii) stimulation of the local release of TNF- α (46) and of IL-8 expression (49) (47), (iv) transcriptional activation of c-Mos (56), cyclin B1 (56)(55), and the pro-apoptotic Bak (57)(39), Bax (88), the gene *krox-24*, and several enzymes (2'5'-oligoadenylate synthase, cyclooxygenase-2, and an I κ B kinase termed *chuk*) that may regulate apoptosis (69), and (v) downregulation of the anti-apoptotic Bcl-X_L (57).

Furthermore, substantial evidence indicates that the G₂/M arrest of the cell cycle may not be the only mechanism to induce apoptosis (46)(65)(67)(69); additional phosphoregulatory pathways may be involved in inducing apoptosis (8)(30)(73)(102). In an attempt to discover biochemical effects of PTX on cancer cells, I examined JNK/SAPK that is frequently associated with apoptosis. The initial detection of JNK activation in a variety of human cells treated with PTX (**Part III, Figure 1**) encouraged me to further characterize the upstream regulators involved in the PTX-activated JNK and the downstream functions of activated JNK in the PTX-initiated apoptosis.

MULTIPLE UPSTREAM REGULATORS OF THE PACLITAXEL-INDUCED JNK ACTIVATION

Treatment with PTX activates JNK in a variety of human cells (**Part III, Figure 1**). PTX-activated JNK may exert its transactivation function through c-Jun, as demonstrated by activation of a TRE-CAT reporter that measures AP-1 activity (**Part III, Figure 4**). By using expression vectors for a series of dominant negative (dn) signaling proteins of the JNK pathway and the activated Ras interaction assay (A.R.I.A), as well as the observation of autophosphorylation of wild type (wt) ASK1, I have identified that activation of Ras, Rac, ASK1, JNKK are required for PTX-induced JNK activation (**Figure 1**).



(Figure 1) Multiple upstream regulators of the paclitaxel-induced JNK activation.

Based on the observations that (i) inhibitions by dn Ras and dn ASK are additive (**Part III, Figure 6**), and (ii) activation of JNK by wt ASK1 can be overridden only by dn JNKK, but not by dn Ras or dn Rac, I have concluded that the optimal activation of JNK requires activation of both Ras and ASK1 and that both activate the common downstream JNKK, leading to JNK activation (**Figure 1**). Since JNK activation has been frequently associated with apoptosis (**Part I, Table 17**) and JNK may indirectly modulate apoptosis through phosphorylation of Bcl-2 (63), I would predict that activation of JNK as a consequence of chemotherapy-induced MT dysfunction may play a role in regulation of apoptosis (**Figure 1**).

**ACTIVATED JNK IS REQUIRED FOR THE EARLY PHASE OF
PACLITAXEL-INDUCED APOPTOSIS THAT MAY OCCUR IN
CELLS AT ANY PHASE OF THE CELL CYCLE**

To further elucidate the role of activated JNK in the PTX-initiated apoptosis, I have examined the effects of a series of dn signaling proteins of the JNK pathway on modulation of PTX-initiated apoptosis. Parameters that were used to evaluate apoptosis include: (i) an increase of annexin-V binding, (ii) DNA fragmentation assays by both electrophoretic “ladder” pattern and a quantitative ELISA-based method, (iii) caspase assay, and (iv) cleavage of poly (ADP-ribose) polymerase (PARP).

Without alternation of the cell cycle profiles (**Part IV, Figure 4**), expression of each of dn signaling proteins, including dn ASK1, dn Rac, dn JNKK, and dn JNK, transiently protects ovarian cancer BR cells from PTX-induced apoptosis up to 16 h (**Part IV, Figures 2 and 5, Table I**). The protective effects of these dn signaling proteins decline at 24 h and completely disappear by 36 h of PTX treatment (**Part IV, Figure 5A**). Since expression of dn signaling proteins remains sufficient to completely inhibit endogenous JNK activities at 48-h treatment of PTX (**Part IV, Figure 3**), the disappearance of protection at the later time points does not result from insufficient JNK inhibition. Instead, the later phase of the PTX action may be independent of the JNK pathway.

Since dn signaling proteins had no effects on Bcl-2 phosphorylation, which were confirmed by two experimental procedures (**Part IV, Figure 8**), I have withdrawn my original hypothesis that activated JNK may account for Bcl-2 phosphorylation in PTX-treated cells, a hypothesis based on a report where JNK induced Bcl-2 phosphorylation (63).

In summary, activation of the JNK pathway independently of Bcl-2 phosphorylation is required for the early phase of PTX-induced apoptosis (**Figure 2**), whereas JNK activation may be dispensable for the late phase. Since blockage of the JNK pathway does not interfere with cell cycle profiles and only protects cells at early time points of PTX treatment where mitotic arrest has not become predominant, I would predict that the early phase of PTX-induced, JNK-dependent apoptosis may occur in cells at any phase of the cell cycle, or at least independently of mitotic arrest.

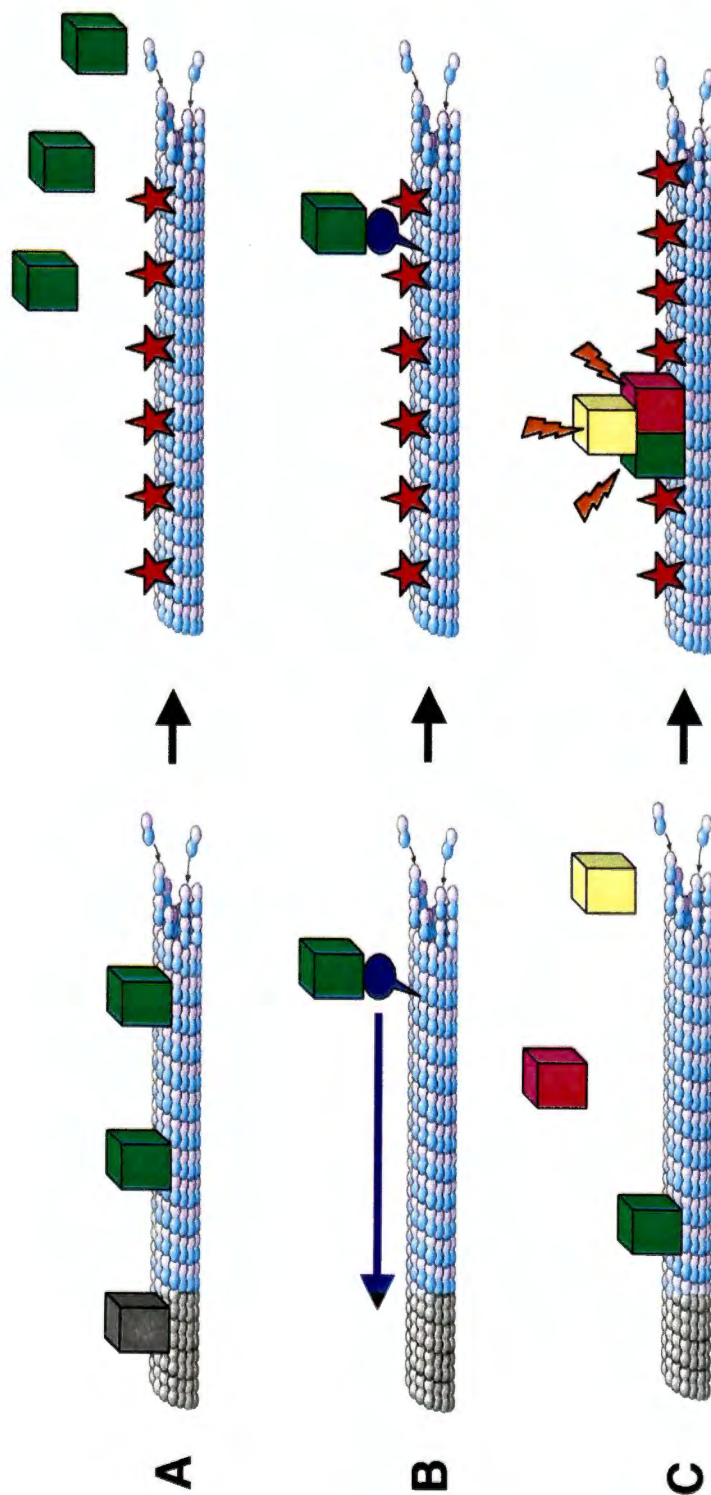
POTENTIAL MECHANISMS FOR ACTIVATION OF THE JNK PATHWAY BY MICROTUBULE DYSFUNCTION

MT dysfunction as a consequence of PTX treatment appears to be required for the JNK activation because that (i) other MT-interfering agents (docetaxel, vinblastine, vincristine, nocodazole, colchicine) also activate JNK (**Part III, Figure 1**), (ii) structural analogs of PTX (10-deacetylbaccatin-III) and of colchicine (lumicolchicine), which do not cause MT dysfunction, cannot activate JNK (**Part III, Figure 3A**), and (iii) PTX cannot activate JNK in the ovarian cancer PTX10 and PTX22 cells containing mutant β -tubulin that abolishes PTX binding (**Part III, Figure 3B**).

Three mechanisms have been proposed for the MT to directly activate its downstream signal transduction pathways (26). (i) Due to its wide distribution over the entire cytoplasm, the MT has a huge protein surface to sequester cellular proteins, including those in various signal transduction pathways. (ii) Signaling proteins can be delivered to specific cellular compartments by MT-dependent motor proteins, such as kinesin and dynein. (iii) MTs may function as a scaffolding structure to facilitate interactions among signaling proteins in a cascade (**Figure 3**). For all of the above mechanisms, interactions between MTs and signaling proteins can be regulated by the MT status, which is altered by PTX binding.

Intriguingly, tubulin itself is a G-protein. GTP binds to β -tubulin reversibly and tubulin, when assembled into the MT lattice, acts as a GTPase activating protein (GAP) (18). Although other small G-proteins such as the Ras, Rac, and Rho family are important signaling proteins (31), it is unclear whether tubulin has a similar role in the JNK signal transduction pathway. Nevertheless, tubulins and MTs have been shown to interact with Rac (6), a Dbl family member Lfc (25)(24), and a MAPK kinase kinase MLK2 (mixed linkage kinase) (71).

Rac can directly bind to tubulin *in vitro* and it has been demonstrated to be co-localized with



(Figure 3) Potential mechanisms of microtubule-regulated signal transduction. *A*, sequestering and release. *B*, motor protein-dependent delivery of signaling proteins. *C*, microtubules as a scaffolding protein. Signaling proteins (green, purple, and yellow cubes), paclitaxel (orange stars), motor protein (blue ballon), movement of motor protein (blue arrow).

MTs *in vivo* (6). Furthermore, the PH (Pleckstrin homology) domain of Lfc has been proposed to mediate its localization to MT, recruiting Rac to MTs to activate JNK (24). Lfc acts as GEF (guanine nucleotide exchanging factor) that specifically stimulates GDP dissociation from Rho, but not those from Rac, Cdc42, or Ras. Since Lfc binds to Rac equally well when Rac is nucleotide-depleted, or in the GDP- or GTP-bound state (25), Lfc may collaborate with MT as scaffolding proteins. On the other hand, Lfc can activate JNK and this activation is blocked by dn Rac, suggesting that Lfc still functions through Rac (24). It is possible that PTX treatment may modulate interactions between MTs, Lfc, Rac, and JNK. Further elucidation of effects of PTX on the Rac-MT interaction may provide explanation to the observation that dn Rac protects BR cells from PTX-induced apoptosis (**Part IV, Table I and Figure 2**).

A kinesin-like KIF3 family member, KIF3A, and its putative target KAP3A have been shown to be colocalized with MLK2 as punctate structures on MTs, suggesting a formation of complex (71). When expressed in fibroblasts, MLK2 colocalizes with active, dually phosphorylated, endogenous JNK to punctate structures along MTs (71). The kinesin-like identity of KIF3A raises the possibility that MTs may regulate the JNK pathway when the function of motor proteins is disrupted by PTX binding (**Figure 3B**). The complex formation between MTs and signaling proteins of the JNK pathway also suggests that MTs may function as a scaffolding structure (**Figure 3C**).

HIGH CONCENTRATIONS OF PACLITAXEL MAY BE REQUIRED FOR ITS FUNCTIONS OTHER THAN MITOTIC ARREST

The fate of cells exposed to PTX is determined by: (i) the cell type (for example, transformed vs nontransformed) (93), (ii) concentrations of PTX (40)(53), and (iii) duration of exposure (53). For example, short times (1 h) of exposure of leukemia HL-60 cells to low concentrations (20 nM) of PTX induce a reversible mitotic block without apoptosis, whereas either 60 nM PTX for 1 h or 10 nM for 12 h induces apoptosis (53).

It has been well documented that PTX concentrations required for blocking mitotic progression are low ($IC_{50} = 8\text{nM}$ in HeLa cells), whereas high concentrations of PTX ($IC_{50} = 330\text{ nM}$ in HeLa cells) are required for stabilizing polymerization (measured by MT mass) and massive MT damage (5)(19)(40)(74). Furthermore, in the studies where PTX has been demonstrated to exert functions other than mitotic arrest, the concentrations of PTX used were often high, ranging from 200 nM to 30 μM

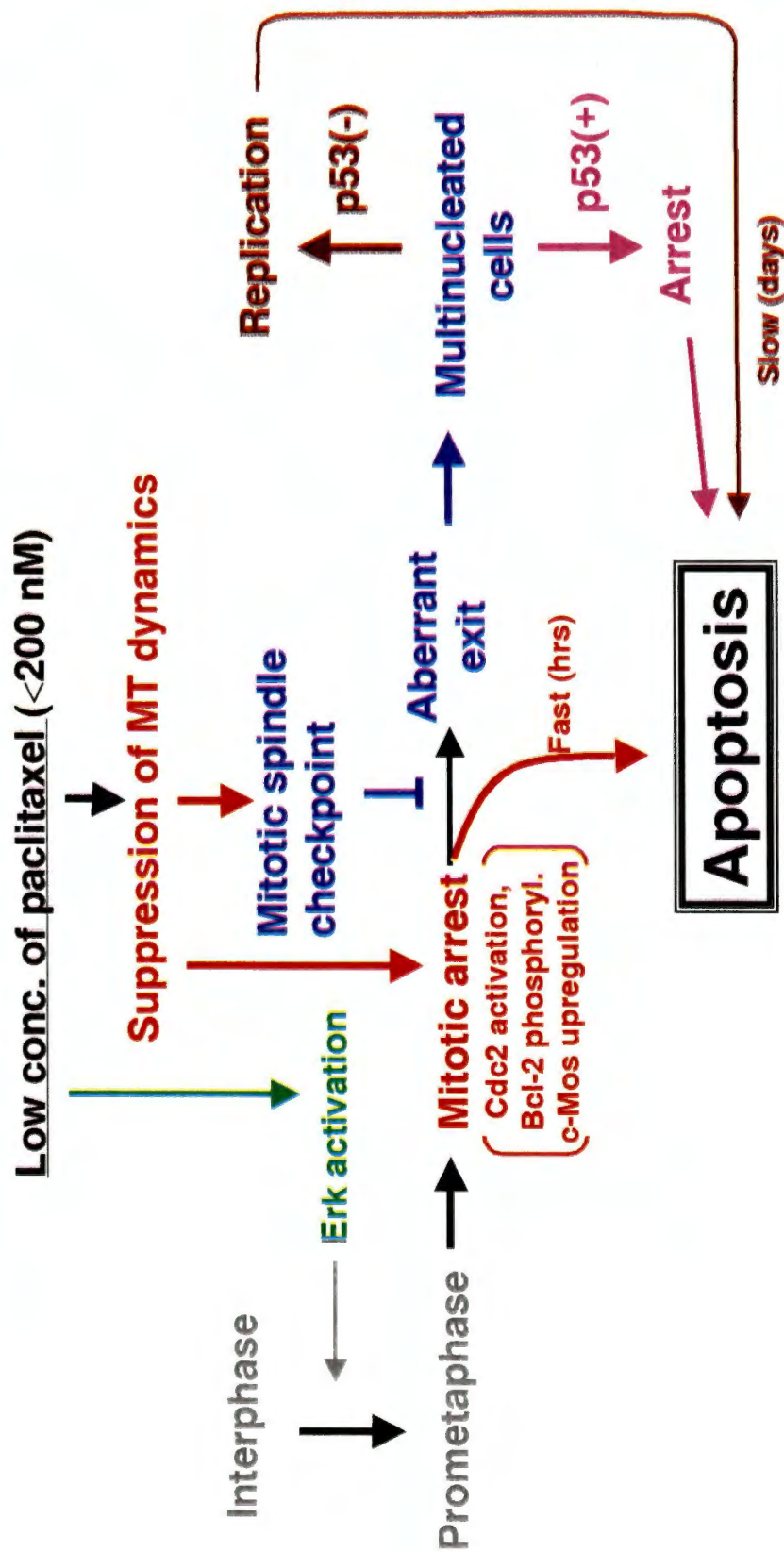
(9)(8)(12)(36)(46)(49)(47)(48)(58)(69). In agreement with the notion that higher concentrations of PTX may be required to exert functions in addition to mitotic arrest, my dose-effect data also exhibit a positive correlation between fold activation of JNK and PTX concentrations from 10 nM to 10 μ M (**Part III, Table I**). Therefore, the activation of JNK pathway may be more associated with MT damage (induced by high concentrations of PTX) than with mitotic arrest (by low concentrations of PTX).

CELLS TREATED WITH LOW CONCENTRATIONS OF PACLITAXEL

Sensitivity of MTs to PTX may differ depending on the cell type, however, it is believed that mitotic spindles are the most sensitive to PTX (41)(53). Possible cellular events in cells treated with low concentrations of PTX are schematically summarized in **Figure 4**. When cells are treated with low concentrations of PTX (such as between 10 and 100 nM), suppression of MT dynamics inhibits formation of mitotic spindles, resulting in mitotic arrest. Dysfunctional mitotic MTs may trigger the mitotic spindle checkpoint. The mitotic spindle checkpoint can be considered as a signal transduction pathway that links a kinetochore-associated sensor, which monitors the appropriate connections between mitotic spindles and chromosomes, to an output that arrests cell cycle (87). It is noteworthy that PTX at low concentrations did not affect the overall architecture of the MT cytoskeleton (40). Interestingly, low (20 nM) concentrations of PTX have been shown to be able to accelerate the progression of the cell cycle to the mitotic phase (54).

Cells arrested at the G₂/M phase are characterized with Cdc2 activation (19)(36)(38)(78) and Bcl-2 phosphorylation (8)(30)(29)(76), both contributing to apoptosis. The duration of normal mitosis varies from 45 min to about 3 h (75) and, after a prolonged period (several hours), cells usually undergo apoptosis through unclear mechanisms (40). In some situations, the mitotic spindle checkpoint fails to sustain the mitotic arrest and cells undergo aberrant exit from the abnormal mitosis, becoming multinucleated (60). The wt p53-containing multinucleated cells arrest and directly undergo apoptosis because chromosomal DNA is damaged during the formation of multinuclei (103). Those multinucleated cells with mutant p53 may continue to cycle and eventually die through apoptosis (87).

The mechanism by which PTX-treated cells exit mitosis (**Figure 4**) remains unclear. A process of adaptation has been proposed that the cell may progressively become less sensitive to the checkpoint signal as the mitotic delay lengthens (68)(92)(99). According to data from yeast, adaptation through *cdc55* and *cdc28* (68)(92)(99) occurs, in which cells override the checkpoint despite the persistence of MT



(Figure 4) Possible events leading to apoptosis in cells treated with low concentrations of paclitaxel.

damage. For instance, *cdc55* mutant *S. cerevisiae* are able to inactivate mitosis-promoting factor and exit mitosis, through inhibitory phosphorylation of *cdc28*. However, the human homologs of these yeast genes are yet to be identified (87). It is also unknown whether PTX has effects on modulation of these gene products.

CELLS TREATED WITH HIGH CONCENTRATIONS OF PACLITAXEL

In cultured cells, high concentrations of PTX have been shown to cause massive MT damage (40)(41)(101), regulate gene expression (39)(46)(49)(47)(56)(55)(57)(69)(88), activate JNK (2)(48)(98), Cdc2, and other kinases (19)(36)(38)(73)(77)(78) (Figure 5). So far, the MT is the only known cellular target that physically interacts with PTX; no cell membrane-associated or cytoplasmic receptor has been identified (89)(101). It remains unknown, however, whether MT damage is responsible for all known effects of PTX or whether PTX also directly activates some other cellular proteins.

PTX-induced activations of JNK (97) and Cdc2 (19)(36)(38)(77) have been closely associated with PTX-induced apoptosis. Among genes that have been shown to be upregulated by PTX, Bak (57)(39), Bax (88), and TNF- α (46) are known to promote apoptosis; whereas cyclin B1 (56)(55) may activate Cdc2 activation, leading to apoptosis. PTX-induced activation of Raf-1(9)(8) has been proposed to be involved in Bcl-2 phosphorylation, which may also promote apoptosis. It is noteworthy that apoptosis induced by these pathway does not requires mitotic arrest, suggesting that it may occur in cells at any phase of the cell cycle (Figure 5).

OTHER CONSIDERATIONS FOR HIGH CONCENTRATIONS OF PACLITAXEL

An intriguing observation is that treatment with 10 nM PTX for 20 h resulted in high intracellular drug accumulation (8.3 μ M) (41). Does this mean that, no matter what concentrations of PTX are used, the cell eventually responds in the same way as it were treated with high concentrations of PTX? Not necessarily, because both (i) the differential PTX sensitivity of tubulin at the ends versus in the shaft of MTs and (ii) the temporal factor are also crucial.

Due to a high affinity of PTX for MTs, after a long time of treatment (20 h) even at low concentration (10 nM), most PTX gradually accumulates in the MT-bound compartment, explaining the

high intracellular drug accumulation. The effects of PTX on MTs, however, may be different in the cases where (i) a few molecules of PTX bind to MTs in cells treated with low concentrations of PTX and (ii) high concentrations of PTX bombards MTs. The speculation is based on reports where PTX in the nM concentration range suppresses assembly dynamics of MT without overstabilization of them, suggesting that ends of MTs, where the assembly dynamics are the most active, are more sensitive to PTX than the shaft (17)(40). Recent evidence also indicates the sensitivities to PTX even differ at the plus and minus ends of MTs (16)(100). Therefore, the effects by gradually added MT-bound PTX at 8.3 μ M concentration on MT or other targets could be very different from those exerted by a sudden increase of cytosolic PTX to the same μ M concentration range.

Furthermore, while low concentrations of PTX may only suppress assembly dynamics of MTs, its high concentrations have been shown to exhibit many more functions (discussed in p. 199) and some cellular responses, such as JNK activation (**Part III, Figure 2**), only occur during a limited time window. When treated with nM concentrations of PTX, by the time cells accumulate PTX to μ M concentrations, the time window for transient activation processes, such as JNK, may have passed. Alternatively, the cell populations responding well to high concentrations of PTX may be limited to non-mitotic cells. After treatment with 10 nM PTX for 20 h, however, the majority of cycling cells would be blocked at the mitotic phase. These are speculations yet to be tested.

The use of high concentrations (>200 nM) of PTX, the concentrations within the clinically achievable plasma concentrations (ranging from 0.1 to 10 μ M) (86), may have various impacts on cancer therapy. Since high concentrations of PTX may induce apoptosis independently of mitotic arrest, a potential advantage is that high concentrations of PTX may be able to kill dormant cancer cells. Cancer dormancy is a state where the population of tumor cells is not progressively increasing in number. It can result from a situation where the majority of tumor cells are in cell-cycle arrest or that there is a balance between proliferation and death (94). On the other hand, the potential disadvantage is that PTX may kill normal, differentiated cells as well. For instance, it is important to study whether the use of higher concentrations of PTX aggravates side effects such as neurotoxicity and cardiotoxicity.

USEFUL METHODOLOGY

Insufficient transfection efficiency perhaps is the most common problem encountered by

researchers when using transient transfection procedures. Several methods have been developed to circumvent this problem. For instance, in assays of CAT reporter, luciferase reporter, HA-JNK (59), and HA-Akt activities (70), a “reporter” construct is coexpressed with the gene(s) to be studied. Based on a commonly accepted assumption that co-transfected genes express within the same cell, activities of reporters thereby reflect the effects of coexpressed gene of interest. Another way to overcome the low transfection problem is to isolate only cell populations that successfully express a cotransfected marker on the cell membrane, such as CD20, by using a fluorochrome-conjugated anti-CD20 antibody and flow cytometric sorting (96).

In my study, the use of pEGFP proved to be straightforward and useful in analyses for apoptosis and in flow cytometric sorting (97). Two experimental procedures used have successfully circumvented the limited transfection efficiencies (20 % in BR cells and 15% in MCF-7 cells at best), allowing me to be able to examine the effects of various dn signaling proteins of the JNK pathway on the PTX-initiated apoptosis.

(i) Flow cytometric sorting: I have cotransfected cells with pEGFP and expression vectors for dn signaling proteins and isolated green cells with flow cytometric sorting for further Western blot analyses, cell cycle analysis, DNA fragmentation assays, or caspase-3 activity assay (**Part IV, Figures 3-8**). Coexpression of pEGFP and dn expression vectors was verified by Western blots showing the presence of dn signaling protein in green cells but not in non-green cells (**Part IV, Figure 3**). Separation by this method appears to be specific, however, the worst drawback is that this method is very time-consuming. The highest acceptable sorting speed in the FACStar^{PLUS} that I have used is 2,000 cells/second, at that speed the “aborted” rate is about 10 %. When two or more cells reside together in a single drop of normal saline passing through the nozzle of flow cytometer, the computer detects them and aborts the collection of this drop. Further increasing flow speed to more than 2,000 cells/second in that machine usually greatly elevates aborted rates, without a much higher collecting efficiency. Newer flow cytometric sorters designed to sort at speed higher than 10,000 cells/second should alleviate this time-consuming disadvantage.

(ii) Three-color analysis for early apoptosis in cells expressing transfected genes: I have cotransfected cells with pEGFP and genes of interest, then done flow cytometric analyses for apoptosis using PE-conjugated AV and 7-AAD. Three fluorochromes, green protein as a result of pEGFP expression, PE-conjugated AV, and 7-AAD, are excited by a laser tuned to 488 nm and emissions are

detected at 507, 575, and 650 nm, respectively (**Part IV, Figure 1**). Intensities of these three parameters of every cell can be acquired and analyzed by flow cytometry. The combined use of AV and 7-AAD can differentiate the early apoptotic cells (AV positive/7-AAD negative) from dead cells (7-AAD positive). This procedure is quick because no sorting is required. I would predict that it shall become a popular screening method to test the apoptotic effects of any gene.

DIRECTIONS FOR FURTHER RESEARCH

Results from my study have suggested a linkage between MT dysfunction and activation of the JNK/SAPK pathway (98). A pro-apoptotic role of JNK/SAPK in PTX-induced apoptosis has also been demonstrated (97). However, these data have raised more questions than they have answered. Three obvious questions extending from what I have observed are: (i) how does MT dysfunction signal Ras, Rac, and ASK1? (ii) how does the PTX-activated JNK promote apoptosis? and (iii) what are the mechanisms of the later phase of PTX-induced apoptosis? The ultimate direction for clinically related research on PTX mechanisms, however, is to develop strategies of using taxanes more efficiently.

MECHANISTIC CONNECTIONS AMONG MICROTUBULES, RAS, RAC, AND ASK1

Exploration of these connections should be focused on proteins that may directly interact with MTs. Known MT-interacting proteins include Rac (6), MLK2, KIFs, JNK (71), Lfc (24), and several MAPs.

The first step is to examine how PTX treatment changes the interactions among these proteins and MTs. This can be achieved by co-staining of these proteins followed by fluorescent microscopy. Furthermore, comparisons of these patterns with or without PTX treatment among the PTX-resistant cell lines, PTX10 and PTX22, and the PTX-sensitive parental 1A9 cells should be able to verify the role of PTX binding to MTs in regulation of these changes. Alternatively, since the amino acid residue mutations in β -tubulin that abolish PTX binding to MTs are known, the genes for wt or mutant β -tubulin can be constructed to be tagged with EGFP and expressed in the cells. If EGFP does not interfere with tubulin functions, we can follow the dynamics of MT assembly before and after PTX treatment. Expression of EGFP also facilitates our observation on MT-interacting proteins using fluorescent microscopy.

Among proteins that have not been identified to be associated with MTs, one candidate is ASK1. ASK1 has been demonstrated to be activated by PTX treatment (98). Similar to MLK2, ASK1 is also a MAPK kinase kinase. It would be of great interest to examine whether ASK1 directly interacts with MTs and how PTX treatment modulates the interactions. Investigation on this hypothesis can be achieved by colocalization under fluorescent microscopy, coimmunoprecipitation, and the GST-fusion protein pull down assay. To further discover novel tubulin-interacting proteins, the yeast two-hybrid assay with the tubulin gene as the bait can be used to screen cDNA library for other candidates.

MAPs are believed to be important in regulating assembly and stability of MT. Although I am not aware of any study demonstrating that PTX-bound MTs have effects on MAPs, it would not be surprising if dysfunctional MTs are proved to be able to signal through MAPs.

All of the approaches described above apply for studies in cells at any phase of the cell cycle. Some proteins interacting with MTs, however, may only function in cells arrested in the mitotic state. For example, is the spindle assembly checkpoint involved in the PTX-induced JNK activation? Several mammalian spindle assembly checkpoint genes, such as MAD2 (51)(52), BUB1 (91), BUB3 (90) (above are kinetochore-localized), PP2A, and MAPK have been identified (87), and overexpression of dn Bub1 in HeLa cells reduces the nocodazole-triggered apoptosis (91)(87). It would be interesting to examine whether PTX (and, if so, at what concentrations) has any effect on these checkpoint gene products and whether these gene products interact with signaling proteins of the JNK pathway.

HOW DOES ACTIVATED JNK CAUSE APOPTOSIS IN THE EARLY PHASE OF PACLITAXEL-INDUCED APOPTOSIS?

JNK has been shown to regulate activating protein-1 (AP-1) transcriptional activity *in vivo* (104)(105). The transactivation activity of AP-1, however, may be regulated by: (i) different dimerization of AP-1 factors from members of the Jun family (c-Jun, JunB, JunD) (15), Fos family (c-Fos, FosB, Fra1, Fra2) (62)(107), and ATF family (ATF2, ATF3/LRF1, B-ATF) (20)(35), thereby eliciting differential binding to the TRE or CRE (28)(44)(84), (ii) phosphoregulation of subunits of AP-1 by specific protein kinases and phosphatases (1)(10)(27)(34)(95), and (iii) further interactions between AP-1 and transcriptional coactivators (3)(4)(14)(43)(44)(45).

It is not surprising, therefore, that the role of AP-1 in apoptosis may be completely opposite,

depending on the cell type and stimuli (44), despite the frequent associations between JNK and apoptosis (**Part I, Table 17**). An obvious question is "Is AP-1 transcriptional activity required for PTX-induced apoptosis?"

Results from my study (98) and others (2)(48) demonstrate that activation of functional AP-1 result from PTX-induced activation of the JNK pathway (**Part III, Figure 4**). It remains unknown, however, whether: (i) transcriptional activation of AP-1 activity is required for PTX-induced apoptosis, (ii) it has no role in this apoptosis, or (iii) it may even protect the cell from apoptosis.

The involvement of transcriptional activation in PTX-induced apoptosis can be tested by using available inhibitors for RNA and protein synthesis. A caveat is that these inhibitors by themselves may induce apoptosis, confounding apoptotic analysis. For example, a commonly used transcriptional inhibitor, actinomycin-D, efficiently induces apoptosis (**Part IV, Figure 7**). It is also worth searching database, such as EMBL/GenBank, for apoptosis-related genes and examine whether there are TRE and/or CRE in the promoter region. The bioinformatic search should narrow down the scope of candidate genes.

The expression vector for dn c-Jun (pCMV-TAM67) is very effective in inhibiting AP-1 activity (**Part III, Figure 4B**) and can be used to examine whether inhibition of c-Jun can protect cells from, at least the first phase of, PTX-induced apoptosis. However, careful titrations to determine an optimal plasmid concentration for cotransfection experiments appear to be critical. In cotransfection experiments using pEGFP and TAM67 at ratio 1:3, I have observed high cytotoxicity of TAM67 overexpression. To determine the appropriate concentration of TAM67 that is high enough to express enough dn c-Jun to block JNK-activated AP-1 activity but low enough to avoid undesired cytotoxicity, two experimental parameters should be carefully monitored during the titration procedures. (i) The lowest concentration of TAM67 that still can sufficiently suppress AP-1 activity can be determined by using a TRE reporter and PTX treatment. (ii) The highest possible concentration of TAM67 should be limited by monitoring percentage of surviving green cells that are cotransfected with pEGFP and increasing amount of pCMV-TAM67.

A systematic way to examine gene regulation by treatment with PTX is screening different pools of mRNA isolated from either PTX-treated cells or control cells with biochips which are coated with thousand of oligonucleotides representing the human cDNA library (33)(72)(83)(85). Briefly in this method, mRNAs are isolated from treated cells, subjected to reverse transcription to generate cDNAs in

the presence of fluorochrome-conjugated dNTP, and the labeled cDNAs are hybridized with biochips. The intensities of fluorescence among thousands of oligonucleotides can be recorded by special readers and analyzed by computer. Upregulation and downregulation of genes by PTX treatment can be easily detected by this method. Biochips provide a powerful way to study: (i) what genes are regulated by PTX treatment, (ii) what concentration ranges of PTX are able to regulate gene expression, (iii) the cell type specificity, such as proliferating tumor cells versus normal differentiated cells, and even (iv) the cell cycle phase specificity, if it exists, for PTX-induced gene regulation. For the last aim, mRNA can be isolated from either G₁ phase or G₂/M phase PTX-treated cells separated by centrifugal elutriation (see Part IV, Appendix).

On the other hand, it remains possible that transactivation through AP-1 may not be required for the PTX-initiated apoptotic pathway. Instead, activated JNK may activate caspases either directly or through interactions with other proteins. For instance, JNK may interact with p21 (81), which in turn may interact with Cdc2 (5)(13)(106) or other CDKs (79). Recent studies suggest an increasingly important role of CDKs as an effector of apoptosis (22)(32)(36)(50)(64)(80)(82). In addition to c-Jun and ATF2, JNK has been shown to be associated with a variety of proteins, including p53 (66), MAPK-APK-3 (MAPK-activated protein kinase-3 (61) (these two are its substrates) and non-substrate such as Jun B (21). Likewise, it remains possible that JNK may turn out to be able to interact with some regulators of the apoptotic pathway, including AIF, Apaf-1, cytochrome *c*, caspases-8, -9, -10, or some yet-to-be-identified apoptogenic regulators and effectors. The use of GST-JNK to affinity precipitate its interacting proteins can be used for this screening.

WHAT ARE THE MECHANISMS OF THE LATER PHASE OF PACLITAXEL-INDUCED APOPTOSIS?

The observation on involvement of the JNK pathway in the early phase of PTX-induced apoptosis only provides a partial answer for the fundamental question: "how does PTX kill cancer cells?". Apoptotic mechanisms for the later phase still remain unknown. Prolonged mitotic arrest remains the most likely culprit, although the mechanism remains unknown. Nevertheless, other factors may also be involved.

Since endogenous JNK activity can be sufficiently suppressed by expression of dn signaling proteins of the JNK pathway up to 48 h (**Part IV, Figure 3**), the use of expression vector for dn JNK can

dissect out the JNK effects, allowing us to study the JNK-independent, PTX-induced apoptosis. For instance, to examine the role of G₂/M arrest in the later phase of apoptosis, we can test whether PTX can still induce apoptosis in the dn JNK-expressing cells that are arrested in the G₁ phase by the use of adenoviral vectors for p16 and/or those for p27 (**Part III, Appendix**). If the G₂/M arrest is the only cause for the JNK-independent late phase of PTX-induced apoptosis, cell death induced by PTX treatment should be markedly inhibited in the dn JNK-expressing G₁-arrested cells. If so, the following phase of research should focus on identification of apoptogenic factors that are responsible for apoptosis following G₂/M arrest. Apoptogenic factors, which are activated in cells arrested in the G₂/M phase, might be identified from cells expressing dn JNK that are arrested in the G₂/M phase by PTX treatment for 36 to 48 h. Apoptogenicity of these factors can be verified by overexpression in the G₁-arrested cells, followed by measurement for apoptosis. Centrifugal elutriation (**Part IV, Appendix**) can be used to isolate G₁ and G₂/M phase cells.

Two methods are useful for screening for potential apoptogenic factors. The first method is the yeast two-hybrid assays using known apoptotic regulators as baits, such as the Bcl-2 family members, Apaf-1, the caspase family members, and various CDKs. The second is to use biochips (described in p. 205) to analyze genes that are upregulated or downregulated in this particular population of cells.

Alternative approach to examine apoptogenicity of the JNK-independent G₂/M arrest is the use of low concentrations of PTX, such as 10 nM, which did not significantly activate JNK (**Part III, Table 1**). In the present study, I have not examined the effects of PTX on JNK activation in cells treated for more than 24 h. However, I would predict that, at 36 to 48 h treatment, the majority of BR cells should be arrested in the G₂/M phase and still undergo apoptosis without the apoptogenic influence by activated JNK. From those apoptotic cells, we may be able to characterize the apoptotic mechanism after a prolonged mitotic arrest or even identify some novel factors responsible for the mitotic arrest-induced apoptosis.

Since PTX has been known to stimulate expression of *mdr* gene (7)(37), one potential pitfall of this approach is that treatment with low concentrations of PTX for several days favors for PTX-resistant cells with upregulated *mdr*. This may explain the observations that one out of four cell lines treated with 12 nM of PTX for four days survived and continued growing (11). Cotreatments with verapamil (5 µg/ml) has been reported to be able to inhibit the P-glycoprotein functions (23) and should be added into culture media in these experiments.

On the other hand, if cells can not be protected from PTX-initiated apoptosis by simultaneous G₁ phase arrest of the cell cycle and suppression of JNK activity, apoptogenic mechanisms independent of G₂/M arrest are indicated and novel apoptogenic factors could be subsequently identified from these cells.

DIRECTION TO THE ULTIMATE GOAL

Our improved understanding of mechanisms of PTX action should allow development of more efficient strategies of using taxane-based chemotherapy. Demonstration of a role for JNK activation in PTX-initiated apoptosis in this study only contributes a tiny share to our understanding on action mechanisms of PTX and, by inference, the role of MTs as regulators of apoptosis. To achieve the ultimate objective of using taxanes more efficiently to treat patients with cancers, we should continue working on: (i) how to increase the specificity of taxanes against cancer cells, (ii) how to enhance its effects by adjuvant agents, (iii) how to decrease the resistance of cancer cells to taxanes, and (iv) how to lessen its adverse side effects.

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