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I am submitting herewith a dissertation written by Cheryl Barbati Bast entitled "Chromosomal and Molecular Alterations Associated with Fibrosarcoma Development in the Mouse." 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 Biomedical Sciences.

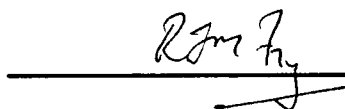


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We have read this dissertation
and recommend its acceptance:







Accepted for the Council:



Vice Provost
and Dean of The Graduate School

CHROMOSOMAL AND MOLECULAR ALTERATIONS
ASSOCIATED WITH FIBROSARCOMA DEVELOPMENT
IN THE MOUSE

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

Cheryl Barbatl Bast

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DEDICATION

TO MY PARENTS FOR MAKING IT POSSIBLE

TO MIKE FOR MAKING IT ENJOYABLE

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Now that this thesis is written, I come to the most pleasurable task of thanking the many people who have helped me during my stay in the Biology Division.

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ABSTRACT

By the time solid tumors are clinically observed, many chromosomal changes are present. Observing tumors early in development and following chromosome changes with the progression of tumors may help determine if specific chromosome changes are associated with tumors at different stages of development and if changes are dependent upon a particular inducing agent. As one approach to studying this, silastic disks were implanted subcutaneously into C₃H female mice, and fibrosarcomas developed at the site of the disks. The disks and associated tissue were removed from 28 weeks post-implantation until mature sarcomas were observed. The associated cells or tumors were then both cultured and karyotyped, and direct chromosome preparations were made from 80% of the tumors. G-banding analysis was performed to look for specific structural and numerical chromosomal changes, and C-banding analysis was performed to look for homogeneously staining regions (HSRs). In some cases the animals were treated with X rays (2.5 Gy x 4), in addition to the disk, and the sarcomas analyzed for chromosome changes. The chromosome numbers per cell varied from 38 to 350 in all tumors analyzed. There were wide variations in chromosome number between sarcomas, with much less variation within a particular sarcoma. Double minutes (DMs) and HSRs, both indicative of gene amplification, were present in many tumors at later stages of development (longer times post-implantation of the disk). Consistent chromosomal trends including duplications of chromosomes 10, 16, and 19 were observed in sarcomas from animals that received both the disk

treatment and the disk + X ray treatment. Non-specific metacentric chromosomes were observed in many tumors from animals that received the disk treatment alone, and of these 55% were isochromosomes. Aneuploidy was a very early change observed in all tumors analyzed. It occurred even before a histological diagnosis of malignancy could be made with confidence (personal communication, R. J. M. Fry).

Southern analysis of 8 tumor cell lines has shown no correlation between the amplification of the c-myc, c-h-ras, c-raf, c-rel, c-mos, c-myb, c-abl, c-erbA, c-erbB, and c-sis oncogenes and the presence of DMs and/ or HSRs. However, Southern analysis has shown a correlation between the presence of DMs and amplification of the multiple drug resistance (MDR) gene. Cells containing DMs were more resistant to methotrexate, cytosine arabinoside (ara C), actinomycin D, and colchicine than tumor cells lacking DMs. The presence or absence of DMs (and thus MDR) had no effect on the X ray sensitivities of these fibrosarcoma cell lines.

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CHAPTER 1

INTRODUCTION

Chromosome Changes and Cancer

Human Studies

The presence of chromosomal changes in neoplastic cells has been recognized for decades. In 1914, Boveri hypothesized that an abnormal chromosome pattern was closely associated with the malignant phenotype of the tumor cell (Boveri, 1914). Since then major advances in cell culture and techniques for recognizing individual chromosomes have enabled Boveri's hypothesis to be tested. An important fact that has emerged from recent cytogenetic studies is that the chromosomal alterations that occur in tumor cells often are specific for a particular tumor. The presence of chromosomal changes appears to be a general phenomenon and thus provides convincing evidence for a fundamental role of chromosomal abnormalities in the neoplastic process. (Mitelman and Heim, 1986).

The analysis of chromosomal alterations in human leukemias can be broken into three periods. The first period lasted from 1960 to 1970. During this period chromosomal alterations were observed without banding, and thus they include only numerical changes and structural rearrangements that resulted in a morphological change (LeBeau and Rowley, 1986). The most important observation during this period was the identification of what appeared to be a deletion of about half of

the long arm of a G-group chromosome in myeloid precursors in patients with chronic myelogenous leukemia (CML) (Nowell and Hungerford, 1960). It was not known whether chromosome 21 or 22 was involved. This chromosome was termed the Philadelphia chromosome (or Ph¹), and this observation led to a search for similar chromosome changes in other types of leukemias and in lymphomas. However, these results were disappointing since specific abnormalities were often present in malignant cells from particular patients, but did not appear to be consistent between patients with a particular lymphoid malignancy (LeBeau and Rowley, 1986). Therefore, it was believed that the Philadelphia chromosome was a unique example of a specific, consistent karyotypic change.

The second period lasted from 1970 to 1980, and provided evidence that the earlier assumptions were not correct. The apparent deletion of chromosome 22 was shown to be a translocation between chromosomes 9 and 22 (Rowley, 1973). With the advent of improved banding techniques, other specific structural abnormalities were found to be associated with specific leukemias and lymphomas. The G-banding technique, whereby metaphase chromosomes are treated with trypsin and stained with Giemsa blood stain, produces chromosome specific banding patterns and thus allows identification of specific chromosomes (Seabright, 1971). Furthermore, banding revealed that chromosome gains and losses were frequently non-random in many lymphoproliferative diseases. It was also observed during this period that specific chromosomal abnormalities corresponded with many subtypes of leukemias.

Several types of chromosomal abnormalities have been identified in human leukemias and lymphomas. These include the gain of an entire chromosome (such as +8 in acute non-lymphocytic leukemia), the loss of an entire chromosome (such as -7 in acute non-lymphocytic leukemia), the gain of a portion of a chromosome (such as 14q+ in B-cell acute lymphocytic leukemia), and consistent translocations [such as the t(9;22)(q34;q11) in CML and the t(8;14)(q24;q32) in Burkitt lymphoma (Croce, 1987).] This diversity of chromosome alterations suggests diversity in the ways genetic change may be related to malignant transformation. The loss of all or part of a chromosome suggests that the loss of certain components that regulate cell growth may allow recessive genes to be expressed. The gain of all or part of a chromosome suggests that an increase in a gene product may also be important for the malignant process. The presence of a translocation suggests a more complex mechanism. Rearrangement of one or more genes may alter gene regulation. For each type of aberration, certain chromosomes are preferentially affected. For example, the gain of chromosome 8 or the loss of chromosome 7 are most common in lymphoid malignancies (LeBeau and Rowley, 1986).

The third period ranges from 1981 to the present. During this period, cytogenetic observations have been correlated with molecular alterations. These molecular analyses, a great majority involving oncogenes, will be discussed later.

Cytogenetic studies of solid tumors have lagged considerably behind studies of the leukemias and lymphomas, and there are several factors responsible for this. The majority of solid tumors have a very low

mitotic index, and the morphology of chromosomes obtained from solid tumors is often more fuzzy than the chromosomes obtained from leukemias and lymphomas (Sandberg and Turc- Carel, 1986). Additionally, in most cases solid tumors contain numerous chromosome changes at diagnosis, making it quite difficult to ascertain if there is a primary, specific cytogenetic change. Often, however, in leukemias and lymphomas only one cytogenetic change is encountered at diagnosis. This is perhaps due to the fact that the clinical signs of leukemias become apparent at an earlier stage of disease than those of solid tumors. Thus, the leukemic cells may be examined at a time when they contain only the primary karyotypic change (Sandberg and Turc- Carel, 1987). However, since solid tumor cells contain complex structural and/or numerical chromosomal alterations, the primary karyotypic change may be masked. Obviously, it is important to determine which of the plethora of cytogenetic changes are intimately involved with tumor development. The alterations should consist of two types: primary changes that are directly involved in neoplastic initiation and secondary changes that may or may not influence tumor growth and progression (Mitelman and Heim, 1986).

As with the leukemias and lymphomas, several classes of cytogenetic change can be observed in solid tumor cells. These include the gain or loss of an entire chromosome (such as +8 in colonic adenomas and -9 in bladder adenocarcinomas), the gain or loss of part of a chromosome (such as 1q- in ovarian adenocarcinomas and 17q+ in colon adenocarcinomas), and translocations [such as t(12;16)(q31;q11) in liposarcomas (Sandberg and Turc- Carel, 1987).] Metacentric marker

chromosomes, double minute chromosomes (DMs), and homogeneously staining regions (HSRs) (see pages 6 and 7 for further discussion) are also often observed in solid tumor cells.

A number of solid tumor cells contain metacentric marker chromosomes, in particular, isochromosomes (Atkin and Baker, 1988). Some of these marker chromosomes may be of sporadic or random origin and of little significance, while others may represent important steps in the development of a particular tumor type. This would be especially true of marker chromosomes that occur with a high frequency in particular tumor types. Isochromosomes could perhaps produce an effect through gene dosage since extra copies of these chromosome arms would introduce provide extra gene copies. This would be especially true where multiple copies of a particular isochromosome are present. However other isochromosomes, such as the i(11q) that is present in bladder carcinomas may exert their effect through loss of material from 11p (Atkin and Baker, 1985). It is as yet unclear whether metacentric marker chromosomes are a primary karyotypic change involved in tumor initiation or if they occur later in development and are perhaps involved in progression. However the presence of two copies of i(12p) as the only chromosomal abnormality in malignant teratomas of the testis, suggests that this isochromosome could represent a primary event in the production of this tumor (Gibas et al., 1984).

Double minutes and homogeneously staining regions are cytological manifestations of DNA sequence amplification. These structures have been observed in direct preparations from tumors, in cell lines derived from tumors, and in cells showing resistance to metabolic inhibitors.

These structures, however, are rarely observed in normal cells (Holden et al., 1987).

Double minutes are acentric, extrachromosomal structures, that are believed to be circular and to replicate approximately once per cell cycle (Von Hoff et al., 1988). They range in size from 100 to 2000 kilobases (Wahl et al., 1984) and are believed to arise from submicroscopic, extrachromosomal elements. These submicroscopic elements are supercoiled and replicate autonomously. They were first reported in tumor cell lines derived from patients who had not undergone chemotherapy or radiation therapy and were found to contain amplified myc oncogene (Von Hoff et al., 1988). Double minutes themselves have been shown to contain myc (Alitalo, 1985; Bigner et al., 1987), as well as numerous drug resistance genes (Wahl et al., 1984), including the multidrug resistance gene (Larcher et al., 1988).

Homogeneously staining regions exist as amplified areas of DNA and can be arranged as both inverted and direct repeats (Passanti et al., 1987). They range in size from 250 to 1000 kilobase pairs (Von Hoff et al., 1988) and stain uniformly in G-banded preparations (Garson et al., 1987). Three major mechanisms have been proposed for the origin of HSRs. These are uptake of DNA from dead cells, multiple cycles of unscheduled DNA synthesis followed by recombination (leading to integration of the copies into the chromosome) (Roberts et al., 1983), and unequal crossing over (Holden et al., 1987). A great majority of the data suggests that unequal crossing over between sister chromatids and/or non-homologous chromosomes bearing HSRs could lead to the amplification phenomenon observed with HSRs (Holden et al., 1987). As

with DMs, some HSRs have been correlated with amplified oncogenes in tumor cells. For example, N-myc has been implicated with HSRs present in human neuroblastomas (Garson et al., 1987).

A number of theories involving gene amplification, regulation, and mutation have been put forth to explain the role of chromosomal changes in malignant disease. However, while each hypothesis incorporates some of what is known about the neoplastic process, so far no model has been successful in combining and explaining all data from the many malignancies that have been studied.

Rodent Studies

Studies of experimentally induced rodent tumors indicate that the same general types of chromosomal alterations that occur in human tumors also occur in rodent systems. Thus, the loss of all or part of a chromosome, the duplication of all or part of a chromosome, translocations, DMs, and HSRs are all observed in rodent cancers.

The most commonly observed chromosomal alterations present in murine leukemias and lymphomas are trisomies. In C57BL/6J mice 70% of leukemias and lymphomas contain trisomy 15 whether or not the tumor is induced by x-irradiation or chemical treatment. Also, trisomy 15 is observed in 60% of preleukemic animals without overt disease (McMorrow et al., 1987).

Chromosome gain (such as an additional copy of chromosome 1 in murine melanomas) (Kendal et al., 1987), partial chromosome loss (such as a deletion of a small portion of chromosome 13 in transformed tumor cells in culture) (Kodama and Sasaki, 1987), and double minute

chromosomes (in many solid tumor cell lines) (Cahilly-Snyder et al., 1987) all occur in cells of murine solid tumors. However, the most frequent chromosomal aberration observed in murine solid tumors is the Robertsonian translocation (Dhaliwal et al., 1987) which appears as a metacentric chromosome in mouse cells. These translocations between two homologous or non-homologous acrocentric chromosomes might be produced in two different ways. They could arise through misdivision of a centromere during a previous mitosis or as a result of breakage followed by rejoining of two homologous centromeres that were lying close to each other because of prior endoreduplication of the cell or association of the homologs. However the metacentric chromosomes may arise, it is possible that these translocations are important in either murine tumor initiation or progression since specific metacentric marker chromosomes occur in specific tumor types. For example, iso 8q and iso 2q are preferentially observed in murine myxosarcomas and appear to be related to metastatic potential (Dhaliwal et al., 1987).

In addition to the specific chromosomal changes that occur in murine solid tumors, many chromosomes are present in variable numbers. Trisomy, tetrasomy, and pentasomy of autosomes with apparently normal morphologies are not uncommon. Furthermore, the loss of the Y chromosome from tumor cells in the male is not uncommon (Dhaliwal et al., 1987).

The Role of Oncogenes

Human Studies

Carcinogens and oncogenic viruses have DNA as their target. Therefore, it seems likely that the carcinogenic process may involve alterations of one or more genes. The existence of cellular oncogenes was first suggested from observations of retroviruses and RNA tumor viruses. The tumorigenic potential of some of these viruses is due to the presence of specific genes (oncogenes) in their genomes. The retroviral genes represent normal cellular genes that exist in an altered form. These altered genes are termed cellular oncogenes (c-onc) or proto-oncogenes (Karens and Hanafusa, 1981).

Oncogenes have Counterparts in Normal Cells

Viral (v-onc) and cellular (c-onc) oncogenes are highly conserved and code for proteins that are involved in the control of cellular differentiation and proliferation (Ascione et al., 1986). The function of some of the oncogenes is unknown; however, for many oncogenes a function has been defined, and the functions of oncogenes tend to fall into four classes (Ascione et al., 1981). These are growth factors, growth factor receptors, transducing signals, and nuclear proteins, and since these are involved in normal cellular growth, the alteration or misregulation of oncogenes can lead to transformation.

Perhaps the most thoroughly described example in the growth factor category is that of platelet derived growth factor (PDGF) and its oncogenic equivalent c-sis. V-sis is the oncogene of the Simian

sarcoma virus. This virus contains only part of the c-sis gene and uses viral genes for transcription and translation of the oncogene product. The c-sis gene shows extensive sequence homology with the gene coding for PDGF (Chiu et al., 1984), and c-sis exists as a B-B dimer of PDGF, while PDGF itself exists as an α -B dimer. PDGF stimulates tyrosine kinase activity (Nishimura et al., 1982) and in vitro treatment of cells with PDGF leads to an increase in c-fos expression and in c-myc RNA (Helden et al., 1985). This suggests that PDGF can induce expression of other genes and that c-sis could lead cells to uncontrolled growth. Furthermore, it also indicates that some oncogenes might be activated by other oncogenes.

The oncogene erbB is included in the growth factor receptor category. Growth factors act by binding to cell surface receptors, and the epidermal growth factor (EGF) receptor shows 89% sequence homology with the v-erbB oncogene product. However, the v-erbB oncogene lacks the extracellular EGF binding domain, while the cytoplasmic portion containing the kinase domain is conserved (Downward et al., 1984). Since the external binding domain provides regulation of kinase activity, the lack of this domain may lead to constitutive expression of kinase activity. The cells would behave as if EGF were always present, and thus be constantly stimulated to grow and divide. V-erbB is only one of several oncogene proteins that carry the tyrosine kinase domain, and it is to be expected that the other oncogene proteins that also carry these domains may be shown to be homologous to various growth factor receptors.

H-ras and K-ras refer to the oncogenes of the Harvey and Kirsten sarcoma viruses, and are an example of the transducing signal category. The H-ras protein localizes to the inner surface of the cell membrane, binds GTP, and has GTPase activity (Shih et al., 1980). The H-ras protein belongs to a family of nucleotide-binding G proteins, that are involved in signal transduction from receptors on the outside of the cell membrane to the adenylate cyclase system on the inside of the membrane (Gilman, 1984). Also, the G proteins, and most probably the ras proteins, are capable of initiating or suppressing signals generated by the binding of a ligand to a receptor at the membrane. Finally, c-ras genes from tumor DNAs code for proteins that have lost the ability to be regulated by the cell, and this suggests that signal transduction may be constitutive, with the cell behaving as if it were constantly receiving a growth stimulus (McGrath et al., 1984).

Another way in which proto-oncogenes function in normal cells is as nuclear proteins. The c-myc proto-oncogene is the cellular homolog of v-myc of the avian myelocytomatosis virus. The myc protein binds DNA, and can positively and negatively regulate the expression of other genes (Cole, 1986). Thus, c-myc expression appears to represent an additional step in the cascade of events that starts with growth factor/ receptor interaction, carries through the signal transduction system, and leads to the activation that most likely occurs in the nucleus.

The Role of Oncogenes in Malignant Cells

Alterations involving cellular oncogene function are involved in the carcinogenic process by disturbing normal cell growth mechanisms. Alterations involving cellular oncogenes have been found in a variety of tumors, and these alterations either alter the regulation or structure of the oncogene. The three main mechanisms involved in oncogene "activation" are specific mutations, tumor-specific chromosomal aberrations, and gene amplification.

Mutations in the c-Ha-ras oncogene have been associated with transforming activity in a human bladder carcinoma cell line (Parada et al., 1982). Hybridization studies have shown that the DNA sequences responsible for transformation in this cell line are homologous to the c-Ha-ras gene (Der et al., 1982). Comparative sequence analysis between the cell line and the normal c-Ha-ras clone revealed that there was a base change of G to T at codon 12. This substitution resulted in the protein product containing a valine in place of a glycine (Tabin et al., 1982). Other studies have revealed that mutations that occur within the ras family are conserved and involve either codon 12 or 61 (Taparowsky et al., 1983). Furthermore, the replacement of glycine at position 12 by any residue, except for proline, leads to transforming activities. Structural studies suggest that the loss of glycine 12 may alter the conformation of the ras protein (McCormick et al., 1985). Finally, the protein formed from the mutated ras gene has defective GTPase activity. Since GTPase activity is required to terminate the growth signal, the protein would not be capable of regulating its own activity through GTP hydrolysis (McGrath et al., 1984).

As discussed previously, specific chromosomal alterations occur in many tumor types. In some cases, these alterations involve structural or functional alterations of oncogenes. Two of the most thoroughly characterized cases involve chronic myelogenous leukemia and Burkitt lymphoma.

The c-abl locus is located near the region of chromosome 9 that is translocated to chromosome 22 in the formation of the Philadelphia chromosome. The breakpoint on chromosome 22 clusters in a 5 Kb. area termed the breakpoint cluster region or bcr (Groffen et al., 1984). In CML cell lines the c-abl protein is abnormally large, and an abnormal 8 Kb. mRNA containing sequences from chromosome 22 is fused to the c-abl transcript (Stam et al., 1985). The abnormal protein has protein kinase activity like v-abl but unlike c-abl. The breakpoint on chromosome 9 is not as specific as the breakpoint on chromosome 22 in that no specific region is involved. However, the first exon of the abl oncogene is always involved in the translocation (Groffen et al., 1984).

Common features of Burkitt lymphoma include translocations involving chromosomes 2, 14, and 22 with band q24 of chromosome 8. In these cases the c-myc gene is translocated from chromosome 8 to chromosome 14 near to the immunoglobulin heavy chain locus, from 8 to 2 near to the immunoglobulin light chain (Igk) locus, or from 8 to 22 near to the immunoglobulin light chain (Igλ) locus (Croce et al., 1979). However, even though the breakpoints involved in Burkitt lymphoma appear to be homogeneous at a cytogenetic level, molecularly they are heterogeneous and thus lead to different structural

alterations of the immunoglobulin and myc loci. However, the fact remains that consistent chromosomal alterations are closely related to oncogene changes. This molecular heterogeneity can be correlated to myc mRNA expression in various Burkitt lymphoma patients. In some patients, high levels of myc mRNA are observed, while in other cases myc mRNA levels are lower than in control cells (Taub et al., 1984). These data suggest that deregulation of myc expression, rather than simply an increase, may be critical for myc activation, and thus transformation, in Burkitt lymphoma.

Oncogene amplification occurs in many tumor cells. As mentioned previously, in most cases the presence of amplified oncogenes is represented by double minutes and homogeneously staining regions. It is likely that in some tumor types DMs and HSRs may involve as yet unidentified oncogenes, and that amplification may represent a mechanism that is involved in the establishment or progression of the transformed phenotype by increased expression of the oncogene through increased expression.

Rodent Studies

Oncogene studies in rodent systems have concentrated on molecular transformation and activation as well as tumor progression and metastasis. Guerreiro et al. (1984) induced thymomas in mice with both N-nitrosomethylurea and gamma radiation, and these tumors exhibited activation of specific oncogenes dependent upon the inducing agent. Tumors induced with the chemical carcinogen showed activation of N-ras, and the radiation-induced tumors exhibited activation of K-ras.

Furthermore, expression of the oncogene mRNA and protein products were also increased. Also, when tumor DNA was isolated and transfected into either NIH 3T3 cells or rat 2 cells, transformants from the chemically-induced tumor DNAs exhibited an increase in N- ras transcripts and those from the radiation-induced tumor DNAs exhibited an increase in K- ras transcription. It is possible that the increase in these activated oncogene transcripts may be due to gene amplification, an increase in the rate of transcription, or a more stable message (Guerreo et al., 1984).

K- ras activation was also observed in rat tumors of varying histological types that were induced by ionizing radiation (Sawey et al., 1987). However, in contrast to the mouse thymomas, activation and amplification of c- myc was observed in some tumors. Concurrent activation of K- ras and c- myc was observed in 25% of tumors. The tumors exhibiting concurrent activation of both oncogenes were all clear cell carcinomas, whereas 80% of those exhibiting only K- ras activation were squamous cell carcinomas, suggesting that activation of particular oncogenes may be dependent on cell type (Sawey et al., 1987).

Not all radiation-induced malignant rodent cells exhibit K- ras activation. Borek et al. (1987) did not observe activation of any known oncogenes in NIH 3T3 cells that had been transformed with DNA from x- irradiated hamster embryo cells. However, all transformants appeared to exhibit activation of an as of yet unidentified oncogene.

A study of 15 c-onc genes in nontumorigenic and low and high malignant potential rat cells, suggests a complex relationship between

c-onc expression and tumor progression. When the nontumorigenic cells were induced to differentiate, the levels of c-myb, c-abl, and c-H-ras transcripts remained unchanged, the level of c-N-ras increased, and the level of c-erbB decreased. Other c-onc genes (c-erbA, c-sis, c-fms, c-fos, c-myc, and c-K-ras) were not detectable. When the three cell types were proliferating at similar rates, c-mos was not expressed in the nontumorigenic cell, c-fos and c-erbA were not expressed in the low malignant cells, and c-erbB and c-sis were not expressed in the high malignant cells. C-K-ras was increased in the highly malignant cells and was barely detectable in the other cell lines (Leibovitch et al., 1986). These data suggest that tumor progression can be associated with decreased or increased expression of c-onc genes, but a direct relationship with the c-onc gene expression cannot be established in rodent systems.

In animal tumor systems progression to the metastatic phenotype has also been examined in an attempt to establish a correlation with increases or decreases in oncogene expression. Mouse lymphoma cells are tumorigenic but not metastatic until they express activated c-K-ras (Vousden and Marshall, 1984). In Ableson virus- transformed mouse lymphomas, v-abl oncogene was not expressed during progression (Grunwald et al., 1982), suggesting that the loss of this oncogene may have led to a loss of regulation. In some solid tumors, such as murine melanomas and fibrosarcomas, c-onc expression appears to be unrelated to the metastatic phenotype (Kris et al., 1985). However, variations in oncogene expression have been observed in N- methyl- N- nitrosourea- induced metastatic mammary carcinomas in the rat (Sukumar et al.,

1983). Southern analysis revealed that the primary tumors had a 10 times greater DNA level of the c-H-ras gene than the metastases. Although modifications in oncogenes or their expression are associated with metastasis of some tumors, it is difficult to conclude that such alterations are required for progression to or maintenance of the metastatic phenotype. It is more likely that such oncogene changes contribute in part to progression of murine solid tumors.

In rodent systems, as in human tumors, cytogenetic abnormalities can be correlated with oncogene alterations. In some murine myxosarcomas a translocation between chromosomes 6 and 15 is present. In this case the proximal one-third (including the centromere) of chromosome 6 is deleted. As a result of this translocation c-myc, which normally resides on mouse chromosome 15, is placed in close proximity to the Ig kappa light chain genes present on chromosome 6 (Banerjee et al., 1985). Also, translocations involving chromosome 15 with chromosome 12 and 15 with 6 occur in some murine B-cell leukemias and lymphomas (Klein, 1983). Because of these translocations, highly active Ig genes activate c-myc. Recent rodent studies have also suggested that chromosomal alterations may play a role in suppression of oncogenes during neoplastic development. Studies by Oshimura et al. (1988) have shown that the loss of one copy of chromosome 15 suppresses neoplastic progression of ras- induced Syrian Hamster tumors. These studies support the idea that the loss of chromosome 15 results in the loss of a gene that suppresses a phenotypic change necessary for tumor development.

Multidrug Resistance

Human Studies

The use of chemotherapeutic agents for the treatment of cancer has resulted in successful treatment for many patients. However, many cancers do not respond to chemotherapy, and other cancers that do initially respond later become resistant. Drug transport studies in various multidrug-resistant (MDR) cell lines suggest that decreased drug accumulation in the resistant cells results in part from an increased rate of drug efflux (Chen et al., 1986). These studies have led to the concept of an efflux pump responsible for the removal of many naturally occurring cytotoxic drugs, such as plant alkaloids, from multidrug-resistant cells. The most common biochemical characteristic of multidrug-resistant cells is the increased expression of a 170 kilodalton (Kd), trans-membrane glycoprotein, that is termed the p-glycoprotein (Kartner et al., 1983; Cornwell et al., 1986; Willingham et al., 1987). The p-glycoprotein gene is amplified in multidrug-resistant cell lines (Scotto et al., 1986), and this gene amplification is accompanied by an increased expression of a 4.5 to 5.0 kilobase p-glycoprotein mRNA (Chen et al., 1986). It is speculated that this p-glycoprotein may be directly involved in drug transport in multidrug-resistant cells either as an efflux pump or by changing the permeability of the lipid bilayer. P-glycoprotein consists of approximately 2 equal parts that are homologous to each other, and both parts contain a hydrophilic and hydrophobic domain. Each hydrophobic domain includes 6 transmembrane segments. The hypothesis that p-

glycoprotein is directly involved in drug transport is supported by the fact that the hydrophilic domains share 80% sequence homology with peripheral membrane components of bacterial transport systems (Chen et al., 1986).

High levels of the MDR gene product are found in many apparently normal human tissues. These include colon, kidney, liver, and adrenal cells (Thiebaut et al., 1987). In tumor cells the amplification of the MDR gene is often indicated cytologically by the presence of double minute chromosomes and homogeneously staining regions. However, the presence of double minutes and homogeneously staining regions alone is not sufficient to prove MDR amplification. These cytological structures might also represent other amplified genes, such as oncogenes. These cytological manifestations are often found in tumors derived from the normal tissues that express high levels of the MDR protein. Such tumors include adenocarcinomas of the colon and kidney, hepatomas, and adrenocortical carcinomas (Thiebaut et al., 1987). However some tumors, such as skin papillomas, are derived from normal tissues that express little or no MDR gene product, but contain DMs that correlate with MDR amplification (Larcher et al., 1988).

Some tumors, such as leukemias and lymphomas, express low or barely detectable amounts of MDR gene product, and are sensitive to chemotherapy. However other tumors, such as melanomas and lung carcinomas, that are relatively resistant to chemotherapy also express only low levels of MDR (Fojo et al., 1987). Furthermore, colon carcinomas and neuroblastomas, both of which express high levels of MDR, exhibit different responses to chemotherapeutic agents.

Neuroblastomas are relatively sensitive, while colon carcinomas are not. It remains to be determined whether the level of MDR expression is sufficient to explain the drug resistant phenotype, or whether other mechanisms are responsible for the resistance of tumor cells to chemotherapy.

Rodent Studies

The majority of MDR studies have been done on human cells; however, it is known that the plasma membranes of hamster and mouse cell lines that display multiple resistance to drugs have an increased expression of a 170 Kd surface protein (Kartner et al., 1983). Also, increased MDR gene expression has been observed in mouse and Chinese hamster cells that were resistant to numerous drugs in culture. The mouse and hamster gene share approximately 95% homology (Scotto et al., 1986).

Similarities and Differences between Rodent and Human Tumors

Chromosomes and Oncogenes

The same general classes of chromosomal alterations occur in both human and rodent tumors, and the majority of known oncogenes have been mapped in both human and rodent systems. However, the examination of particular tumor types indicates that while many cytogenetic and oncogenic similarities exist between human and rodent malignancies, not all individual tumor types contain similar alterations. For example, in murine B- cell leukemias and lymphomas t(15;12) and t(15;6) are common (Klein, 1983), while in Burkitt lymphoma, a human B-cell

disorder, t(8;14) is commonly observed (Croce et al., 1979). In the cases of the murine translocations, c-myc , normally residing on mouse chromosome 15, is activated through translocation to a location near to immunoglobulin genes. In Burkitt Lymphoma, c-myc is translocated from chromosome 8 near to the immunoglobulin heavy chain locus on chromosome 22 and is activated. Thus, in these B- cell malignancies, very similar chromosomal and molecular alterations are observed between humans and mice. Another similarity, although not as striking, is observed when comparing human and rodent myxosarcomas. A t(9;22) involving the translocation of c-abl from chromosome 9 to chromosome 22 is observed in the human tumor (Sandberg et al., 1988). Isochromosome 2 is observed in the murine tumor (Dhaliwal et al., 1987), and c-abl is again involved since this oncogene is located on chromosome 2 in the mouse. However, t(6;15), eliciting the translocation of c-myc near to the immunoglobulin kappa promoter on chromosome 6, is also commonly observed in murine myxosarcomas. Involvement of this oncogene has not been reported in the human tumor.

In the case of some tumors, such as malignant melanoma and mesothelioma, there appears to be no similarity between the human and rodent tumors. In human melanomas t(1;17) and a deletion of 6q are commonly observed (Sandberg, 1988), while in the mouse trisomy of chromosome 1 is observed. Thus, the chromosomal alterations observed in melanomas of humans and mice are not even of the same general type. Human mesotheliomas contain a deletion of chromosome 3p as their consistent chromosomal alteration (Popescu et al., 1988), while translocations involving chromosomes 5, 10, and 13 are observed in rat

mesotheliomas (Libbus and Craighead, 1988). Again, the chromosomal alterations observed in the human and rodent are completely different even though the tumors are of the same histological type. It is possible that the different chromosomal alterations alter cellular functions that result in similar phenotypic responses, but this hypothesis would require further research.

Multiple Drug Resistance

As was discussed earlier, expression of the multidrug resistance gene is an important consideration in the treatment of human malignancies even though it is not yet clear if the MDR phenotype is fully responsible for a tumor's response to chemotherapy. There is 90% homology between the human and mouse MDR genes (Scotto et al., 1986), and both species express the 170 kd. p- glycoprotein (Kartner et al., 1983). Normal human tissues express MDR between 1 and 500 fold, where 1 has been defined as baseline expression (Fojo et al., 1987). For example, skin, skeletal muscle, heart, and bone marrow all have expression values between 1 and 5, while liver has a value of 25 fold, kidney a value of 50 fold over baseline, and adrenal expresses MDR 500 fold over baseline. The normal function of MDR is unclear. However, many MDR substrates are naturally occurring, charged ring structures (Udea et al., 1987). These substances are found in plant seeds, and perhaps animals could protect themselves from these substrates by transporting them back into the lumen of the colon. If they did reach the blood, they would be filtered and transported into the bile or urine (Pastan and Gottesman, 1987). The function of MDR in the adrenal

is quite unclear. However, the fact that MDR is expressed in human adrenals, but not in rodent adrenals (Pastan and Gottesman, 1987) may eventually provide further clues to the normal function of MDR.

Previous studies have indicated chromosomal, oncogenic, and multiple drug resistant similarities as well as differences between human and rodent malignancies. It is therefore possible that specific rodent tumor models may provide information about chromosomal alterations in corresponding human tumors. Even if the animal tumor models do not provide information on which specific human chromosome might be involved, perhaps they may indicate the type of chromosomal alteration important in causation or early development of the tumor.

CHAPTER 2

CHROMOSOME CHANGES IN MOUSE FIBROSARCOMAS

Summary

By the time solid tumors are clinically observed, many chromosome changes are already present, and it is virtually impossible to determine if any specific ones are common from tumor to tumor. Thus, it is important to observe chromosome changes early in tumor development in order to determine whether these are involved in the development of the tumor. We have utilized a system that makes this possible. It essentially provides a "petri dish" in an animal that can be removed at different times after it is implanted for evaluation of the cells growing on it. Since the fibrosarcomas develop on the disk, it is possible to analyze those cytogenetic changes that occur early in the development of the tumor and to follow additional chromosome changes that might be associated with the tumor's development. Some mice were treated with X rays in addition to the disks. The disks and associated tissues were then removed from tumors at a very early stage of development and at several subsequent times until mature sarcomas were observed. Chromosomal analysis of both direct preparations of sarcoma cells and of cultured cells derived from the sarcomas showed duplications of chromosomes 10, 16, and 19 in cells from tumors that developed in mice that received both the disk alone as well as in those mice that received the disk + X rays. Additionally, metacentric

chromosomes were observed in animals that received the disk alone. Double minute chromosomes were observed in 41% of all tumors analyzed, while homogeneously staining regions were present in 22% of tumors. These gene amplifications were not observed in all cells from any particular tumor.

Introduction

Foreign Body Induced Tumors in Human and Rodent Species

Tumors in humans and rodent species have been associated with non-degradable foreign bodies have since the late 1800's (Ott, 1870). In humans, both medical implants and foreign bodies acquired by accident, such as bullets, induce chronic tissue reactions that involve scarlike encapsulation of the foreign bodies. This condition may then lead to a variety of types of neoplasms, predominantly sarcomas. The latency period of foreign body induced tumors in humans appears to range from 10 to 50 years (Bischoff, 1972).

Foreign bodies are much more tumorigenic in mice and rats than in humans, and also induce tumors in dogs, but not in guinea pigs, chickens, nor in hamsters. Studies of hybrid and inbred mice have indicated that this species difference is most likely due to genetically determined differences in macrophage activities that influence tumor latency (Brand et al. , 1977). The majority of foreign body induced tumors that arise in rodents are, as for humans, sarcomas, and these appear as various histological types including rhabdomyosarcomas, liposarcomas, reticulosarcomas, plasmocytomas, and,

most frequently as fibrosarcomas. Most of the foreign body induced sarcomas are composed of cells that are poorly differentiated, and that have a high mitotic rate and hyperchromatic nuclei. Also, many of the tumors have areas of necrosis and hemorrhage (Johnson et al., 1970).

Properties of Tumorigenic Implants

Physical irritation, not chemical factors, appears to be responsible for foreign body tumorigenesis. Foreign bodies of many chemical compositions are able to induce murine tumors, provided that a smooth, uninterrupted surface is present. If foreign bodies with rough surfaces are used, latency increases (Bates and Klein, 1966). Likewise, if the material is ground into a powder, or if it is perforated with holes, no tumor develops (Bischoff and Bryson, 1964). Also, the size and shape of the foreign body influences tumorigenicity and latency period. Tumor frequency is directly proportional to the surface area of the implanted foreign body; however, if multiple pieces are implanted, with a surface area equal to one large piece, the latency period increases (Brand et al., 1973).

The Mechanism of Foreign Body Tumorigenesis in the Mouse

Foreign bodies probably do not induce carcinogenic events per se (Brand, 1982), but by stimulating proliferation of fibroblasts, they can increase the likelihood of a spontaneous event. The cellular activity involved in foreign body tumorigenesis was described by Brand (1982). Within 2 weeks after subcutaneous implantation of a plastic disk in a mouse, granulocytes and monocytic macrophages infiltrate the

implant site. The macrophages then settle on the implant surface and form a monolayer. At this time a very thin membrane, consisting of fibroblasts and collagen fibers, begins to develop around the implant. Fibroblasts then cover the inner surface of the membrane and are separated from the outer surface of macrophages by capillaries that have invaded the area. Between 4 and 8 weeks post-implantation of a plastic disk, cellular infiltration and proliferation of macrophages, granulocytes, and fibroblasts, as well as capillary outgrowth, occur in the tissue. At the implant surface, macrophages, giant cells, and granulocytes predominate. From 8 to 12 weeks post-implantation, there are more fibroblasts in the tissue and collagen production increases, while macrophages and giant cells predominate at the implant surface. From 12 to 28 weeks post-implantation, collagen is deposited in the tissue, capillaries are no longer present, and macrophages are inactivated. Inactive macrophages and a few giant cells are present at the implant surface. After 28 weeks post-implantation, there is continued fibroblast proliferation and collagen deposition, and the fibrosarcoma increases in size.

Chromosome Changes in Foreign Body Induced Sarcomas

Chromosomal analysis of murine foreign body induced sarcomas has shown both structural and numerical abnormalities. The most frequently observed alteration was hyperdiploidy (Rachko and Brand, 1983; Banerjee and Bates, 1966). G-banding analysis revealed that aneuploidy in different tumors involved virtually every mouse chromosome, but that no particular chromosome was involved consistently in foreign body-

induced sarcomas (Banerjee and Bates, 1966). Structural alterations, especially metacentric chromosomes, were also present. However, these alterations were not specific, occurring with equal probability among each individual chromosome (Rachko and Brand, 1983). Double minute chromosomes, of unknown identity and origin, were also observed in several foreign body induced murine sarcomas (Buoen and Brand, 1968).

Rationale for These Experiments

As has been previously discussed, alterations of chromosome structure and/or number are associated with most, if not all, tumors. By the time solid tumors are clinically observed, many chromosome changes are present. Thus, to determine if specific chromosome changes are associated with tumorigenesis, it is important to observe those cytogenetic changes that are present in tumors early in development and to follow chromosome changes with the progression of the tumors. In addition, it is important to determine if such changes are dependent upon a particular inducing agent. As an approach to studying this, plastic disks were implanted subcutaneously into mice, and the disks and associated tumor tissue were removed at various stages post-implantation. Thus, chromosomal changes could be analyzed at various stages of tumor development.

Materials and Methods

Plastic Disk Implantation/ Removal (Sarcoma Production)

Silastic, medical grade plastic disks (Dow Corning) measuring 14 mm. in diameter and 1 mm. thick were implanted subcutaneously (laterally) into C₃H female mice. Some mice, in addition to the disk implant, received X ray treatments of 2.5 Gy, 4 times for a total exposure of 10 Gy. These X ray treatments were absorbed to the skin and subcutaneous tissue and were delivered at 3 day intervals, using a Norelco MG 150 X ray unit. The X ray machine settings were 25 kv, 3.5 mA with a beam filtration of 0.01 mm. of aluminum. The X rays were delivered at a dose rate of 0.77 Gy/ min. at 32.5 cm. from the filter. Mice were restrained in a lucite holder that was centered under the beam and rotated during exposure. The disks and associated tumor tissue were palpated and then removed at one week intervals (regardless of tumor size) from 28 weeks post-implantation of the disk (when a capsule could first be observed around the disk) until mature sarcomas were observed (approximately 53 weeks post-implantation). A general flow diagram of the basic procedure is shown in figure 2.1.

Culturing Solid Tumors- The Spillout Technique

The method for separating cells for culture was modified from that described by Liebovitz (1986). The tissues associated with the disk implants were placed in sterile petri dishes and were completely covered with L-15 medium (American Biooragynics) containing penicillin (Squibb) (300 ug/ml), streptomycin sulfate (Lilly) (300 ug/ml),

Figure 2.1. General procedure for chromosomal analysis of sarcoma cells.

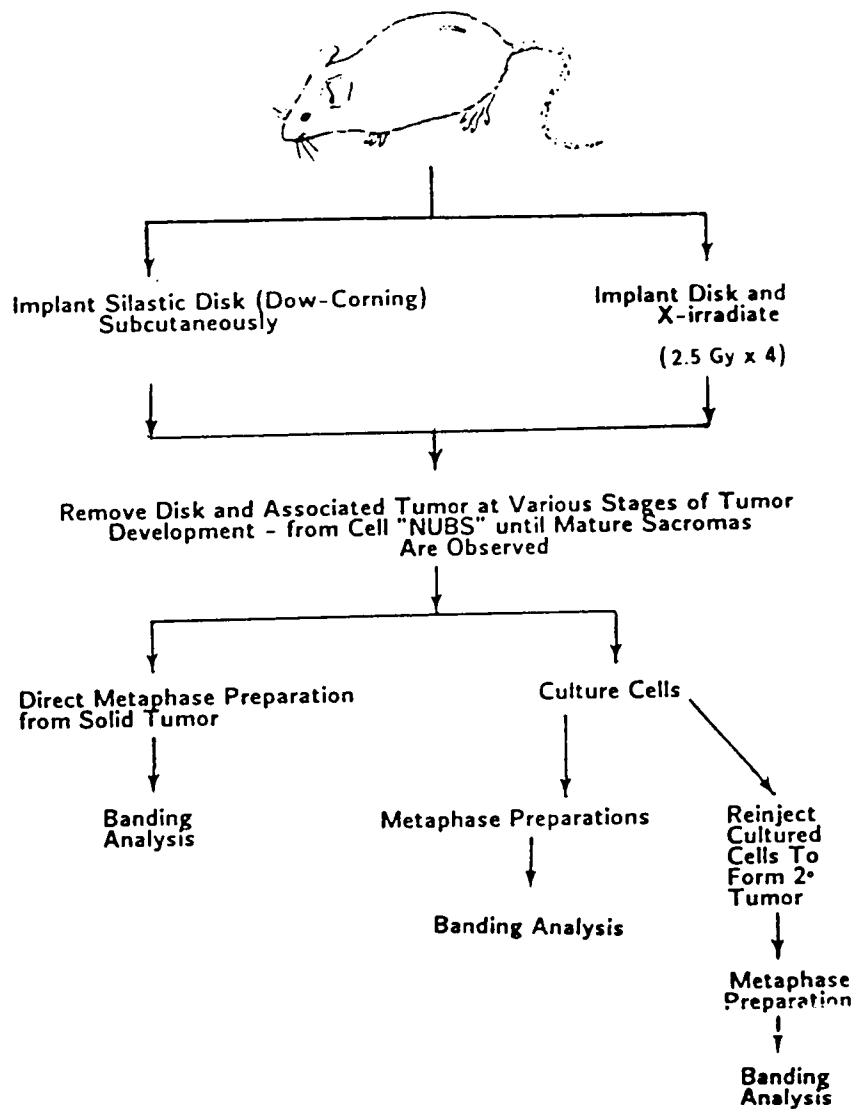


Figure 2.1

amphotericin B (Sigma) (3 ug/ml), transferrin (Sigma) (0.005 ug/ml), epidermal growth factor (Sigma) (0.01 ng/ml), and 15% fetal bovine serum (Gibco). All necrotic areas, connective tissue, fat, and blood vessels were cut away with sterile scissors. The tissue was washed in a second petri dish containing L-15 medium and was then transferred to a third petri dish containing the same media for disaggregation into 1 mm. cubes with sterile scissors. The medium surrounding the tissue cubes was harvested with a 5 ml. pipette and transferred to a 15 ml. sterile screw capped centrifuge tube. The fine minces were then allowed to settle for 3 minutes, and the supernatant over the settled minces was transferred to another 15 ml. centrifuge tube. Both the supernatant and fine minces were then washed 3 times in Hanks balanced salt solution. The supernatant pellet and fine minces were cultured separately in 5 ml. of L-15 in 25 mm. tissue culture flasks (Falcon). Cultures were maintained in a 37°C. incubator humidified 95- 100% in an atmosphere of 5% CO₂ in air.

Metaphase Preparation From Cultured Tumor Cells

To obtain metaphase preparations from tumor cell cultures, colchicine was added to 75% confluent cell cultures at a concentration of 1×10^{-7} M. The cells were incubated at 37°C for 5 hours. Cells were removed from flasks by scraping, centrifuged, resuspended in 10 ml. of 0.075 M KCl and incubated at 37° for 20 minutes. The fixation, slide preparation, and staining methods followed our standard procedure (Brewen and Gengozian, 1971).

Direct Metaphase Preparation From Solid Tumors

Direct metaphase preparations from the tissue associated with the disk implants were obtained by mincing the tissue into 1 mm. pieces with sterile scissors. These pieces were then incubated in 10 ml. of dispersing solution [0.0025% trypsin (Sigma), 0.0025% collagenase (Sigma), 0.001% hyaluronidase (Sigma), and 0.005% bovine serum albumin (Miles) dissolved in Hanks balanced salt solution] for 2 hours at room temperature with mild agitation. Colchicine was added at a concentration of 1×10^{-7} M, and the incubation was continued for 1 additional hour (Aldaz et al., 1986). Cells were centrifuged, resuspended in 10 ml. of 0.075 M KCl and incubated at 37° for 20 minutes. The fixation, slide preparation, and staining methods followed our standard procedure (Brewen and Gengozian, 1971).

G- Banding and Karyotyping

To determine whether or not specific chromosomal alterations could be correlated with different stages of tumor development, the metaphase spreads (from cultured cells and/or direct preparations) were G-banded. Slides between 1 and 2 weeks old were microwaved for 2 minutes and allowed to cool. The banding method was modified from that described by Seabright (1971). The cells were treated for 10 to 20 seconds with 0.02% trypsin/EDTA in phosphate buffered saline. Immersion in 5% fetal bovine serum stopped the action of the trypsin. The slides were rinsed with phosphate buffered saline and stained in 5% Giemsa in Gurr buffer (pH 7.6) for about 6 minutes, until suitable banding was obtained. Slides were then air dried and mounted in

Permout. Karyotypes were arranged according to a standard mouse nomenclature system (Nesbitt and Francke, 1973).

C- Banding

To analyze cells for the presence of HSRs, slides were C- banded to stain for centric heterochromatin, using a modification of the method of Salamanca and Armendares (1976). Preparations were treated with 0.2 N HCl for 30 minutes at room temperature. They were then treated with 0.07 N barium hydroxide for 7 minutes at 37°C and incubated in 2X SSC at 65°C for 1 hour. Slides were stained in 5% Giemsa stain for about 20 minutes until suitable C- banding was obtained, air dried, and mounted in Permout.

Results

Chromosomal Analysis of Sarcomas Induced by Disk Implantation

Table 2.1 lists the chromosomal trends that were observed in direct preparations of cells from sarcomas that developed in animals receiving disks. Tumors were obtained from 26 to 53 weeks post-implantation of the disk, and the numbers of chromosomes per cell in different tumors varied from 38 to 350. There were considerable variations in the number of chromosomes per cell between sarcomas with much less variation within each sarcoma. Generally, there was an increase in the number of chromosomes per cell as the time after disk implantation increased. However, this was only a general trend, and did not hold true for the 53 week sarcomas, where the number of chromosomes per cell

Table 2.1 Chromosomal Analysis of Sarcoma Cells that Developed in Animals Receiving the Disk Treatment

<u>Sarcoma</u>	<u># Weeks after Implantation</u>	<u># Chromosomes/Cell*</u>		<u>% Cells Containing:</u>		
		<u>Mean</u>	<u>Range</u>	<u>DMs</u>	<u>HSRs</u>	<u>Metacentrics</u>
88-7576	26	59	42-118	0	0	30
88-7983	31	63	46-81	0	0	30
88-7984	31	58	41-100	0	0	40
85-11424	34	58	38-120	0	0	40
85-11365	34	52	41-59	0	0	50
85-11420	36	62	41-92	0	0	30
85-11409	38	66	52-85	32	0	0
85-11486	38	67	52-116	40	0	0
85-11426	40	70	45-113	0	0	80
85-11485	40	69	44-89	40	0	40
87-215	42	77	40-94	45	0	80
86-7638	42	80	42-95	45	0	30
86-1504	43	83	50-200	0	36	100
85-11364	43	84	41-119	0	0	0
85-11333	44	117	90-161	0	44	30
85-11305	48	159	102-195	0	0	80
85-11304	50	288	287-290	75	0	0
85-11391	50	301	252-350	70	0	0
87-296	53	42	40-48	0	0	100
87-297	53	71	60-77	0	50	60

* a minimum of 25 metaphases (direct preparations) per tumor were scored

was much lower than expected. The two 53 week sarcomas analyzed had means of 42 and 71 chromosomes per cell respectively. If the general trend held, greater than 300 chromosomes per cell would have been expected in these tumors.

Double minute chromosomes and homogeneously staining regions, both indicative of gene amplification, were present in 50% of the sarcomas analyzed, and these structures were observed in tumors at later stages of development (based on the time post- implantation of the disk. Between 32% and 78% of the cells in a particular sarcoma contained DMs and HSRs (Table 2.1). Additionally, 80% of these sarcomas contained at least 1 metacentric chromosome, that was easily observed due to the fact that the normal mouse karyotype contains only acrocentric chromosomes.

Representative distributions of the chromosome number per cell for different tumors are shown in figure 2.2 (a 34 week post-implantation tumor), figure 2.3 (a 36 week post-implantation tumor), figure 2.4 (a 40 week post-implantation tumor), figure 2.5 (a 42 week post-implantation tumor), and figure 2.6 (a 43 week post-implantation tumor). Again it can be seen that as the time after disk implantation increases, the number of chromosomes per cell generally increases. Furthermore, as the time post- implantation increases, the range of chromosomes per cell generally increased. Also, it appears that more than one population of cells may be present in some sarcomas, and it is possible that a non-clonal tumor origin might account for the lack of continuous chromosomal distributions. This is not unprecedented since Reddy and Fialkow (1984) observed a small number of cases of non-clonal

Figure 2.2. Chromosomal distribution of sarcoma 85-11424, a 34 week post-implantation tumor from a mouse that received the disk implant alone.

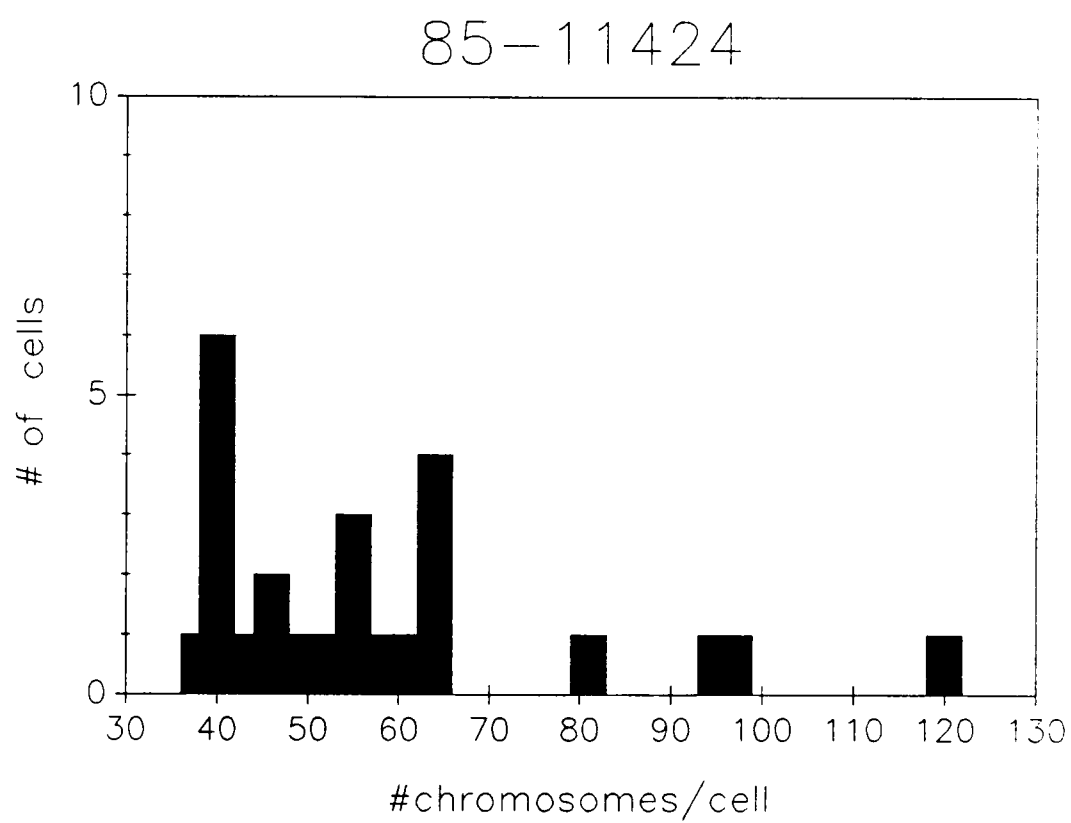


Figure 2.2

Figure 2.3. Chromosomal distribution of sarcoma 85-11420, a 36 week post-implantation tumor from a mouse that received the disk implant alone.

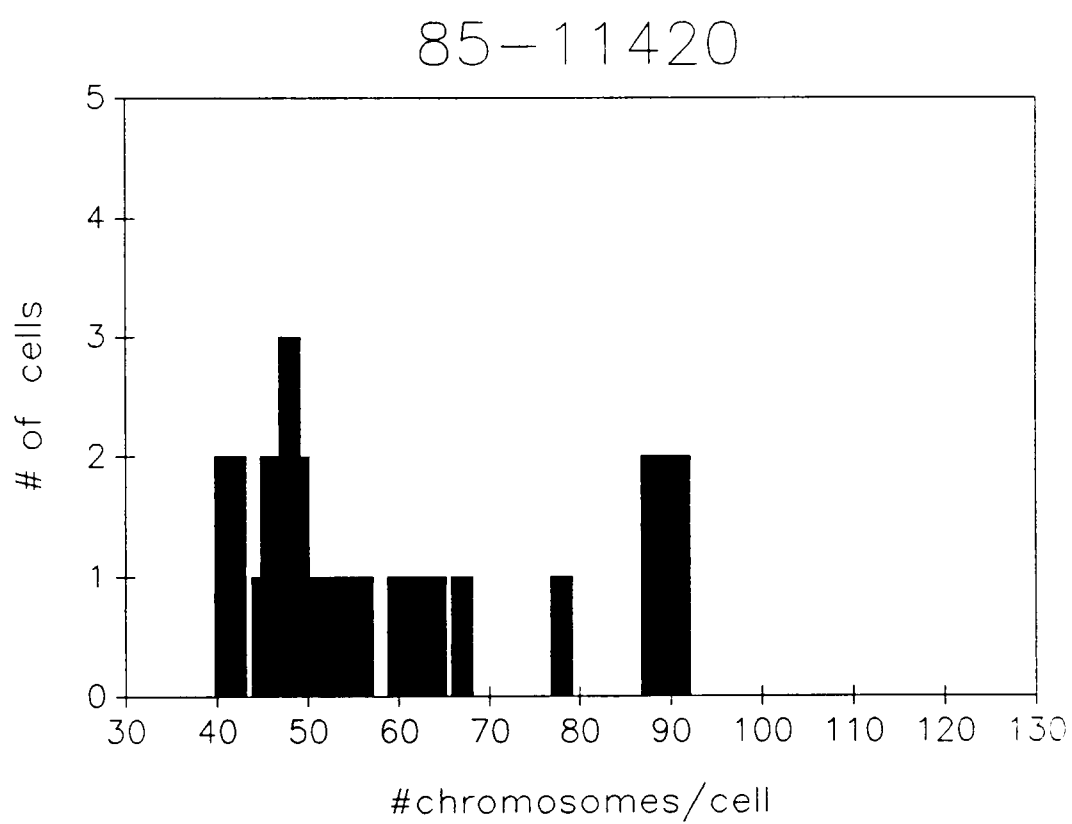


Figure 2.3

Figure 2.4. Chromosomal distribution of sarcoma 85-11485, a 40 week post-implantation tumor from a mouse that received the disk implant alone.

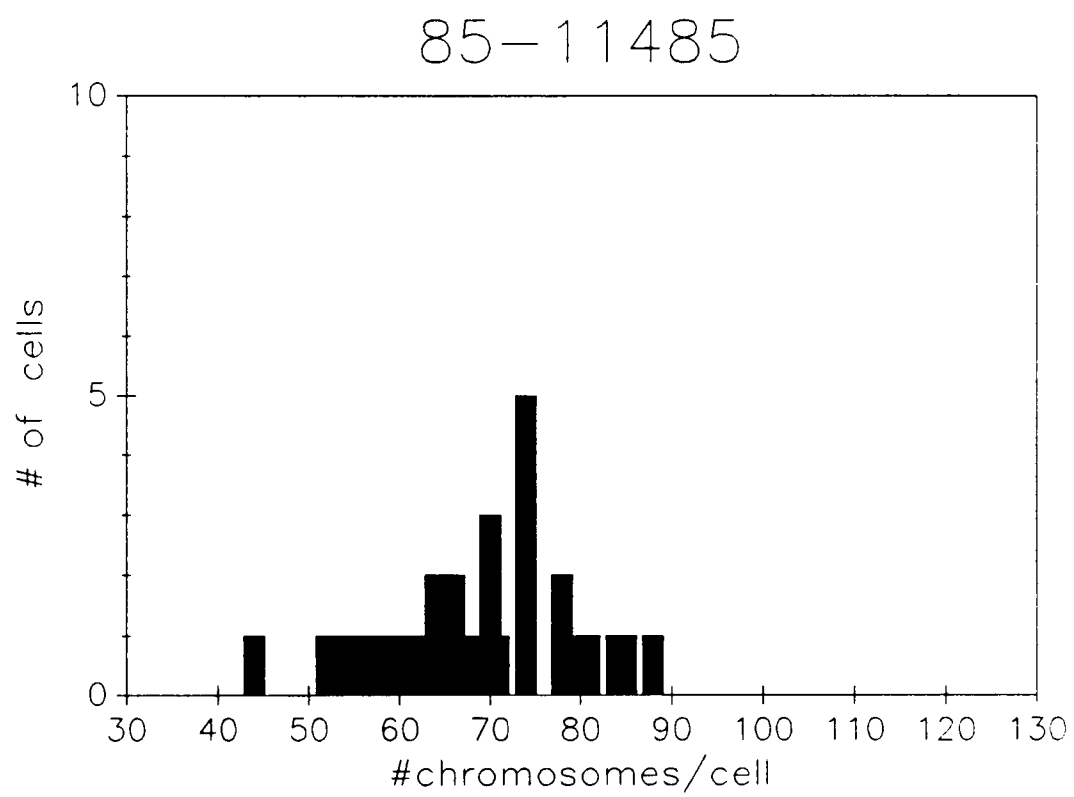


Figure 2.4

Figure 2.5. Chromosomal distribution of sarcoma 87-215, a 42 week post-implantation tumor from a mouse that received the disk implant alone.

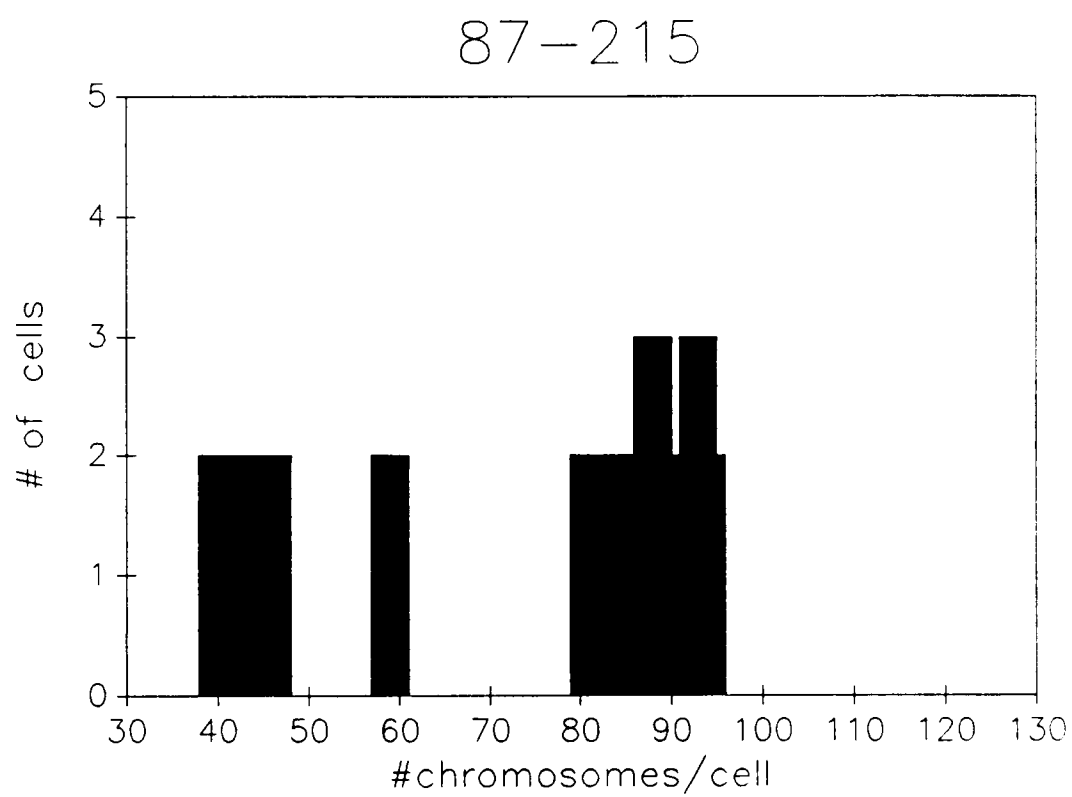


Figure 2. 5

Figure 2.6. Chromosomal distribution of sarcoma 85-11364, a 43 week post-implantation tumor from a mouse that received the disk implant alone.

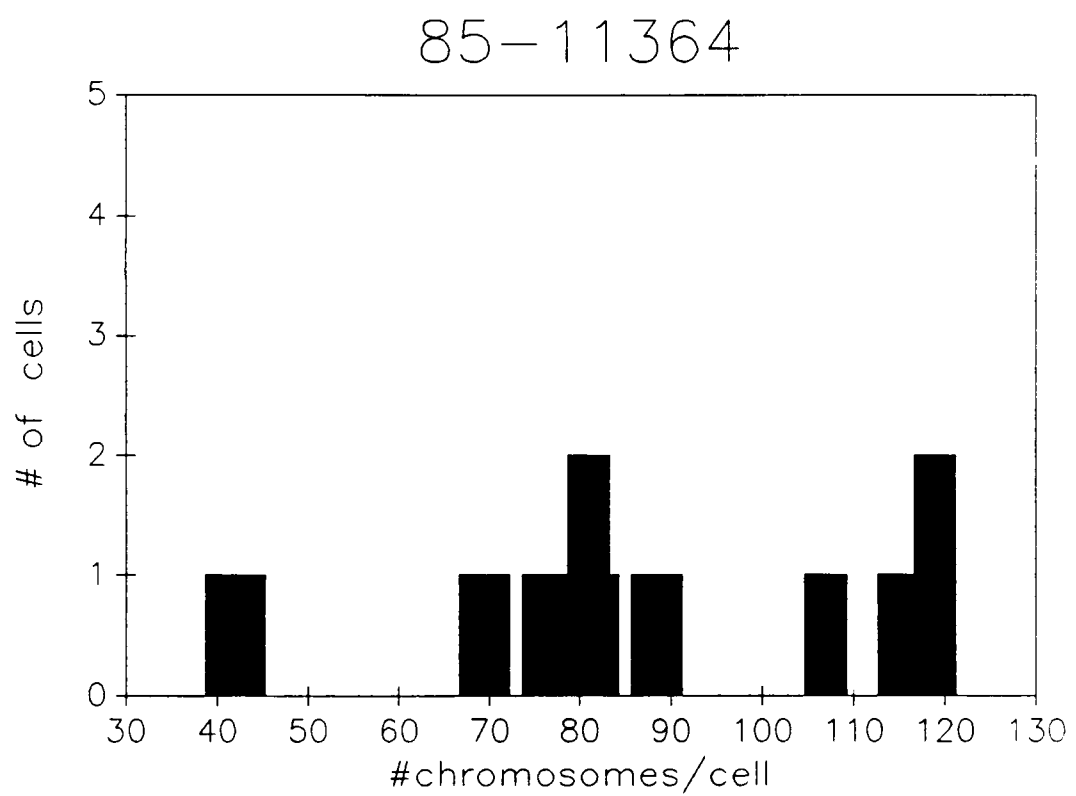


Figure 2.6

development in foreign body- induced murine sarcomas. G-banding analysis revealed that there were many different karyotypes for the different tumors and within a tumor. However, some alterations consistently occurred in particular sarcomas, and in many cases these changes were observed in several of the sarcomas from animals that received the disk treatment alone (Table 2.2). The most common chromosomal alterations (those present in 50% or more of cells analyzed in a particular tumor) were multiple copies of chromosomes 10 and 16 , as well as metacentric chromosomes that were present in 75% of sarcomas. Multiple copies of chromosome 16 were present in 50% while multiple copies of chromosome 10 were present in 66% of the sarcomas analyzed. The multiple copies of chromosome 10 included trisomy in 17% of tumors, tetrasomy in 25% of tumors, and pentasomy in 17% of tumors. In all tumors analyzed that contained multiple copies of chromosome 10 (66%), trisomy 10 was most frequently observed. Overall, 92% of the tumors analyzed contained metacentric chromosomes, and although these metacentrics were not identical between tumors, only four chromosomes were involved. These were chromosomes 4, 15, 17, and 19. Isochromosome 19 was present in 58% of the sarcomas, while metacentrics involving (4;17), (4;15), and (1;17) were present in 17%, 17%, and 8% of tumors analyzed, respectively. Metacentric chromosomes were observed in tumors at all stages of development; however, isochromosome 19 was present in earlier tumors, while (4;15), (4;17), and (1;17) were observed in later tumors. Other alterations that were common (present in 25- 49% of cells analyzed in a particular tumor) were nullisomy 8 in 42%, trisomy 5 in 33%, and trisomy 7 in 17% of sarcomas analyzed.

Table 2.2 Frequent Chromosome Changes in Tumors from Animals
Receiving the Disk Treatment Alone

<u>Sarcoma</u>	<u># Weeks after Implantation</u>	<u>Chromosome Alterations</u>	
		<u>Most Common</u> (≥50% of cells)	<u>Less Common</u> (25%-49% of cells)
85-11424	34	iso 19 tetrasomy 16	nullisomy 8 trisomy 10
85-11365	34	iso 19 trisomy 10 tetrasomy 16	nullisomy 8
85-11420	36	iso 19 trisomy 10 tetrasomy 16	nullisomy 8 trisomy 5
85-11426	40	iso 19 trisomy 10 trisomy 16	nullisomy 8 trisomy 5
85-11485	40	iso 19 trisomy 10 trisomy 16	nullisomy 8 trisomy 5 trisomy 7 trisomy 9
87-215	42	iso 19 trisomy 10	nullisomy 8 trisomy 5 trisomy 7
86-7638	42	metacentric (4;15)	-----
85-11364	43	trisomy 10 tetrasomy 19 pentasomy 16	-----
86-1504	43	metacentric (4;17) trisomy 10 pentasomy 19	Del 1q 3 unidentified markers
85-11333	44	metacentric (4;17) trisomy 10 tetrasomy 19	-----
85-11305	48	metacentric (4;15) monosomy X	-----
87-296	53	metacentric (1;17)	-----

Trisomy 9 was observed in one tumor (8%) and a deletion of 1 was present along with 3 unidentified marker chromosomes in cells from one other tumor. Representative G- banded karyotypes are shown in figure 2.7 (a 34 week post- implantation tumor), figure 2.8 (a 36 week post- implantation tumor), figure 2.9 (a 40 week post- implantation tumor), figure 2.10 (a 42 week post- implantation tumor), figure 2.11 (a 43 week post- implantation tumor), figure 2.12 (a 43 week post- implantation tumor), figure 2.13 (a 48 week post- implantation tumor), and figure 2.14 (a 53 week post- implantation tumor).

Chromosomal Analysis of Sarcomas Induced By Disk + X ray Treatment

Table 2.3 lists the chromosomal trends that were observed in cells from sarcomas that developed in animals that received the disk + X ray treatment. Tumors were obtained from 18 to 60 weeks post-implantation of the disk, and the number of chromosomes per cell in different tumors ranged from 38 to 102. As with the sarcomas from animals receiving the disk treatment alone, there were considerable variations in the number of chromosomes per cell between sarcomas with much less variation within each sarcoma. The early cell "nubs" that initially formed around the disk implants (18 to 34 weeks post-implantation) consisted of cells with low chromosome numbers, close to the normal 40. Again, there was an increase in the mean number of chromosomes per cell as the time after disk implantation and X rays increased and DMs were observed in 50% of sarcomas, while HSRs were observed in 25% of sarcomas analyzed. No metacentric chromosomes were observed in sarcomas that developed in animals that received the disk + X ray treatment.

Figure 2.7. Karyotype of sarcoma 85-11424, a 34 week post-implantation tumor from a mouse that received the disk implant alone.

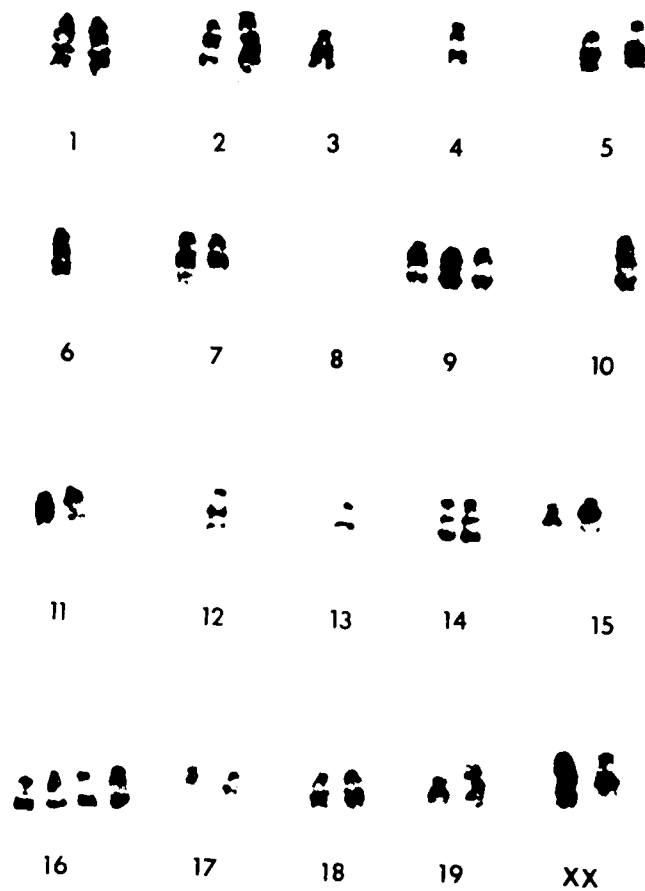


Figure 2.7

Figure 2.8. Karyotype of sarcoma 85-11420, a 36 week post-implantation tumor from a mouse that received the disk implant alone.

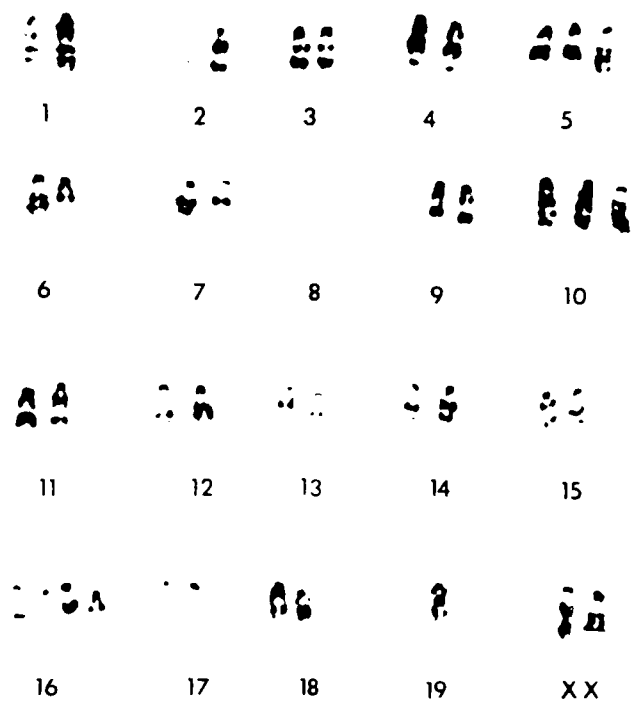


Figure 2.8

Figure 2.9. Karyotype of sarcoma 85-11485, a 40 week post-implantation tumor from a mouse that received the disk implant alone.



Figure 2.9

Figure 2.10. Karyotype of sarcoma 87-215, a 42 week post-implantation tumor from a mouse that received the disk implant alone.



Figure 2.10

Figure 2.11. Karyotype of sarcoma 85-11364, a 43 week post-implantation tumor from a mouse that received the disk implant alone.



Figure 2.11

Figure 2.12. Karyotype of sarcoma 86-1504, a 43 week post-implantation tumor from a mouse that received the disk implant alone.

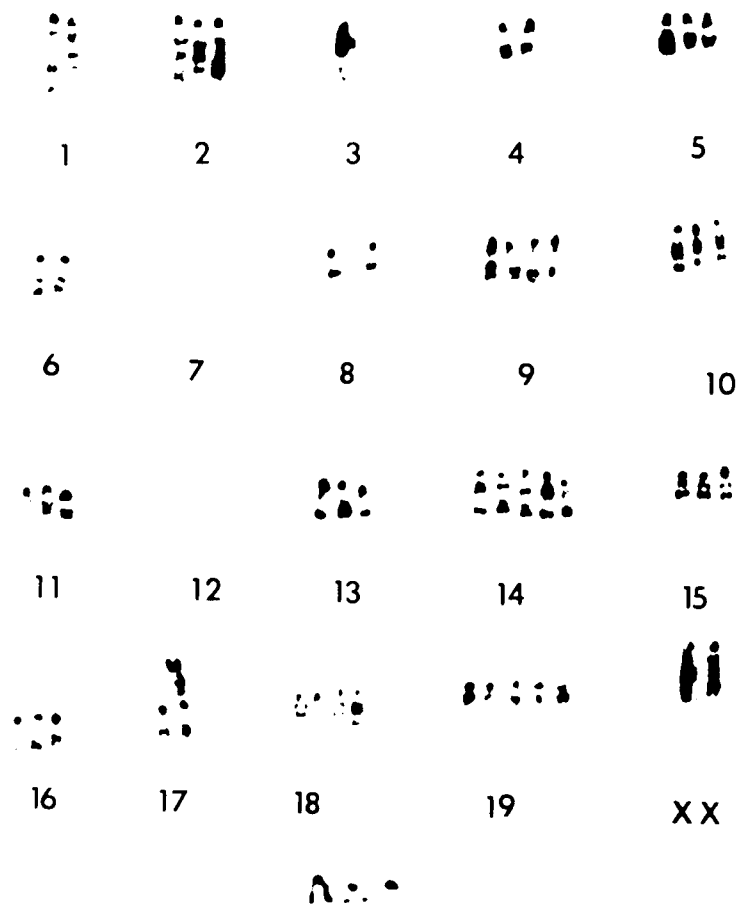


Figure 2.12

Figure 2.13. Karyotype of sarcoma 85-11305, a 48 week post-implantation tumor from a mouse that received the disk implant alone.



Figure 2.13

Figure 2.14. Karyotype of 87-296, a 53 week post-implantation tumor from a mouse that received the disk implant alone.



Figure 2.14

Table 2.3 Chromosomal Analysis of Sarcoma Cells that Developed in Animals Receiving the Disk + X Ray (2.5Gyx4) Treatment

<u>Sarcoma</u>	<u># Weeks after Implantation</u>	<u># Chromosomes/Cell*</u>		<u>% Cells Containing:</u>		
		<u>Mean</u>	<u>Range</u>	<u>DMS</u>	<u>HSRs</u>	<u>Metacentrics</u>
87-176	18	40	39-41	43	0	0
86-7644	34	41	40-42	0	0	0
86-7645	34	44	38-46	0	0	0
87-214	38	47	37-78	0	20	0
86-7643	42	59	42-86	0	20	0
88-7804	42	61	41-76	0	0	0
86-7661	44	65	44-71	0	30	0
86-7660	44	69	52-130	52	0	0
86-7667	46	69	40-102	52	0	0
87-231	52	65	50-96	0	40	0
87-233	52	65	45-78	0	48	0
87-211	60	68	39-80	50	0	0
87-213	60	71	69-94	78	0	0

* a minimum of 25 metaphases (direct preparations) per tumor were scored

Representative distributions of the number of chromosomes per cell are shown in figure 2.15 (a 34 week post-implantation tumor), figure 2.16 (a 42 week post-implantation tumor), and figure 2.17 (a 44 week post-implantation tumor). Again, more than one population of cells can be observed in the later (42 and 44 week post-implantation) tumors, while an early tumor (34 weeks post-implantation) has a near diploid chromosome complement. As the time after disk implantation and X ray treatment increased, the range of the number of chromosomes per cell broadened. For example, a 34 week tumor had a range of 40- 42 chromosomes per cell (pseudodiploid complement), while a 60 week tumor had a range of 39- 80 chromosomes per cell.

As with the tumors that developed in animals receiving the disk implant alone, G- banding analysis of cells from disk + X ray treated animals revealed numerous chromosome changes. However, again, some of these changes were consistent in several tumors. The most common chromosome alterations (those present in 50% or more of cells analyzed from a particular tumor) include multiple copies of chromosome 10 in 100% of the sarcomas analyzed, multiple copies of chromosome 16 in 57% of the sarcomas, and multiple copies of chromosome 19 in 71% of tumors.

The multiple copies of chromosome 10 existed as trisomy 10 (71%), and tetrasomy 10 (28%). The multiple copies of chromosome 16 also existed as trisomy 16 (43%) and tetrasomy 16 (14%) as did the multiple copies of chromosome 19 (57% trisomy and 14% tetrasomy). However, the multiple copies of chromosome 19 were not present as isochromosomes. In fact, no metacentric chromosomes were observed in animals receiving the disk + X ray treatment. Other common alterations (occurring in 25% to 49%

Figure 2.15. Chromosomal distribution of sarcoma 86-7644, a 34 week post-implantation tumor from a mouse that received the disk + x ray treatment.

86-7644

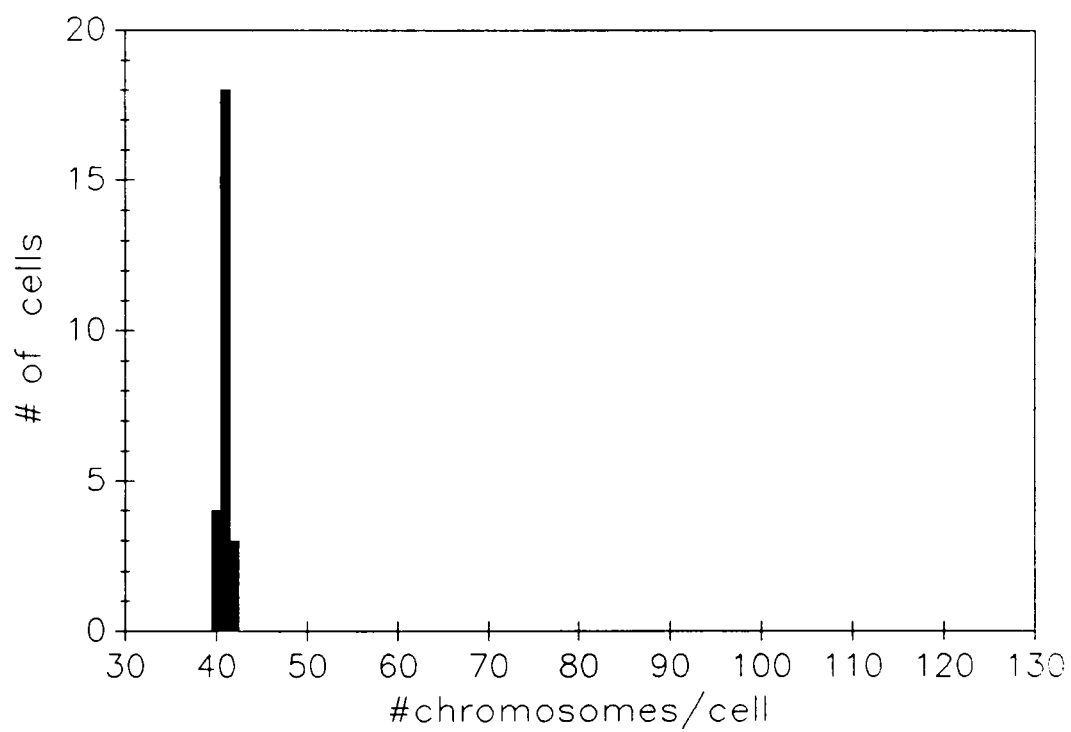


Figure 2.15

Figure 2.16. Chromosomal distribution of sarcoma 86-7643, a 42 week post-implantation tumor from a mouse that received the disk + x ray treatment.

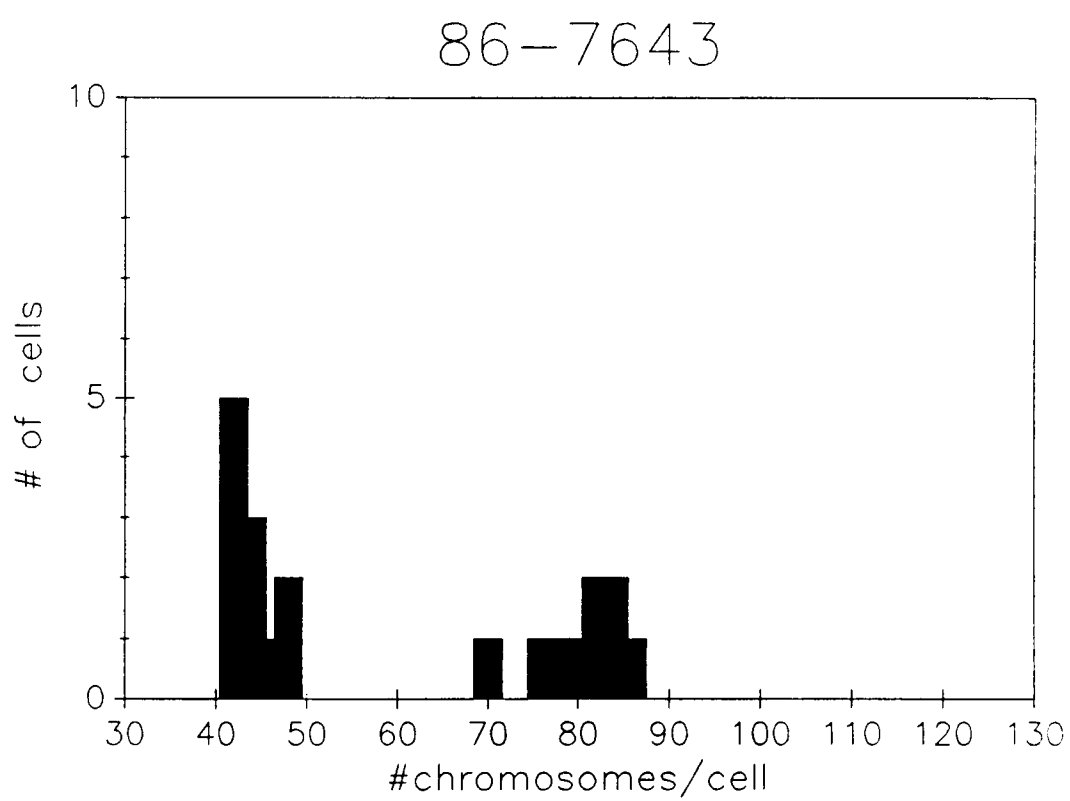


Figure 2.16

Figure 2.17. Chromosomal distribution of sarcoma 86-7661, a 44 week post-implantation tumor from a mouse that received the disk + x ray treatment.

86-7661

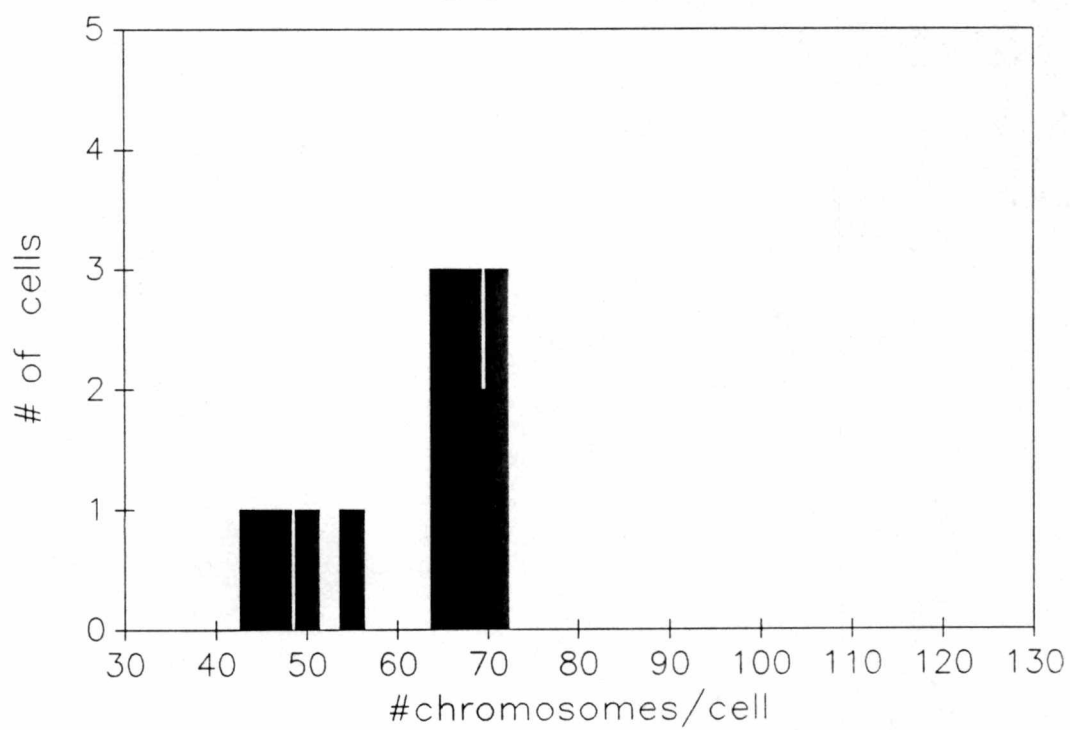


Figure 2.17

of cells analyzed from a particular tumor) include monosomy X in 43% of sarcomas and nullisomy 18 and trisomy 16 in 14% of tumors analyzed (Table 2.4). Representative G- banded karyotypes are shown in figures 2.18 (a 34 week tumor), 2.19 (a 42 week tumor), and 2.20 (a 44 week tumor).

Reinjected Cells

In order to determine whether or not the presence of DMs was related to tumorigenesis, 1,000,000 cells of each of 7 tumor cell lines (with or without DMs) were injected subcutaneously into both thighs and the intrascapular region of C₃H female mice. For each cell line 3 mice were utilized. Cell lines injected that contained DMs were 88-1363, 88-7704, 88-7577, and 87-213. Cell lines lacking DMs were 87-214, 88-7804, and 88-7576. Animals were checked for tumor growth 2 times each week, and time to tumor between mice receiving different injections was compared. When tumors were observed, animals were sacrificed by cervical dislocation, tumors were removed and cultured (as described in the Materials and Methods section: Culturing solid tumors- the spillout technique), and direct metaphase preparations were also obtained from the tissue (as described in the Materials and Methods section: Direct metaphase preparations from solid tumors). At least 25 metaphase spreads per tumor were analyzed and chromosome number and the presence or absence of DMs were compared with the parent cells.

Table 2.5 shows the results from experiments designed to determine whether or not the presence of DMs has any effect on the time to tumor.

Table 2.4 Frequent Chromosome Changes in Tumors from Animals
Receiving the Disk + X Ray(2.5Gyx4) Treatment

<u>Sarcoma</u>	<u># Weeks after Implantation</u>	<u>Chromosome Alterations</u>	
		<u>Most Common (≥50% of cells)</u>	<u>Less Common (25%-49% of cells)</u>
86-7664	34	trisomy 10	nullisomy 18 monosomy X
86-7645	34	trisomy 10	monosomy X
86-7643	42	trisomy 10 trisomy 16 trisomy 19	monosomy X
86-7661	44	tetrasomy 10 trisomy 16 trisomy 19	-----
86-7660	44	tetrasomy 10 trisomy 16 trisomy 19	-----
86-7667	46	trisomy 10 trisomy 19	trisomy 16
87-233	52	trisomy 10 tetrasomy 16 tetrasomy 19	-----

Figure 2.18. Karyotype of sarcoma 86-7644, a 34 week post-implantation tumor from a mouse receiving the disk + x ray treatment.

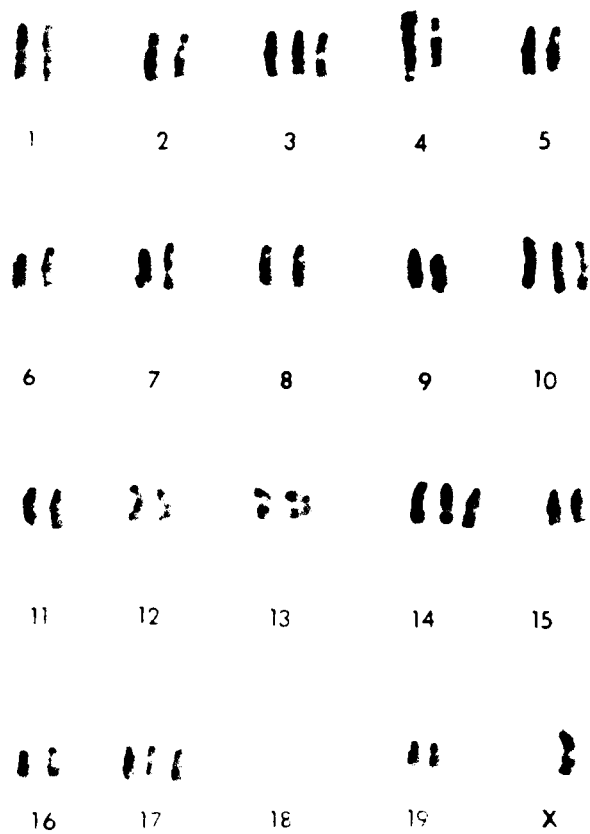


Figure 2.18

Figure 2.19. Karyotype of sarcoma 86-7643, a 42 week post-implantation tumor from a mouse receiving the disk + x ray treatment.

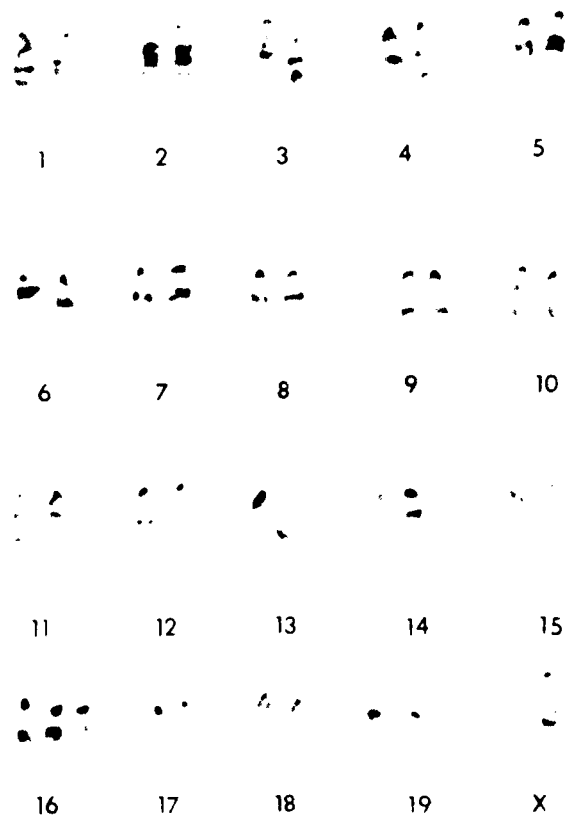


Figure 2.19

Figure 2.20. Karyotype of sarcoma 86-7661, a 44 week post-implantation tumor from a mouse receiving the disk + x ray treatment.



Figure 2.20

Table 2.5 Time to Tumor and Chromosomal Characteristics of Reinjecting Sarcoma Cells

Sarcoma	Age (wks)	Parent Tumor	Parent Tumor			Time to tumor (average wks)	Secondary Tumor			% cells with DM Metacentric
			Mean	Range	# chromosomes/cell		Mean	Range	# chromosomes/cell	
88-7576	26	Disk	59	42-118	0	30	62	40-115	0	0
87-214	38	Disk + X ray	47	37-78	0	0	53	39-80	0	0
88-213	60	Disk + X ray	71	60-94	78	0	72	60-96	0	0
<u>Origin</u>										
87-1363	Reinjected sarcoma 87-213, passage 3	64	45-77	27	28	3	61	40-81	0	30
88-7804	Reinjected sarcoma 87-296, passage 2	61	41-76	0	0	2.8	57	40-75	0	0
88-7704	Reinjected sarcoma 87-296, passage 6	49	40-64	44	40	3.1	44	40-65	0	25
88-7577	Reinjected sarcoma 87-296, passage 8	47	43-52	64	8	3	44	40-51	0	5

Parent sarcomas 88-7804, 88-7704, and 88-7577 were themselves produced by reinjecting cells from passages 2, 6, and 8 of tumor 87-296, respectively. Also, parent sarcoma 87-1363 was produced by reinjecting passage 3 cells from sarcoma 87-213. Thus, the corresponding tumors that developed (2° tumors) are from reinjected- reinjected cells. It appears that the "2° tumors", derived from the "parental tumor cells", had essentially the same time to tumor regardless of whether or not DMs were present in the parental tumor cells. Tumors derived from parent cells containing DMs had an average time to tumor of 3 weeks, while tumors derived from parent cells lacking DMs had an average time to tumor of 2.9 weeks. The range and average number of chromosomes per cell remained essentially the same in the parent cells and the corresponding 2° tumor cells. No DMs or metacentrics were detected in the 2° tumors derived from parent cells that did not originally contain these structures, but metacentrics persisted in 2° tumors when a metacentric was present in the parent cells. Also, interestingly, DMs observed in the parent cells were not present in the corresponding 2° tumor cells. It is possible that some sort of selective pressure, originally responsible for the DMs, was not present under the reinjection conditions. Banding analysis, that has not yet been done, will be necessary to determine if any specific chromosomes were preferentially conserved, lost, or rearranged in the 2° tumors.

Conclusions and Discussion

The chromosome numbers per cell in different tumors varied from 38 to over 300, and there was a wide variation in chromosome number between sarcomas, but much less variation within a particular sarcoma. Generally, there was an increase in chromosome number per cell as time post-implantation increased. In all tumors analyzed, chromosomal abnormalities were observed before a histological diagnosis of malignancy could be made with confidence (personal communication, R. J. M. Fry). DMs were observed in 36% of tumors and HSRs in 24%, most often in tumors at later stages of development.

Sarcomas that developed on the disks in x-irradiated animals have a lower mean chromosome number per cell than sarcomas that developed in mice containing the disk alone. For example, the 34 week post-implantation tumors that developed in animals treated with the disk alone had a mean chromosome number of 55, while the 34 week post-implantation tumors from the animals that received the disk + X ray treatment had a mean of 43 chromosomes per cell. This difference is even more apparent in 44 week tumors where the mean numbers of chromosomes per cell were 117 and 67, respectively. This suggests that perhaps some type of selection is occurring following the X ray treatment.

Duplications of chromosomes 10, 16, and 19 were observed in tumors that developed in both animals treated with the disk alone and those treated with the disk + X ray. In the case of the animals treated with the disk alone, the duplication of chromosome 19 was often present as

an isochromosome 19. While metacentric chromosomes were never observed in tumors from animals that received the disk + X ray treatment, they were often observed in animals that received the disk. These included metacentrics involving (4;17), (1;17), and (4;15) as well as the isochromosome 19. Additionally, nullisomy 8, trisomy 5, and trisomy 7 were observed in 50% of disk alone tumors, while monosomy X was observed in 38% of disk + X ray tumors. The prevalence of metacentric chromosomes in the tumors from animals receiving only the disk suggests that they may be important in tumor development, perhaps by an alteration of the regulation of the genes on the involved chromosomes. Also, the presence of DMs in 36% and HSRs in 24% of tumors suggests that the DNA that is amplified may have an influence on tumor development or maintenance. Identification of the amplified sequences in these structures could reveal important information and will be discussed later.

Although consistent chromosome trends were observed, there was no clear evidence for the involvement of a specific chromosomal alteration in the initiation or progression of the tumor. Due to technical limitations, the earliest sarcomas analyzed were 26 weeks post-implantation from animals that received the disk alone and 18 weeks post-implantation from animals that received the disk + X ray. Both 18 and 26 weeks are fairly late into the developmental process of the tumor, and it is thus possible that specific alterations were involved in the formation of the fibrosarcomas and were not detected. It is hypothesized that the disk implant is not itself directly tumorigenic but that it stimulates cell proliferation (Brand, 1982).

If this is the case, chromosomal alterations could result from segregation errors at a number of points in a number of different cells, leading to a mixed population of tumor cells with diverse karyotypes. This might imply that disk- induced mouse fibrosarcomas are multicellular, rather than monoclonal in origin (Reddy and Fialkow, 1984). In this way, diverse karyotypes might be observed, and the presence of consistent chromosome trends might be due to cells with selective growth advantages. The fact that differences in disk vs. disk + X ray induced tumor chromosome compliments were observed also implies that a variety of cells are initiated. Some fraction of these cells may be X ray sensitive and thus may not contribute to the karyotypic diversity observed in the disk only tumors.

It is also possible that the macrophages that are so prevalent around the disk might form sufficient amounts of free radicals that could interact (either randomly or non- randomly) with the chromosomes to produce alterations. A causal relationship between specific chromosomal alterations and tumor formation in this sarcoma system has not yet been demonstrated, and this is in agreement with much of the published literature describing chromosomal alterations in rodent tumor systems.

Reports of asbestos- induced solid tumors in rats indicated translocations in 12 of 15 tumors studied (Libbus and Craighead, 1988). These translocations all involved chromosomes 5, 10, and 13, indicating that specific chromosomal alterations might be involved in asbestos- induced rat tumors. However, translocations were not observed in all tumor cells, and many tumor cells contained apparently

random structural and numerical alterations. Furthermore, studies by Kendal et al. (1987) suggest that chromosomal abnormalities in murine melanomas cell lines may be related to metastasis rather than to tumor induction. Although numerous, apparently random, structural and numerical changes were present in all cells analyzed, two specific chromosomal alterations were associated with a more metastatic phenotype. These were extra copies of chromosome 1 and an unidentified metacentric marker chromosome.

A terminal deletion of chromosome 13 was involved in the X ray-induced transformation of mouse embryo fibroblast cells in vitro (Kodama and Sasaki, 1987). 17 of 24 transformants analyzed contained this deletion that was apparently necessary for the formation of foci. However, the deletion alone was not sufficient to provide for tumorigenicity, indicating that this chromosomal alteration may be only in part responsible for the transformation of these cells. Finally, karyotypic analysis of vinylacetate polymer- induced sarcomas in mice failed to reveal any specific chromosomal alterations (Rachko and Brand, 1983). Although no particular chromosomal characteristic could be identified as being specific to foreign body- induced sarcomas, Rachko and Brand most frequently observed ploidy deviations in chromosomes 1, 6, 7, 13, 15, 18, and 19. However, these changes were in fully developed tumors and thus may not represent primary changes. It is, however, interesting that mouse chromosome 19 was also consistently involved in the ploidy alterations from the plastic disk-induced sarcomas described in this chapter.

It is still possible that karyotypic changes and tumor induction are two separate phenomena, such that the chromosomal alterations are a consequence of tumor induction or progression. However, it is probable that the consistent trends that were observed here and in the published literature are not entirely random, but are related to tumor initiation and progression.

CHAPTER 3

THE ROLE OF ONCOGENES IN THE FIBROSARCOMA SYSTEM

Summary

Southern analysis was used to determine whether or not the presence of double minutes, homogeneously staining regions, and metacentric chromosomes could be correlated with the amplification or rearrangement of any of 10 oncogenes in tumor cell DNA. The oncogenes examined were *c-myb*, *c-h-ras*, *c-rel*, *c-raf*, *c-abl*, *c-erbA*, *c-erbB*, *c-sis*, *c-myc*, and *c-mos*. DNA was isolated from fibrosarcomas, and normal mouse cell DNA was used as a control. No amplification of the DNA of these oncogenes was observed in any of the tumor cell lines, and thus no correlation between oncogene amplification and the presence of DMs or HSRs could be established. Also, no correlation could be drawn between the presence of metacentric chromosomes and oncogene rearrangement.

Introduction

Since the discovery of drug resistance in eukaryotic cells, amplification of specific genes has been associated with many adaptive responses of cells to environmental stress (Schimke, 1982; Pohjanpelto et al., 1985). Cytogenetic abnormalities, such as double minutes and homogeneously staining regions, were originally found in cells that had been selected for drug resistance (Levan et al., 1968; Biedler and Spengler, 1976). However, both DMs and HSRs have also been observed in

metaphase preparations of cancer cells before the start of chemotherapy. Cellular oncogenes have been shown to be amplified in many tumors, and these amplified oncogenes can, in many cases, be correlated with the presence of DMs and/or HSRs (Bigner et al., 1987; Meltzer et al., 1987). These include the amplification of c-myc in a human colon carcinoma (Quinn et al., 1979). In these tumor cells, c-myc was amplified approximately 30 fold, and the c-myc copies were mapped to double minutes. Similarly, amplified c-k-ras oncogenes were mapped to DMs and HSRs in a mouse adrenocortical tumor (Schwab et al., 1983). Not all oncogene amplification, however, manifests itself as DMs or HSRs. For example, c-myc is amplified between 8 and 32 fold in some colon carcinomas in the absence of any observable cytogenetic abnormalities. Also, in some cases oncogene amplification is accompanied by a rearrangement of the particular oncogene involved (Collins and Groudine, 1982; Ullrich et al., 1984). However, oncogene alterations and chromosomal abnormalities do not correlate in all tumors. For example, in acute myeloid leukemia, the only oncogene amplified was c-myc (located on human chromosome 8) even though the only chromosomal alteration present was trisomy 6 (Alitalo et al., 1986).

Since DMs and HSRs were observed in many of the mouse fibrosarcomas analyzed in our studies, and since many tumors contained metacentric chromosomes, the possibility of the amplification and/or rearrangement of oncogenes was explored. The oncogenes c-mos, c-raf, c-H-ras, and c-sis, were chosen because they have a sarcoma virus as their prototype virus. Additionally, c-mos has been mapped to mouse chromosome 4

(Threadgill, 1988) and c-sis to chromosome 15 (Kozak et al., 1983). Both chromosomes 4 and 15 were involved in the formation of metacentric chromosomes, thus suggesting a possible rearrangement of these oncogenes. The oncogenes c-erbA (Zabel et al., 1984) and c-erbB (Silver et al., 1985), which are both located on mouse chromosome 11, were analyzed because they have been shown to induce sarcomas in chickens. C-myb was analyzed since it maps to chromosome 10 (Sheiness and Gardinier, 1984), and whole copies of chromosome 10 were duplicated in many of the sarcomas analyzed. The c-myc and c-abl oncogenes, which map to mouse chromosomes 15 (Crews et al., 1982) and 2 (Goff et al., 1982) respectively, because they are amplified or rearranged in some human tumors (Quinn et al., 1979; Selden et al., 1983). Furthermore, the presence of c-myc on chromosome 15 again indicated the possible rearrangement of this oncogene with the formation of the 4;15 metacentric chromosome. C-rel, whose chromosomal location and precise function are unknown, was chosen because it is believed to be related to c-raf whose prototype virus is a sarcoma virus.

Materials and Methods

DNA Isolation from Cultured Tumor Cells

The following procedure is a modification of Maniatis (1982). In order to extract DNA from sarcoma cell lines 87-296 (0% of cells containing DMs or HSRs), 87-297 (0% of cells containing DMs and 50% containing HSRs), 87-176 (43% of cells containing DMs and 0% containing HSRs), 87-231 (0% of cells containing DMs and 40% containing

HSRs), 87-233 (0% of cells containing DMs and 48% containing HSRs), 86-7643 (40% of cells containing DMs and 0% containing HSRs), 85-7645 (0% of cells containing DMs and 0% HSRs), and 87-7660 (52% of cells containing DMs and 0% containing HSRs), cells from 10 confluent T-75 tissue culture flasks (per cell line) were removed by scraping with a rubber policeman. (Only two of these cell lines contained metacentric chromosomes. These were 87-296 [100% of cells containing a (1;17)] and 87-297 [60% of cells containing an unidentified metacentric].) The cells were then pooled and were centrifuged for 5 minutes at 2000 RPM. The pellet was then suspended in 10 milliliters (ml.) of solution H, containing 0.5 molar (M) sucrose, 25 millimolar (mM) potassium chloride, 0.5 mM spermidine, 0.15 mM spermine, 2mM EDTA, and 0.5mM EGTA. This mixture was then homogenized in a tissue homogenizer with a teflon pestle until viscosity was achieved and stringy elements were observed (approximately 10 min.). This mixture was then centrifuged for 10 min. at 2000 RPM, and the pellet containing nuclei was resuspended in 10 ml. of solution H containing 5% Triton X-100. This mixture was then centrifuged for 10 min. at 2000 RPM, and the pellet was resuspended in 2 ml. of T10-E10-N100 containing 10 mM Tris, 10 mM EDTA, and 100 mM NaCl. Eight ml. of Hirt's solution (0.01 M Tris-HCl pH 7.6, 10 mM EDTA, and 1% SDS) at 65°C was added in order to lyse the nuclei. The sample was then mixed until it was gelatinous (a few seconds), and was then digested with 1 ml. of pronase (20 mg./ml.) at 55°C overnight. One ml. of 5M NaCl was then added, and the sample was agitated at 37°C to clear the turbidity generated by the salt. In order to remove protein, the sample was extracted 2 times with 8 ml. of

phenol at pH 7.0, and then once with chloroform. The aqueous phase from the chloroform extraction was placed into a 50 ml centrifuge tube, and 25 ml. of 95% ethanol (-20°C) was layered onto this aqueous phase by gently pouring onto the inside of the tube. The DNA fibers that formed at the ethanol-aqueous interface were then spooled out and were frozen in 95 % ethanol overnight. To remove excess water, the spooled DNA was then lyophilized for 30 minutes to insure that the DNA was thoroughly in solution. To remove RNA, two ml. of T10-E10-N100 was added, and the sample was incubated at room temperature for 30 minutes. The gelatinous DNA was treated with RNase A (5 mg./ml.) for 1 hour at 37°C. The sample was then treated with proteinase K (10 mg./ml.) for 1 hour at 37°C. and was then extracted 2 times with 4 ml. of phenol/chloroform/ isoamyl alcohol (25/25/1) to remove more protein and thus further purify the sample. Four ml. of 95% ethanol (-20°C.) was then added, and the sample was incubated for 10 min. at -80°C. before undergoing centrifugation for 10 min. at 7000 RPM. The pellet was lyophilized for 30 min. to remove excess water and was resuspended in 200 microliters of TE buffer at pH 7.4, containing 10mM Tris-Cl (pH 7.4) and 1mM EDTA (pH 8.0) for storage. The final concentration and purity of DNA was measured on a spectrophotometer at 260nm/ 280nm.

Restriction Enzyme Digest, DNA Precipitation, and Electrophoresis

Ten micrograms of tumor cell DNA was digested with 20 units of EcoRI (BRL) at 37°C. overnight. twenty microliters of 20% potassium acetate solution was then added, and the sample was mixed by vortexing. Two hundred fifty microliters of 95% ethanol (-20°C.) was added, and the

sample was incubated for 5 min. at -80°C . The sample was then centrifuged in a microcentrifuge for 10 minutes. The pellet was rinsed 2 times with 1 ml. of 75% ethanol, and was lyophilized for 10 minutes. Forty microliters of TE buffer, 5 microliters of TBE buffer (containing 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA), and 10 microliters of 3% bromphenol blue/20% glycerol were added in preparation for agarose gel electrophoresis. The samples were then incubated for 2 min. at 65°C . and were loaded onto horizontal, 0.7% agarose (BRL) gels. The gels were run at 30 volts for 48 hours to achieve clean separation of the whole genomic DNA. A HindIII cut, DNA marker (BRL) was used as a molecular weight standard. After the 48 hours, the gel was stained with ethidium bromide (0.1% solution) and was examined under an ultraviolet light. The gel was then treated with 0.25M HCl for 30 min. in order to neutralize the ethidium bromide in preparation for gel transfer (Maniatis, 1982; modified)

Southern Blotting

The tumor DNA on the gel was transferred onto a nylon membrane (Zetabind) using the Southern blotting technique (Southern, 1975). The transfer was accomplished in 0.4M sodium hydroxide overnight at room temperature and pressure. After the transfer, the membrane was washed 2 times for 15 min. each with 2X SSC, that contained 0.3M sodium hydroxide and 0.03M sodium citrate. The nylon membrane was washed for 1 hour in 0.1X SSC/0.5% SDS at 65°C . It was then treated with hybridization solution containing 50% deionized formamide, 6X SSC, and 0.25% non-fat dry milk for 1 hour at 42°C .

Preparation of Oncogene Probes

Plasmids containing desired oncogene sequences were obtained from the American Type Culture Collection, except for myc and mos which were commercially prepared (BRL). The plasmids were digested at 37°C. overnight with the appropriate restriction enzymes that would release the oncogene DNA from the plasmid. The specific plasmids and restriction enzymes utilized are listed in Table 3.1. The samples were then ethanol precipitated and were loaded onto a 5% acrylamide (BRL) gel. OX174 DNA was used as a molecular weight marker, and the gel was run at 300 volts for 2 hours. The gel was then stained with a 0.1% solution of ethidium bromide and the desired bands were cut from the gel. The bands containing the oncogene DNA were then placed in microcentrifuge tubes and were ground with a teflon pestle. Six hundred microliters of DNA extraction buffer containing 0.5M ammonium acetate, 1mM EDTA, 5mM magnesium acetate, and 0.1% SDS were added. The samples were then mixed until a uniform white color was observed. Samples were incubated at 37°C. for 15 min., centrifuged for 5 min., and the pellet was washed with 95% ethanol (-20°C.). The extraction was repeated , and the samples were lyophilized for 10 minutes. The oncogene DNA fragments were resuspended in TE buffer and were ready for Nick translation.

Nick Translation and Membrane Hybridization

The oncogene DNA fragments were nick translated. The reaction mixture contained 5 μ g 32P (dCTP), 5 microliters 5X Nick buffer

TABLE 3.1

PLASMIDS AND RESTRICTION ENZYMES UTILIZED TO OBTAIN DESIRED
ONCOGENE DNA FRAGMENTS

Plasmid	Restriction Enzyme	Size of Insert (kb)	Size of Vector (kb)
pmyb	EcoRI	1.2	4.3
pHa-ras	EcoRI/PvuII	0.7	2.1
pkw101rel	EcoRI	0.87	4.3
p171raf	EcoRI/AvaI	1.1	2.8
pHB1abl	HindIII/BamHI	0.51	4.0
pAEPst.45erbA	PstI	0.45	4.3
pAEBam.5erbB	BamHI	0.5	4.3
pv-sis	PstI	1.2	4.3

(Amersham), 1 microgram oncogene DNA, 2.5 microliters E.coli DNA polymerase I, and 9 microliters water. The reaction mixture was incubated at 15°C. for 1.5 hours. 0.2M EDTA was added to stop the reaction, and the labeled oncogene DNA was separated from unincorporated nucleotides with a Nick-column (Pharmacia) packed with sephadex G-30 (Maniatis, 1982). The probe was denatured by boiling for 5 min., and an amount of probe equivalent to 1,000,000 counts per minute (CPM, as determined by Cherenkov radiation on a scintillation counter) was added to the hybridization solution for each 10 ug. of DNA that had been loaded onto the original gel. The membrane was exposed to this probe/ hybridization solution at 42°C. overnight.

The nylon membrane was washed with 10X SSC/50% deionized formamide, followed by 5X SSC/ 0.1%SDS at 50°C., 0.2X SSC/ 0.1% SDS at 50°C., 2X SSC/ 0.1% SDS, 2X SSC, 20X SSC, and finally 0.2X SSC. The membrane was exposed to Kodak X-OMat X ray film in a Kodak X ray exposure holder for 4 to 7 days at -80°C. and was then developed.

Results

DNA Isolation from Cultured Tumor Cells

DNA preparations were obtained from cultured tumor cells. The DNA was of good quality having an A 260/280 ratio of 1.8 and was not sheared (see uncut DNA in lanes 6- 10 of figure 3.1). Figure 3.1 shows an ethidium bromide stained agarose gel containing both DNA that had been cut with EcoRI and uncut DNA. Lanes 1-5 represent cut DNA of sarcoma cell lines 87-296, 87-297, 87-7660, 87-7667, and a normal mouse

Figure 3.1. Ethidium bromide stained agarose gel of EcoRI cut
(lanes 1-5) and uncut (lanes 6-10) fibrosarcoma DNA.

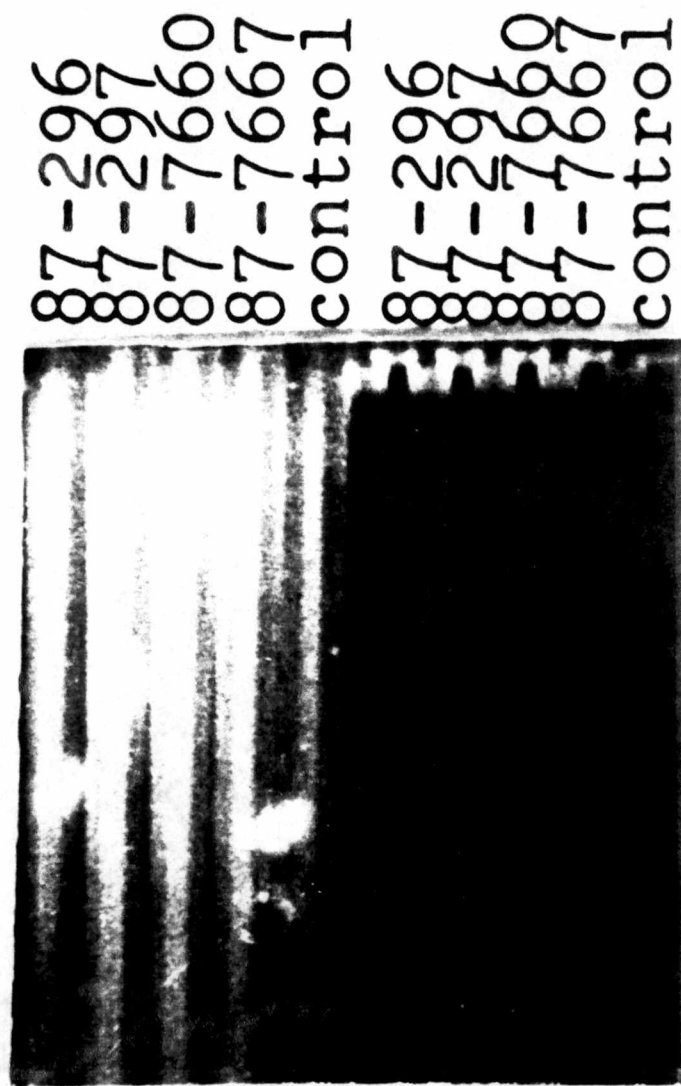


Figure 3.1

cell DNA control (derived from the heart and lung of a normal C₃H female mouse), respectively. Lanes 6-10 represent the uncut DNA of the same cell lines. It is evident that the restriction enzyme EcoRI was effective in cutting the tumor DNA.

Preparation of Oncogene Probes

An ethidium bromide stained acrylamide gel showing the separation of oncogene DNA inserts from plasmid DNA is shown in figure 3.2. Lane 1 represents a ϕ X174 DNA molecular weight marker, while lanes 2-9 represent the oncogenes myb, h-ras, rel, raf, abl, erbA, erbB, and sis. Myc and mos fragments were commercially prepared (BRL). These oncogene DNAs were removed from the plasmid DNA using the restriction enzymes listed in table 3.1. The lower band in each lane represents the oncogene insert, while the upper (and larger) band in each lane represents the plasmid DNA. These oncogene DNAs were then purified from the gel and were nick translated and used as probes in Southern analysis as described in the Materials and Methods section. Results of experiments utilizing these DNA fragments can be found in table 3.2.

Absence of Oncogene Amplification or Rearrangement

Figures 3.3, 3.4, and 3.5 are representative of the Southern analyses that were obtained. The 23.7 kb. band in figure 3.3 represents the myc gene. In all lanes, those containing tumor DNA (lanes 1-5) and the one containing the normal control DNA (lane 6), the bands are of the same intensity, indicating no myc amplification. Also, only one band is present, and it is present at the same location,

Table 3.2
PRESENCE (+) OR ABSENCE (-) OF ONCOGENE AMPLIFICATION/REARRANGEMENT

% OF CELLS CONTAINING													
SARCOMA	DMS	HSRS	METACENTRIC	*myc(15)	h-ras(7)	raf(6)	rel(?)	mos(4)	myb(10)	abl(2)	erb-A(11)	erb-B(11)	sis(15)
87-296	0%	0%	100%	-	-	-	-	-	-	-	-	-	-
87-297	0%	50%	60%	-	-	-	-	-	-	-	-	-	-
87-176	43%	0%	0%	-	-	-	-	-	-	-	-	-	-
87-231	0%	40%	0%	-	-	-	-	-	-	-	-	-	-
87-233	0%	48%	0%	-	-	-	-	-	-	-	-	-	-
86-7643	40%	0%	0%	-	-	-	-	-	-	-	-	-	-
86-7645	0%	0%	0%	-	-	-	-	-	-	-	-	-	-
87-7660	52%	0%	0%	-	-	-	-	-	-	-	-	-	-

*onco gene (chromosomal location)

Figure 3.2. Ethidium bromide stained acrylamide gel showing separation of oncogene DNA inserts from plasmid DNA.

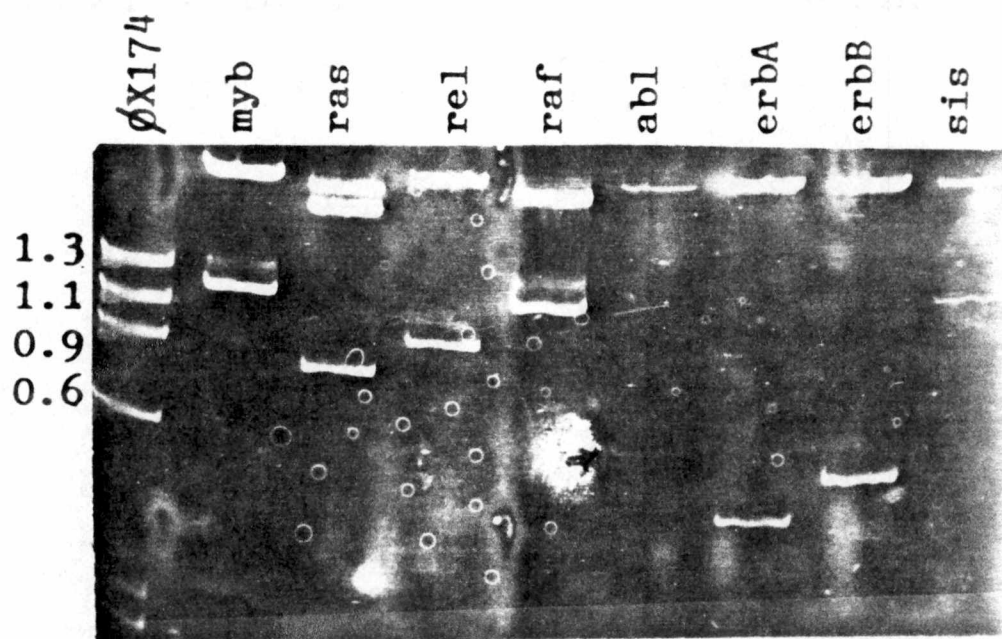


Figure 3.2

Figure 3.3. Southern analysis of tumor DNA using the myc probe.

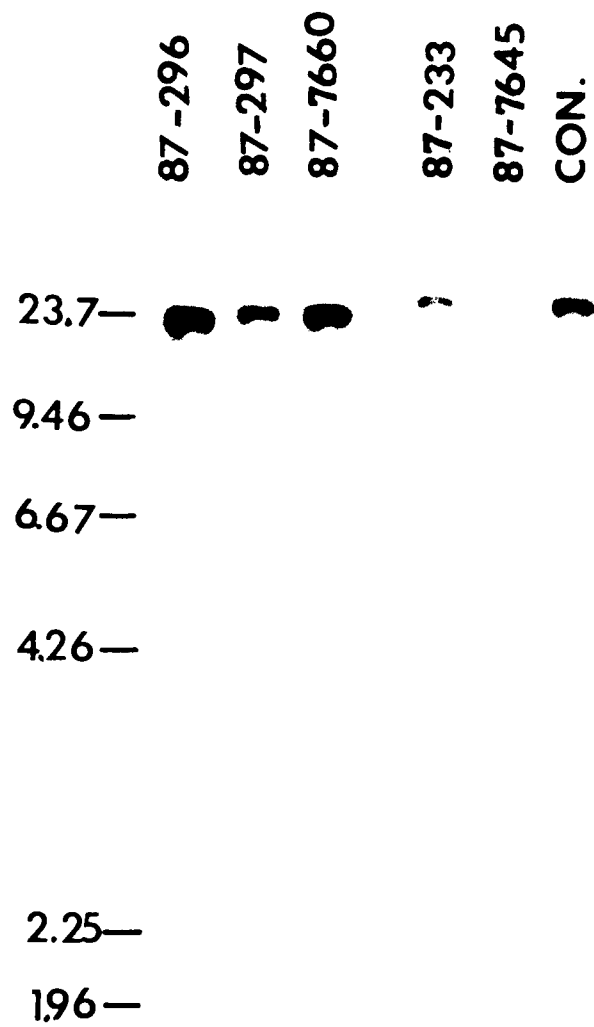


Figure 3.3

Figure 3.4. Southern analysis of tumor DNA using the raf probe.

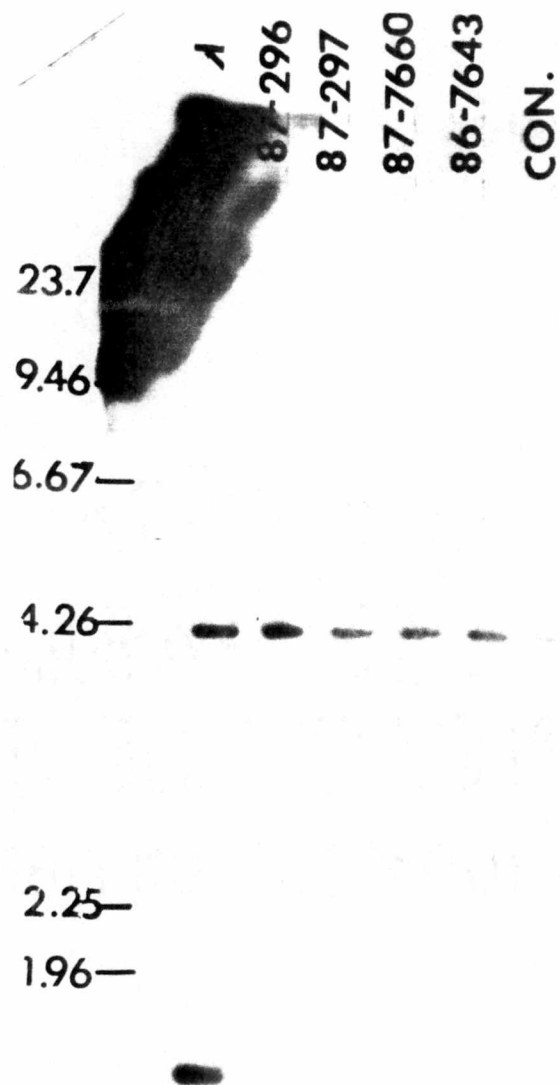


Figure 3.4

Figure 3.5. Southern analysis of tumor DNA using the rel probe.

87-296	- 23.7
87-297	- 9.46
87-7660	- 6.67
86-7643	- 4.26
CON.	
	- 2.25
	- 1.96

Figure 3.5

indicating no myc rearrangement. In figure 3.4 lane 1 represents a molecular weight marker, lanes 2-5 tumor DNA, and lane 6 control DNA probed with raf. The 4.26 kb. band indicates no amplification or rearrangement of raf. In figure 3.5 (for rel) lanes 1-4 represent tumor DNA, and lane 5 represents control DNA. Again, no amplification or rearrangement of the rel oncogene was observed. In fact, as shown in table 3.2, irregardless of the presence or absence of either DMs or HSRs, no amplification or rearrangement of any of the 10 oncogenes examined was observed.

Conclusions and Discussion

From the data obtained in these experiments, it is evident that there is no correlation between the amplification of c-myb, c-h-ras, c-raf, c-rel, c-abl, c-erbA, c-erbB, c-sis, c-myc, and c-mos and the presence of double minutes and/or HSRs. Also, no correlation was observed between oncogene amplification or rearrangement and the presence of metacentric chromosomes. Thus, it appears that in these mouse sarcomas the amplification and rearrangement of the selected oncogenes are of little or no importance in fibrosarcoma development. It is possible that an extensive search for changes in other oncogenes might reveal amplification or rearrangements that may correlate with DMs, HSRs, and/or metacentric chromosomes. It is also possible that such a search would reveal no amplification or rearrangement. Furthermore, it is quite likely that oncogene changes that do not manifest themselves though cytogenetic change may be present. This

possibility has not been ruled out. It is also possible that the DMs and HSRs may represent a new, unidentified oncogene, such as that observed in mouse 3T3 cells (Cahilly- Snyder et al., 1987) and in transformed NIH 3T3 and C3H/101/2 cells (Borek et al., 1987), or that these cytogenetic abnormalities may not represent the same amplified DNA sequence. These possibilities were not explored since the most likely oncogenes to be involved were examined. Instead, alternative explanations were explored and will be described in chapter 4.

Double minute chromosomes and homogeneously staining regions have been observed in many experimental systems involving selection for drug resistance in cell culture. However, in nonselective conditions (when the drug is removed from culture) the cytogenetic manifestations of DNA amplification are lost (Schimke et al., 1981). In the fibrosarcoma system, the DMs and HSRs persisted in culture for 5 to 10 passages after which they were no longer observed. Thus, it is possible that even though these tumors were never exposed to drugs, the DMs and HSRs may represent some type of drug resistance gene.

Whatever the source of the DMs and HSRs, it is probable that the amplified DNA sequence or gene is important to the establishment, development, or persistence of the tumor in vivo, since they were present in such a high percentage of the fibrosarcomas analyzed.

CHAPTER 4

THE ROLE OF THE MULTIPLE DRUG RESISTANCE GENE IN FIBROSARCOMAS

Summary

Southern analysis and cell survival studies were performed on fibrosarcoma cells containing or lacking DMs in order to determine if the DMs were cytological manifestations of multiple drug resistance (MDR) gene amplification. X ray survival studies were also done to determine if the presence of DMs had any effect on the response to x-irradiation. Southern analysis with the MDR probe revealed that cell lines containing DMs had detectable MDR DNA, while in those cells lacking DMs no hybridization was observed with the MDR probe. Drug survival studies indicated that those cell lines containing DMs were generally resistant to AraC, methotrexate, actinomycin D, and colchicine relative to the cells lacking DMs and to the normal controls. Cells from different fibrosarcomas exhibited a variety of sensitivities to X rays, but these sensitivities could not be correlated with the presence or absence of DMs in the different cell lines.

Introduction

In our studies, the presence of double minutes and homogeneously staining regions could not be correlated with oncogene amplification.

However, DMs and HSRs had been correlated with amplification of the multiple drug resistance (MDR) gene in other tumors (Scotto et al., 1986; Pastan and Gottesman, 1987).

Double minute chromosomes have been observed in mouse skin papillomas which did not correlate with oncogene amplification. However, these DMs were eventually shown to be cytological manifestations of MDR amplification (Larcher et al., 1988). Also, many normal human tissues such as liver, kidney, and adrenal express MDR in higher than expected levels (Fojo et al., 1987). Furthermore, it has recently been determined that the 170 Kd transmembrane glycoprotein transport system responsible for multidrug resistance is functioning in non-selected, non-resistant dog kidney cells in culture (Neyfakh et al., 1988).

When cells in culture are selected for resistance to a specific drug, for example, the resulting surviving cells are not only resistant to the selecting drug but are also cross-resistant to other drugs (Pastan and Gottesman, 1987). Studies with hamster cells have shown that this type of multiple drug resistance is accompanied by a decrease of intracellular drug accumulation and an increase in the 170 Kd transmembrane glycoprotein (Kartner et al., 1983). Studies involving drug uptake and release kinetics have revealed that the selected cells had acquired the ability to pump drugs out of the cells. The MDR gene product confers resistance to such diverse drugs as adriamycin, actinomycin D, colchicine, and vinblastine. However, a few drugs, such as methotrexate, AraC, cyclophosphamide, and bleomycin, seem to be able to overcome multiple drug resistance by allowing the chemicals to

accumulate within the otherwise resistant cells (Pastan and Gottesman, 1987).

Although the fibrosarcoma cells had not been exposed to chemical agents, it was plausible that MDR amplification might be involved in the generation of DMs and HSRs. In order to investigate this possible correlation between MDR amplification and DMs, both Southern analysis and cell survival studies were done with various sarcoma cell lines containing or lacking DMs. Normal mouse heart/lung cells were used as controls. Drug survival studies employed actinomycin D and colchicine (for which MDR is reported to confer resistance) and methotrexate and AraC (for which MDR is reported not to confer resistance). X ray survival studies were also performed to investigate any possible connection between DMs and resistance to X rays.

Materials and Methods

The MDR Sequence

The MDR sequence (from a Chinese hamster fibroblast cell line) in plasmid p5L-18 (Scotto et al., 1986) was kindly provided by Dr. Peter Melera (The University of Maryland Cancer Center, Baltimore, MD.). This Chinese hamster sequence is reported to share approximately 90% homology with both the mouse and human MDR sequence.

Large Scale Preparation of the MDR Plasmid

In order to obtain sufficiently pure quantities of the MDR sequence for nick translation, the MDR plasmid was transformed into E.

coli MV1190 using the method of Maniatis (1982). Transformants were selected from ampicillin agar plates, and plasmid DNA was extracted from a 5 liter overnight culture of transformants via the alkaline lysis procedure (Maniatis, 1982). The closed circular DNA was then purified by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient (Maniatis, 1982). The sample was then extracted with water saturated N- butanol, resuspended in TE buffer, and ethanol precipitated for further purification. Ten micrograms of the purified DNA was cut with 10 units of PstI (BRL) for 1 hour at 37°C., run on a 2% agarose gel, stained with ethidium bromide, and analyzed for appropriate fragments in order to verify the integrity of the purified DNA.

Use of the MDR Plasmid as a Probe

Methods utilized for DNA isolation from cultured tumor cells, restriction enzyme digest, DNA precipitation, electrophoresis, Southern blotting, and membrane hybridization are the same as those described in detail in the Materials and Methods section of Chapter 3. The method for nick translation is also the same as described in chapter 3 except that the purified MDR DNA was used instead of oncogene DNA.

Drug Survival Experiments

Survival studies were done to determine whether the presence of DMs had an effect on cell survival in the presence of various drugs. Sarcoma cell lines used were 88-7804, 87-214, and 88-7576 (all lacking DMs, although 87-214 does contain an HSR) and 87-1363, 88-7704, 88-

7577, and 87-213 (all containing DMs). Normal fibroblasts (passage 3) cultured from minced samples of heart and lung from a normal C₃H female mouse were used a control.

Cells were removed from petri dishes with 0.05% trypsin, diluted in L- 15 media, and counted on a hemocytometer. Various numbers of cells (as determined from previous plating efficiency studies) were plated on 60 mm. tissue culture dishes (Falcon) in media containing methotrexate (Sigma), colchicine (Sigma), actinomycin D (Sigma), or Ara C (Sigma). The different numbers of cells plated were dependent upon dose and were selected in order to yield approximately 100 colonies per plate. The concentrations of each drug were 0 ug/ ml, 0.001 ug/ ml, 0.01 ug/ ml, 0.1 ug/ ml, 1.0 ug/ ml, or 10 ug/ ml, and each treatment was conducted in triplicate. Plates were incubated undisturbed for 7 days in a 37°C incubator humidified 95- 100% in an atmosphere of 5% CO₂ in air. The plates were then fixed with 70% ethanol at room temperature for 15 minutes, stained with 15% Giemsa blood stain (Carolina Biological) in water for 30 minutes, rinsed with water, and allowed to air dry. Colonies of 50 cells or greater were then counted under a dissecting microscope. Each colony was assumed to represent a single surviving cell.

X Ray Survival Experiments

Survival studies were also performed in order to determine whether or not the presence of DMs had an effect on cell survival after treatment with X- irradiation. Cells were removed from tissue culture dishes with 0.05% trypsin, diluted L- 15 in media, and counted with a

hemocytometer. Different numbers of cells (depending upon dose) were again plated, as determined by prior plating efficiency studies, onto 60 mm. tissue culture dishes (Falcon). Three plates were used for each dose group. The plates were incubated for 4 hours at 37 °C to allow cells to recover from trypsinization. The cells were then X-irradiated with 0, 1, 2, 3, 4, 6, or 8 Gy at a dose rate of 1 Gy/ min. A 320 KVp Gemini Industrial X ray unit was used. The machine settings were 250 kV., 10 mA. with a beam filtration of 1 mm. of aluminum. Cell cultures were centered under the beam and rotated during exposure. The plates were incubated undisturbed for 10 days in a 37° C incubator humidified 95- 100% in an atmosphere of 5% CO₂ in air. The plates were fixed with 70% ethanol at room temperature for 15 min., stained with 15% Giemsa stain (Carolina Biological) in water for 30 min., rinsed with water, and allowed to air dry. Colonies of 50 cells or greater were counted under a dissecting microscope, and each of these colonies was assumed to represent a single surviving cell.

Results

Large Scale Preparation of the MDR Plasmid

An ethidium bromide stained agarose gel showing DNA fragments from the MDR plasmid obtained after cesium chloride purification is shown in Figure 4.1. The left- hand lane represents a 123 base pair ladder (standard) while the right - hand lane represents the purified MDR plasmid cut with PstI. The appropriate fragments of 411 base pair and 88 base pair fragments were observed in the purified MDR plasmid. This

Figure 4.1 Ethidium Bromide stained agarose gel showing the 411 base pair and 88 base pair fragments from the MDR plasmid cut with Pst I.

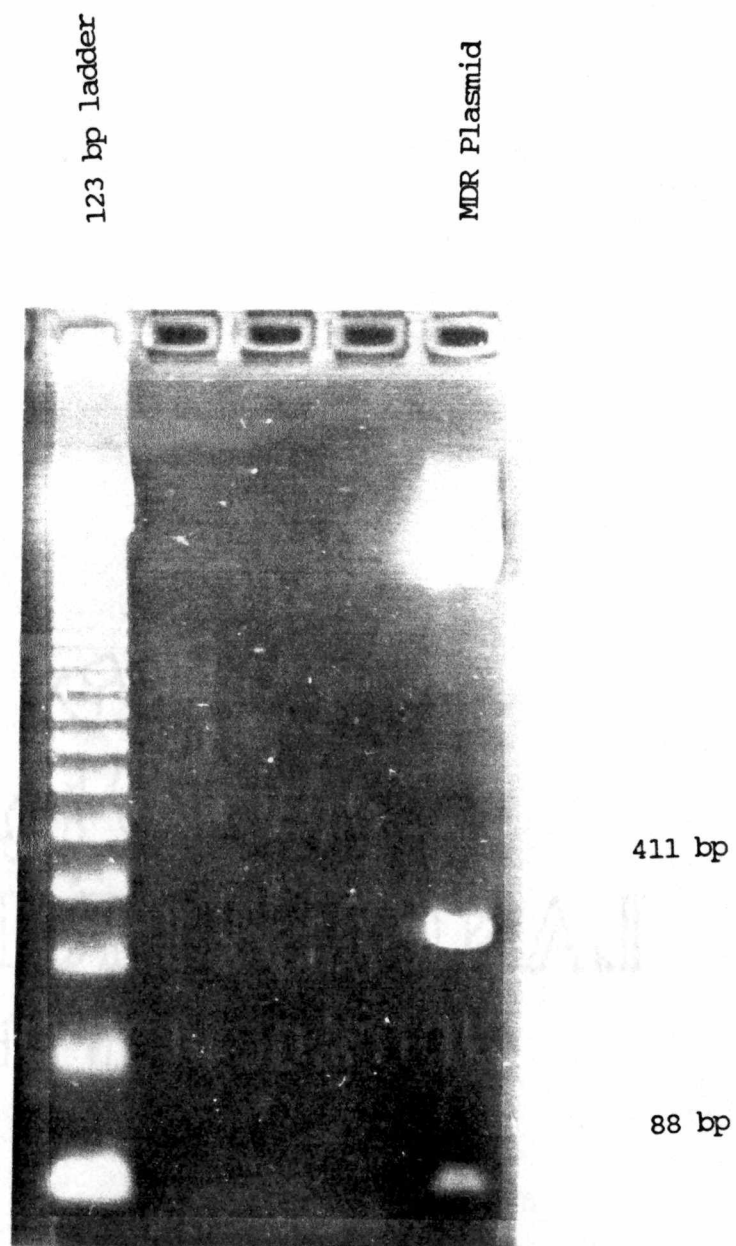


Figure 4.1

indicated that the purified DNA could be used as a probe for the MDR sequence in the fibrosarcoma DNA.

Southern Analysis with the MDR Probe

Figure 4.2 represents a Southern blot analysis of tumor cell DNAs containing DMs, HSRs, or neither. Lanes 1- 5 (87-213, 85-11304, 85-11409, 87-176, 87-211) represent DNA from tumor cell lines that contained DMs, and lanes 6- 10 (86-7643, 86-7661, 87-231, 87-233, 87-297) represent DNA from tumor cell lines that contained HSRs. Lanes 10- 15 (88-7576, 88-7804, 86-7644, 86-7645, 87-213p16) represent DNA from cell lines that contained neither DMs or HSRs. A band at approximately 1 kb. is present in lanes 1, 2, 4, and 5 which all contain DNA from cell lines containing DMs. No band was observed in lane 3 even though 32% of the cells from tumor 85-11409 contained DMs. No hybridization was observed in any of the lanes representing cell lines containing HSRs or in lanes representing cells containing neither DMs or HSRs. Lane 15 contains DNA from tumor 87-213 after 16 passages in culture.

Drug Survival Studies

Survival curves of various sarcoma cells treated with colchicine, actinomycin D, Ara C, and methotrexate are shown in figures 4.3, 4.4, 4.5, and 4.6, respectively. Cell lines containing DMs (87-1363, 88-7577, 88-7704, 87-213) were resistant to colchicine at all concentrations tested. (DNA from cell lines 87-1363, 88-7577, and 88-7704 was not examined for MDR amplification). Cell lines from sarcoma

Figure 4.2 Southern Analysis of tumor cell DNAs containing DMs,
HSRs, or neither probed with the MDR probe

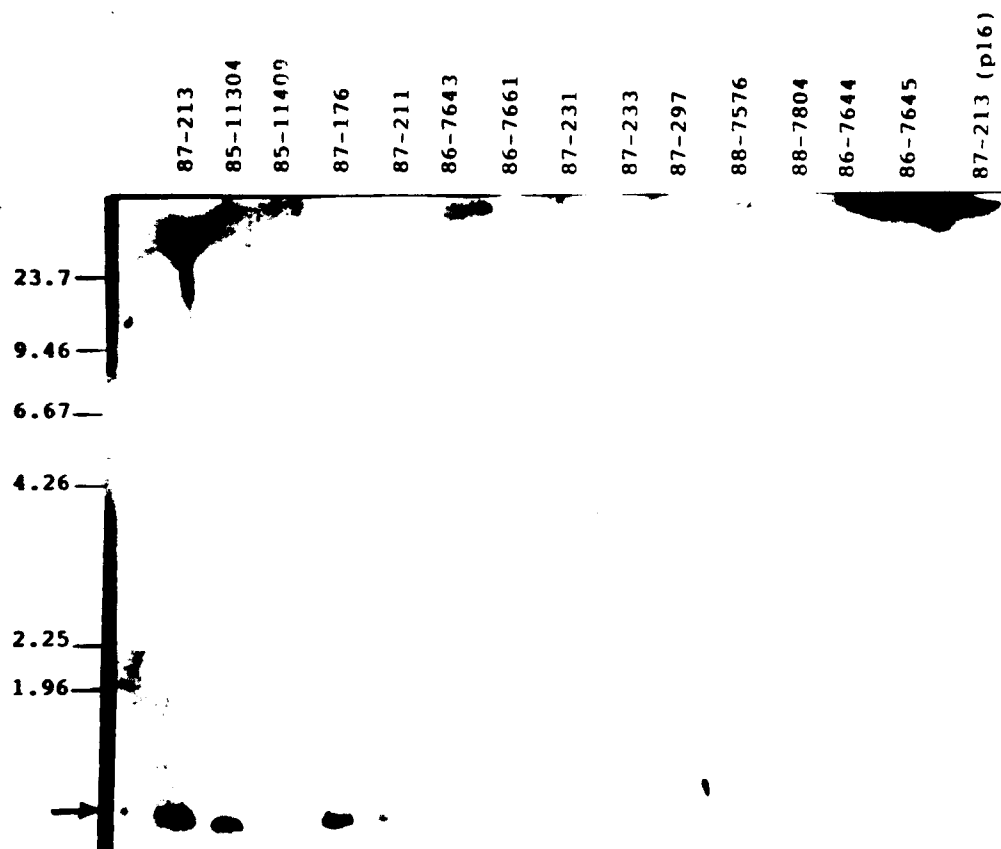


Figure 4.2

Figure 4.3 Survival Curves of fibrosarcoma cells (containing or lacking DMs) in the presence of Colchicine. Cell lines containing DMs were 87-1363, 88-704, 88-7577, and 87-213, while cell lines lacking DMs were 88-7804, 87-214 (contained HSR), and 88-7576.

Colchicine

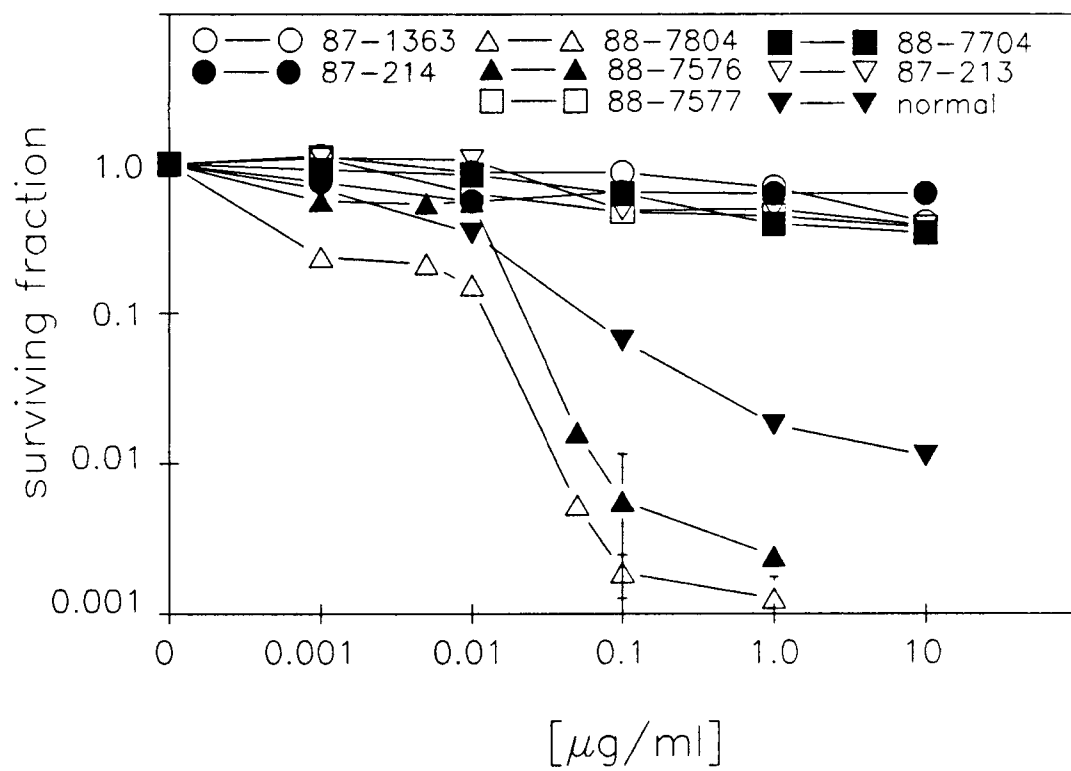


Figure 4.3

Figure 4.4 Survival Curves of fibrosarcoma cells (containing or lacking DMs) in the presence of Actinomycin D. Cell lines containing DMs were 87-1363, 88-704, 88-7577, and 87-213, while cell lines lacking DMs were 88-7804, 87-214 (contained HSR), and 88-7576.

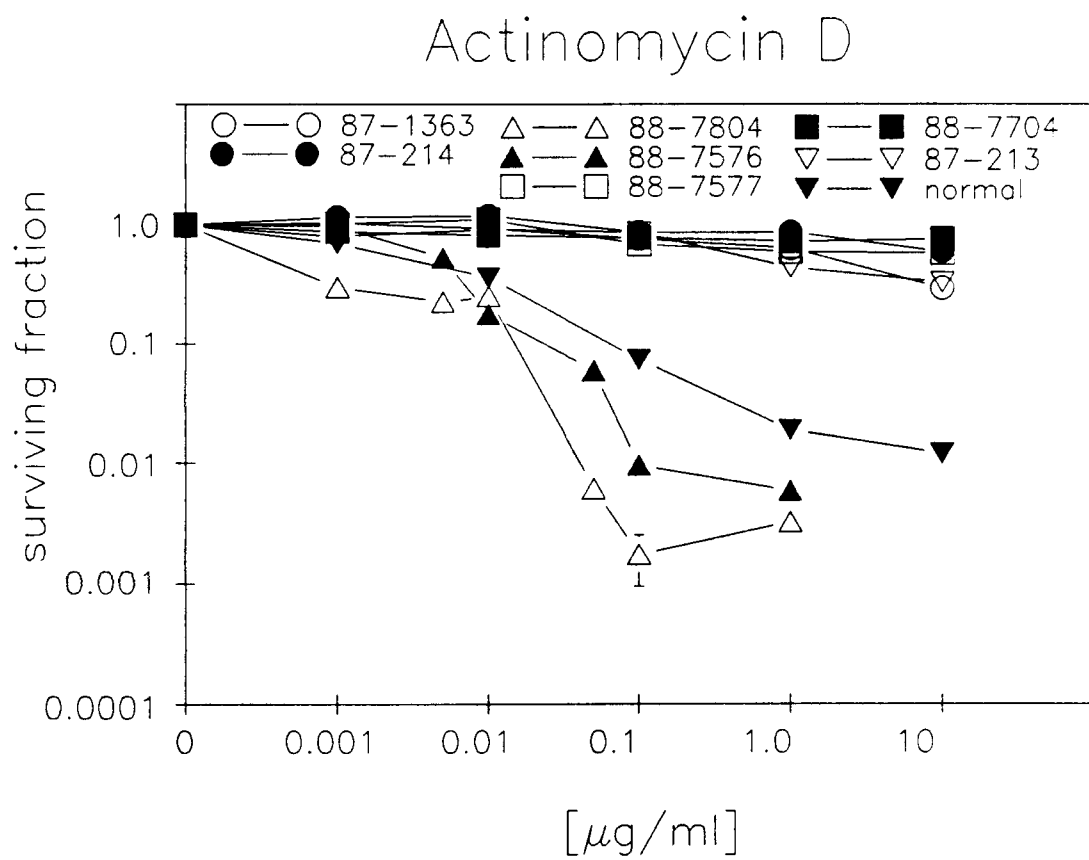


Figure 4.4

Figure 4.5 Survival Curves of fibrosarcoma cells (containing or lacking DMs) in the presence of Ara-C. Cell lines containing DMs were 87-1363, 88-704, 88-7577, and 87-213, while cell lines lacking DMs were 88-7804, 87-214 (contained HSR), and 88-7576.

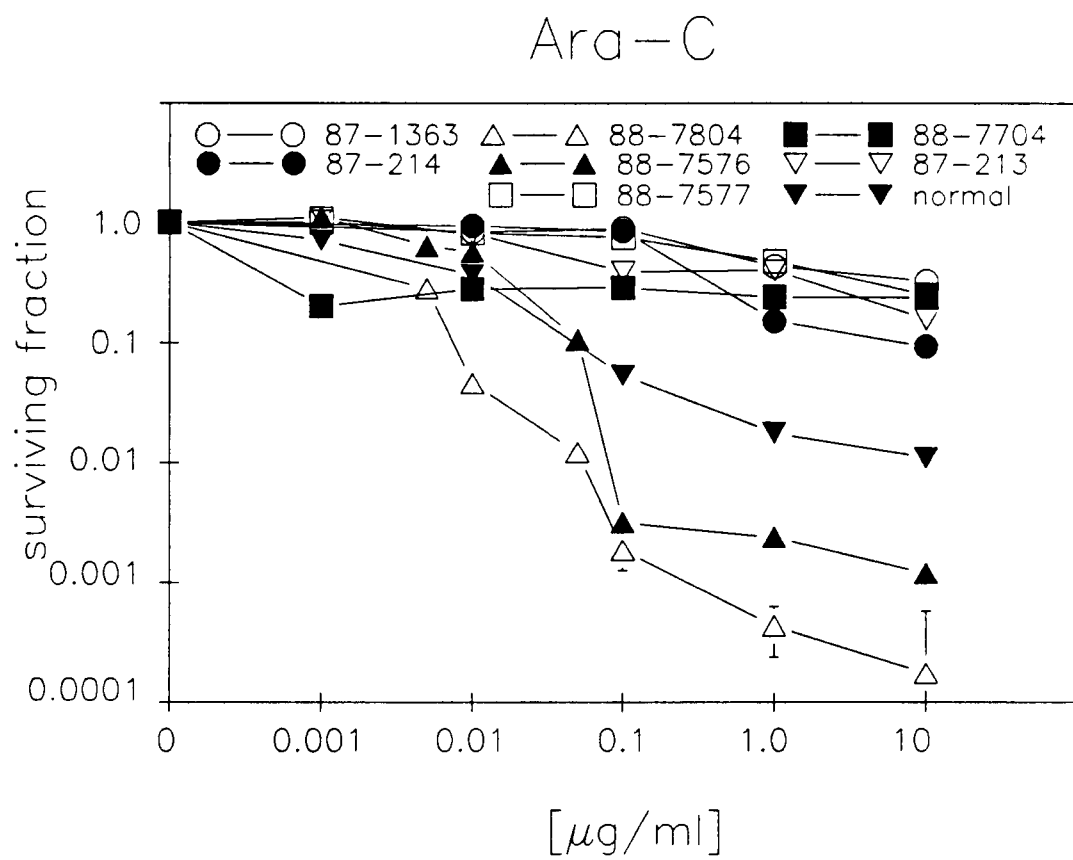


Figure 4.5

Figure 4.6 Survival Curves of fibrosarcoma cells (containing or lacking DMs) in the presence of Methotrexate. Cell lines containing DMs were 87-1363, 88-704, 88-7577, and 87-213, while cell lines lacking DMs were 88-7804, 87-214 (contained HSR), and 88-7576.

Methotrexate

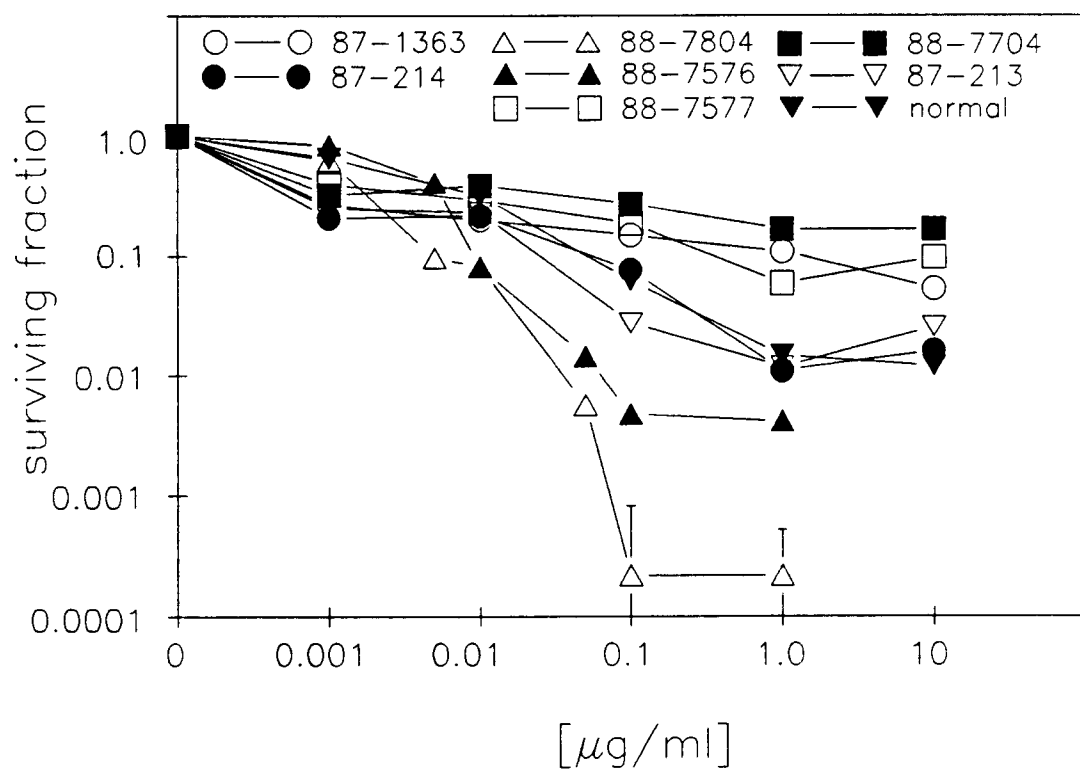


Figure 4.6

87-214 were also resistant to colchicine, and although these cells did not contain DMs, they did contain an HSR. However, the MDR status of 87-214 is not known. Sarcoma cell lines containing neither DMs or HSRs (88-7804, 88-7576) were much more sensitive to colchicine compared to the resistant cell lines at concentrations of 0.1 ug/ ml and greater. The fibroblasts cultured from normal mouse heart and lung tissue were more sensitive to colchicine than the cell lines containing DMs or HSRs, but less sensitive than sarcoma cell lines lacking these abnormal cytogenetic structures.

As with the colchicine treatments, cell lines containing DMs were more resistant than sarcoma cell lines lacking DMs at concentrations of 0.1 ug/ ml and greater of actinomycin D and Ara C. The cell line containing the HSR was also resistant to actinomycin D and Ara C, although the MDR status of this cell line is not presently known (see chapter 5 for future studies). Normal mouse heart/ lung cells were once again more sensitive to actinomycin D and Ara C than sarcoma cell lines containing DMs and the HSR but less sensitive than sarcoma cell lines lacking both of these structures.

Survival studies with methotrexate were not as conclusive as the studies with the other drugs. However, the sarcoma cell lines lacking both DMs and HSRs appear to be more sensitive to methotrexate at concentrations of 0.1 ug/ ml and greater as compared to normal cells and cell lines containing either DMs or the HSR.

X Ray Survival Studies

Data from X ray survival studies are shown in figure 4.7. The sarcoma cell lines exhibited a variety of sensitivities to X-irradiation; however, these sensitivities could not be correlated with karyotype (see Tables 2.1, 2.2, 2.3, and 2.4 for chromosomal changes) or with the presence or absence of DMs. For example, cell lines containing DMs had LD₅₀s of 4.6 Gy (87-1363), 3.8 Gy (88-7704), 2.6 Gy (88-7577), and 3.6 Gy (87-213), while cell lines lacking DMs had LD₅₀s of 4.0 Gy (88-7804), 3.4 Gy (87-214/ also had HSR), and 0.65 Gy (88-7576). Normal fibroblasts cultured from heart and lung tissue had an LD₅₀ of 3.4 Gy. Thus, it appears that there is no specific correlation between the presence of DMs and sensitivity to X rays.

Conclusions and Discussion

The presence of double minutes in 4 of 5 fibrosarcoma cell lines correlated with DNA hybridization of the MDR gene probe, thus indicating amplification of the MDR gene in these cells. In one cell line (85-11409) containing DMs, no hybridization of cellular DNA with the MDR probe was observed. However, only 32% of these cells contained DMs, and it is possible that either not enough cells contained DMs or that these particular DMs were not large enough to exhibit hybridization at the level of sensitivity of Southern analysis. On the other hand, these DMs might not represent amplification of MDR. No hybridization was observed for cell lines containing HSRs or for cell lines that did not contain DMs or HSRs. Particularly interesting is

Figure 4.7 Survival Curves of fibrosarcoma cells (containing or lacking DMs) after treatment with X rays. Cell lines containing DMs were 87-1363, 88-704, 88-7577, and 87-213, while cell lines lacking DMs were 88-7804, 87-214 (contained HSR), and 88-7576.

X Ray

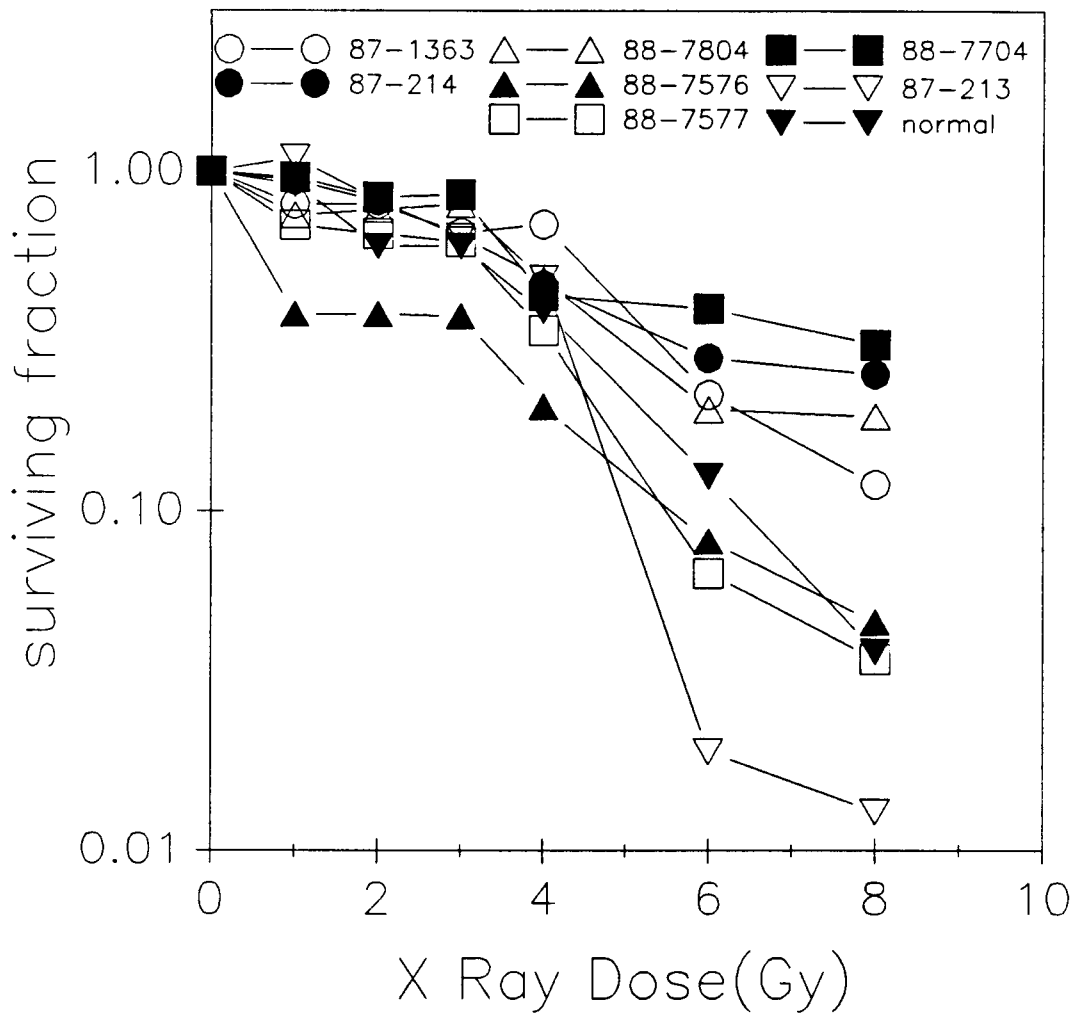


Figure 4.7

the fact that no hybridization was observed in DNA from sarcoma 87-213 after 16 passages in culture. 78% of the cells from early passages of sarcoma 87-213 contained DMs; however, as often is observed with these fibrosarcoma cells in culture, after 16 passages none of the cells analyzed contained DMs. Thus, when the DMs were no longer observed hybridization to the MDR probe was no longer observed. This lends further support to the conclusion that the DMs are cytological manifestations of amplification of the MDR gene.

Data from the drug survival studies support the Southern analysis data suggesting that DMs represent MDR amplification. Cell lines containing DMs were significantly more resistant to colchicine, actinomycin D, Ara C, and methotrexate at concentrations of 0.1 ug/ ml or greater than were cell lines lacking DMs. However, one cell line (87-214) that contained an HSR was also resistant to the drugs, even though no hybridization with the MDR probe was observed in other sarcomas containing HSRs. It is however possible that 87-214 would have MDR amplification, although this has not yet been shown.

There are two broad classes of chemical agents that must be considered when discussing MDR, those agents to which cells expressing MDR are resistant and those agents to which cells expressing MDR are sensitive. Actinomycin D and colchicine belong to the first class, while Ara C and methotrexate belong to the second (Pastan and Gottesman, 1987). The drug survival experiments employing the fibrosarcoma cell lines suggest, as expected, that cell lines with MDR amplification are resistant to actinomycin D and colchicine. However, fibrosarcoma cell lines containing DMs were also resistant to Ara C and

also exhibited a slight resistance to methotrexate. The apparent discrepancy between these data and the published literature might be due to the possibility that all of the DMs in each cell line may not be cytological manifestations of MDR amplification. Furthermore, there is evidence that multidrug resistance is not due to a single gene. MDR genes appear to be part of a multigene family, and two or more full-length cDNA sequences have been isolated from the mouse (Gros et al., 1987) and human (VanDerBliet et al., 1987) genomes. Thus, it is possible that different "members" of the multigene family may be induced by a particular drug in different cell lines. If this were the case, various responses might be observed with one particular drug in different cell lines expressing MDR.

The normal fibroblasts were more sensitive to the drugs tested than sarcoma cell lines containing DMs and less sensitive than sarcoma cell lines lacking DMs. The increased sensitivity compared to DM containing lines is most likely due to the fact that the normal cells are not expressing amplified MDR. In the absence of MDR amplification, the tumor cells could be more sensitive than normal cells because other factors such as unstable karyotype or altered DNA repair would play a role.

The fibrosarcomas analyzed exhibited varying responses to x-irradiation. However, few conclusions can be drawn from the survival curves themselves since the shapes of the curves may be due, in part, to technical difficulties such as cell clumping. A clump of cells would need only one survivor to form a colony, and thus, artificially high survivals might be observed. It can be concluded however, that

the X ray responses did not correlate with chromosome number or specific karyotypic changes. Also, the presence or absence of DMs did not appear to influence sensitivity to X rays. No correlation would be expected if the DMs are indeed indicative of MDR amplification. The p-glycoprotein is purported to function as a pump that does not allow accumulation of numerous chemical agents inside the cell (Fojo et al., 1985), and thus would not seem to be involved in resistance to X-irradiation.

Even though the fibrosarcoma cells had not been exposed to chemical agents, either in the mouse during tumor development or in culture, the MDR gene appears to be amplified in tumor cells containing DMs. This is not completely unexpected since many normal tissues as well as tumors have been found to express MDR (Fojo et al., 1987). Thus, "MDR" may be a sort of misnomer since the protein probably serves a function in normal cells. It is likely that the formation of DMs is not a primary step in fibrosarcoma formation but is involved in or a consequence of progression. This is supported by the fact that DMs occurred only in tumors at later stages of development. Also, in secondary tumors formed by reinjection of parent cells containing DMs (see chapter 2) no DMs were observed in the cells analyzed. It is also possible that MDR in the fibrosarcomas is involved with the metastatic potential of the tumor cells. Natural killer (NK) cells have been shown to play an important role in the prevention of metastases (Herberman and Ortaldo, 1981), and increased drug resistance was associated with both an increased resistance to NK cytotoxicity and increased p-glycoprotein expression (Woods et al., 1988). If p-

glycoprotein does actually function as an efflux pump, preventing intracellular accumulation of some cytotoxic drugs, it is also possible that the MDR gene product could also control the intracellular accumulation of cytotoxic factors produced by NK cells. In fact, it has been reported that the cytotoxic potential of NK cells was reduced in proportion to the level of p- glycoprotein in target cells (Woods et al., 1988). Therefore, it is possible that in this fibrosarcoma system, the MDR amplification is a response to the NK cells of the immune system. Therefore, if the fibrosarcomas were not removed from the mouse until death, autopsy might reveal a greater number of metastases in animals whose tumors contained DMs compared to animals whose tumors did not contain these structures.

CHAPTER 5

SUMMARY

The original objectives of this study were to follow chromosome changes during fibrosarcoma progression, to determine if specific chromosome or oncogene changes were associated with sarcomas at particular stages of development, and to determine if specific chromosome changes were dependent upon the agent that induced the tumor. These studies are presented in chapters 2 and 3. Generally, as the time post-implantation of the disk increased, the number of chromosomes per cell increased in tumors from the animals receiving the disk as well as those receiving the disk + X ray treatments. The mean chromosome number per cell was lower (at comparable times post-implantation of the disk) in sarcomas from animals that received the disk + X ray treatment compared to tumors from animals that received the disk alone. DMs and HSRs were observed in many of the tumors analyzed, and were predominantly present in tumors at later stages of development. Duplications of chromosomes 10, 16, and 19 were present in tumors from animals treated with the disk + X ray and those with the disk alone. Metacentric chromosomes were observed only in tumors that developed in animals that received the disk alone. The most frequently observed metacentrics were (4;17), (1;17), (4;15), and isochromosome 19.

Consistent chromosomal "trends" were observed; however, no particular chromosomal alteration could be identified as being an

initial and perhaps causative event in tumor formation. Also, no specific chromosomal alterations could be correlated with particular stages of tumor development. DMs and HSRs were present in many tumors at later stages of development, suggesting the importance of gene amplification in the later stages of neoplastic progression. It is likely that the DMs, however, are not necessary for tumor maintenance since 2° tumors formed via injection of parental cells containing DMs did not themselves contain double minute chromosomes. The only chromosome change that was specifically associated with the inducing agent was the formation of metacentric chromosomes. These structures were observed only in tumors from animals that received the disk alone. However, no one specific metacentric chromosome was observed in all tumors containing these structures.

Any relationship between the production of specific chromosomal abnormalities and the formation of the fibrosarcomas is uncertain. The karyotypic inconsistencies of individual tumors suggest that more is involved in malignant transformation than a single primary chromosomal lesion. However, Hayflick and Moorhead (1961) observed chromosomal alterations associated with the earliest stages of transformation of human cells in culture. It is quite probable that the chromosomal alterations are involved in the development of the fibrosarcomas. It is feasible that each cytogenetic alteration could produce a metabolic change in the cell, such that the numerous chromosomal changes in fibrosarcoma cells could be responsible for a plethora of as yet unidentified metabolic abnormalities.

Because DMs and HSRs were observed in tumors particularly at later stages of development, experiments were done to investigate if these represented amplification of oncogenes. Several of the oncogenes investigated had been mapped to chromosomes involved in the alterations seen in sarcomas or were associated with sarcoma viruses. Chapter 3 describes studies involving oncogene analysis of c-myb, c-h-ras, c-raf, c-rel, c-abl, c-erbA, c-erbB, c-sis, c- myc, and c-mos. Examination of these oncogenes through Southern analysis yielded no correlation between the presence of DMs and/or HSRs and oncogene amplification. Also, there was no correlation between oncogene rearrangement and the presence of metacentric chromosomes.

Since oncogene amplification did not appear to be related to the presence of DMs or HSRs in sarcomas, the possible involvement of the multiple drug resistance gene was investigated. Chapter 4 describes these studies. Southern analysis utilizing an MDR probe revealed a positive correlation between the presence of DMs and amplification of the MDR sequence. Also, drug survival studies suggested that sarcoma cell lines containing DMs (and one cell line containing an HSR) were resistant to ara C, methotrexate, actinomycin D, and colchicine relative to sarcoma cells lacking DMs. Normal mouse fibroblasts, used as a control, had drug sensitivities greater than sarcoma cell lines containing DMs and less than sarcoma cell lines lacking DMs. These results were generally consistent with the Southern hybridization studies indicating that the DMs were cytological manifestations of MDR amplification.

X ray survival studies revealed that cells from various tumors had a variety of sensitivities to X- irradiation. However, these sensitivities did not correlate with the presence or absence of DMs or with karyotypic changes. Thus, it appears as if the DMs present in the fibrosarcoma cells do indeed represent amplification of the MDR sequence even though the tumors had not been exposed to any MDR-inducing agents either in vivo or in vitro prior to the survival studies. However, as is described in chapter 4, this amplification is possibly an immunological response to the cytotoxic agents produced by natural killer cells.

Although these studies indicate that chromosomal changes occur rather early in the neoplastic process, it still cannot be determined whether any represent the primary neoplastic event. Furthermore, in spite of the fact that a non- random pattern of chromosomal trends was observed, the way in which these changes contribute mechanistically to the neoplastic process cannot be fully explained. It is possible that each cytogenetic change produces at least one alteration of a cellular process. Thus, as a result of chromosomal alterations normal cellular growth and function could be altered. In this system, as well as in human solid tumors, future studies will need to concentrate on the interaction between cytogenetic and molecular changes.

Possible future studies involve the utilization of pulse field gel electrophoresis (PFGE) to identify specific translocation (metacentric) breakpoints. PFGE is an electrophoretic method for the separation of large DNA molecules on agarose gels (Schwartz and Cantor, 1984). Rearrangements or translocations that move a DNA sequence of 5×10^6

bp. or greater are cytogenetically visible. Detection of rearranged sequences through standard Southern blotting is difficult, since only a small portion (approximately 1%) of a chromosome can be scanned with a given gel (Sandberg et al., 1988). PFGE can scan 20% to 40% of a chromosome in a given gel, thus greatly increasing the chances of detecting a breakpoint. In fact, the size range of molecules resolved well by PFGE falls into the gap between standard Southern blotting and cytogenetic analysis (Sandberg et al., 1988).

Southern blots prepared from PFGE gels could be used to generate large scale restriction maps (Herrmann et al., 1987), and these maps, constructed using several closely linked probes, could span a chromosome band. Thus, PFGE could be useful in identifying specific breakpoints involved in translocations, and rearrangements that appear cytogenetically identical could be examined for heterogeneity at the molecular level. For example, it would be possible to determine if the isochromosome 19s observed in various tumors were derived from the same molecular rearrangement.

Future experiments should also involve further MDR studies. First, drug survival curves and Southern analysis should be performed on additional tumor cell lines containing HSRs to determine if the HSRs are in fact involved in the MDR phenotype. Also, some mice should be allowed to die of their fibrosarcomas, and autopsies should concentrate on metastases. If MDR amplification is affecting the ability of natural killer cells to fight metastatic potential, the animals whose tumor cells contained DMs (MDR amplification) would possibly have more metastases than animals whose tumor cells did not contain MDR

amplification. Furthermore, it would be interesting to isolate the DMs, transform sensitive tumor cell lines with these DMs, and determine if the transformed cells would become resistant, more tumorigenic, or more metastatic than cells before transformation.

Knowledge of specific chromosomal and molecular changes involved in mouse fibrosarcomas may eventually lead to a better understanding of human fibrosarcomas. If the specific breakpoints involved in the mouse tumor cell translocations could be elucidated, identification of homologous sequences in humans could provide clues as to the location of preferential breakpoints in human sarcomas. Also, a more complete understanding of the role of MDR amplification in the tumor cell lines may provide information about the cellular processes responsible for neoplastic progression and neoplastic potential. Ultimately, cytogenetic and molecular studies of animal solid tumors may help to provide knowledge that could serve as a supplement to conventional histological diagnosis and prognosis, and thus improved management of human solid malignancies.

REFERENCES

REFERENCES

- Aldaz, C. M., Conti, C. J., Klein-Szanto, A. J., and T. J. Slaga (1986) A direct cytogenetic technique for mouse skin carcinomas and papillomas. *Cancer Genetics and Cytogenetics*, 20: 223-229.
- Alitalo, K. (1985) Acute myelogenous leukemia with c- *myc* amplification and double minute chromosomes. *The Lancet*, 985: 1035-1038.
- Ascione, R., et. al. (1986) Oncogenes: Molecular probes for clinical application in malignant diseases. *Gene. Anal. Techn.*, 3: 25-39.
- Atkin, N. B., and M. C. Baker (1985) Cytogenetic study of ten carcinomas of the bladder: involvement of chromosomes 1 and 11. *Cancer Genetics and Cytogenetics*, 15: 253-268.
- Atkin, N. B., and M. C. Baker (1988) Small metacentric marker chromosomes, particularly isochromosomes in cancer. *Human Genetics*, 79: 96-102.
- Banerjee, M. R., and R. R. Bates (1966) Prevalence of heteroploidy in plastic film induced primary sarcomas. *British Journal of Cancer*, XX no. 3: 555-563.
- Banerjee, M., Wiener, F., and J. Spira (1985) Mapping of the c- *myc*, pvt-1 and immunoglobulin kappa genes in relation to the mouse plasmacytoma-associated variant (6;15) translocation breakpoint. *EMBO J*, 4: 3183-3188.
- Bates, R. B., and M. Klein (1966) Importance of a smooth surface in carcinogenesis by plastic film. *JNCI*, 37:145-151.
- Bigner, S. H., Wong, A. J., Mark, J., Kinzler, K., Vogelstein, B., and D. D. Bigner (1987) Chromosomes and gene amplification in malignant human gliomas. *Cancer Genetics and Cytogenetics*, 28: 32.
- Bigner, S. H., Wong, A. J., Mark, J., Muhlbaier, L. H., Kinzler, K. W., Vogelstein, B., and D. D. Bigner (1987) Relationship between gene amplification and chromosomal deviations in malignant human gliomas. *Cancer Genetics and Cytogenetics*, 29: 165-170.
- Biedler, J. L., and B. A. Spengler (1976) Metaphase chromosome anomaly: association with drug resistance and cell-specific products. *Science*, 191: 185-187.
- Bischoff, F. (1972) Organic polymer biocompatibility and toxicology. *Clinical Chemistry*, 18: 869-894.
- Bischoff, F., and G. Bryson (1964) Carcinogenesis through solid state surfaces. *Progress in Experimental Tumor Research*, 5: 85.

Boveri, R. (1914) Zur Frage der Entstehung Maligner Tumoren. Fischer, Jena: 1-64.

Borek, C., Ong, A., and H. Mason (1987) Distinctive transforming genes in X ray transformed mammalian cells. PNAS, 84: 794-798.

Brand, K. G. (1982) Cancer associated with asbestosis, schistosomiasis, foreign bodies, and scars, in: F. F. Becker (ed.), Cancer 1, A Comprehensive Treatise; Etiology: Chemical and Physical Carcinogenesis, Plenum Press, pp. 661-692.

Brand, K. G., Buoan, L. C., and I. Brand (1973) Foreign body tumorigenesis in mice: most probable number of originator cells. JNCI, 51: 1071-1074.

Brand, I., Buoan, L. C., and K. G. Brand (1977) Foreign body induced tumors of mice: strain and sex differences in latency and incidence. Journal of the National Cancer Institute, 58: 1443-1447.

Brewen, J. G., and N. Gengozian (1971) Radiation- induced human chromosome aberrations, II. Human in vitro irradiation compared to in vitro and in vivo irradiation of marmoset leukocytes. Mutation Research, 13: 383-391.

Buoan, L. C., and K. G. Brand (1968) Double minute chromosomes in plastic film- induced sarcomas in mice. Naturwissenschaften, 3: 135.

Cahilly- Snyder, L., Yang- Feng, T., Francke, U., and D. L. George (1987) Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somatic Cell and Molecular Genetics, 13: 235-244.

Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M., and I. B. Roninson (1986) Internal duplication and homology with bacterial transport proteins in the *mdr1* (p- glycoprotein) gene for multidrug- resistant human cells. Cell, 47: 381-389.

Chiu, I. M., Reddy, E. P., Givol, D., Robbins, K. C., Tronick, S. R., and S. A. Aaronson (1984) Nucleotide sequence analysis identifies the human *c- sis* proto- oncogene as a structural gene for platelet derived growth factor. Cell, 37: 123-129.

Cole, M. D. (1986) The *myc* oncogene: its role in transformation and differentiation. Annual review of genetics, 20: 361-384.

Collins, S. J., and M. T. Groudine (1982) Amplification of endogenous *myc*- related DNA sequences in a human myeloid leukemia cell line. Nature, 298: 679-681.

Cornwell, M. M., Safa, A. R., Felsted, R. L., Gottesman, M., and I. Pastan (1986) Membrane vesicles from multidrug resistant human cancer cells contain a specific 150 to 170 KDa protein. PNAS, 84: 265-269.

Crews, S., Barth, R., Hood, L., Prehn, J., and K. Calame (1982) Mouse c-myc oncogene is located on chromosome 15 and translocated to chromosome 12 in plasmacytomas. Science, 218: 1319-1321.

Croce, C. (1987) Role of chromosome translocations in human neoplasia. Cell, 49: 155-156.

Croce, C., Shander, M., Martinis, J., Cicurel, L., D' Ancona, G., Dolby, T. W., and H. Koprowski (1979) Chromosomal location of the genes for human immunoglobulin heavy chains. PNAS, 76: 3416-3419.

Der, C. J., Krontiris, T. G., and G. M. Cooper (1982) Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of harvey and kirsten sarcoma viruses. PNAS, 79: 3637-3640.

Dhaliwal, M. K., Norman, J. O., Pathak, S., and D. L. Busbee (1987) Cytogenetic analysis of a gossypol- induced murine myxosarcoma. Journal of the National Cancer Institute, 78: 1203-1207.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and M. D. Waterfield (1984) Close similarity of epidermal growth factor receptor and v- erbB oncogene protein sequences. Nature, 307: 521-527.

Fojo, A. Akiyama, S., Gottesman, M., and I. Pastan (1985) Reduced drug accumulation in multiple drug- resistant human KB carcinoma cell lines. Cancer Research, 45: 3002-3007.

Fojo, A. T., Udea, K., Slamon, D. J., Poplack, D. G., Gottesman, M., and I. Pastan (1987) Expression of a multidrug- resistance gene in human tumors and tissues. PNAS, 84: 265-269.

Garson, J. A., VandenBerghe, J. A., and J. T. Kemshead (1987) High resolution in situ hybridization technique using biotinylated NMYC oncogene probe reveals periodic structure of HSRs in human neuroblastoma. Cytogenetics and Cell Genetics, 45: 10-15.

Gibas, Z., Prout, G. R., and A. A. Sandberg (1984) Malignant teratoma of the testis with an isochromosome no. 12, i(12p), as the sole structural cytogenetic abnormality. Journal of Urology, 131: 762-763.

Gilman, A. G. (1984) G- proteins and dual control of adenylate cyclase. Cell, 36: 577-579.

Goff, S. P., D'Eustachio, P., Ruddle, F., and D. Baltimore (1982) Chromosomal assignment of the endogeneous protooncogene c- abl. Science, 218: 1317-1319.

Groffen, J., Stephenson, J., Heisterkamp, N., DeKlein, A., Barton, C. R., and G. Grosveld (1984) Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell*, 36: 93-99.

Gros, P., Ben Neriah, Y., Croop, J. M., and D. E. Housman (1987) Isolation and expression of a complementary cDNA that confers multidrug resistance. *Nature*, 323: 728-731.

Grunwald, D. J., Dale, B., Dudley, J., Lamph, W., Sugden, B., Ozanne, B., and R. Kissen (1982) Loss of viral gene expression and retention of tumorigenicity by Ableson lymphoma cells. *Journal of Virology*, 43: 92-103.

Guerreo, I., Calzava, P., Mayer, A., and A. Pellicer (1984) A molecular approach to leukemogenesis: mouse lymphomas contain an activated c- ras oncogene. *PNAS*, 81: 202-205.

Hayflick, L., and P. S. Moorhead (1961) The serial cultivation of human diploid cell strains. *Experimental Cell Research*, 25: 585-621.

Helden, C. H., Betsholtz, C., Johnsson, A., Nister, M., Ek, B., Ronnstrand, L., Wasteson, A., and B. Westermarck (1985) Platelet derived growth factor: mechanism of action and relation to oncogenes. *Journal of cell science*, 3: 65-76.

Herberman, R. B., and J. R. Ortaldo (1981) Natural killer cells: their role in defenses against disease. *Science*, 214: 24-30.

Herrmann, B. G., Barlow, D. P., and H. Lehrach (1987) A large inverted duplication allows homologous recombination between chromosomes heterozygous for the proximal t complex inversion. *Cell*, 48: 813- 825.

Holden, J. A. A., Hough, M. R., Reimer, D. L., and B. N. White (1987) Evidence for unequal crossing- over as the mechanism for amplification of some homogeneously staining regions. *Cancer Genetics and Cytogenetics*, 29: 139-149.

Johnson, K. H., Buoen, L. C., Brand, I., and K. G. Brand (1970) Polymer tumorigenesis: clonal determination of histopathological characteristics during early preneoplasia: relationships to karyotype, mouse strain, and sex. *JNCI*, 44: 785-793.

Karess, R. E., and H. Hanafusa (1981) Viral and cellular src genes contribute to the structure of recovered avian sarcoma virus transforming protein. *Cell*, 24: 155.

Kartner, N., Riordan, J. R., and V. Ling (1983) Cell surface p-glycoprotein associated with multidrug resistance in mammalian cells. *Science*, 221: 1285-1287.

Kendal, W. S., Wang, R., Hsu, T. C., and P. Frost (1987) Rate of generation of major karyotypic abnormalities in relationship to the metastatic potential of B16 murine melanoma. *Cancer Research*, 47: 3835-3841.

Klein, G. (1983) Specific chromosomal translocations and the genesis of B- cell derived tumors in mice and men. *Cell*, 32: 311-315.

Kodama, S., and M. S. Sasaki (1987) The involvement of chromosome 13 in the x- ray induced in vitro transformation of mouse m5S cells. *Japanese Journal of Cancer Research*, 78: 372-381.

Kozak, C. A., Sears, J., and M. D. Hoggan (1983) Genetic mapping of the mouse protooncogene c- sis to chromosome 15. *Science*, 221: 867-869.

Kris, R. M., Aviv, A., Bar-Eli, M., Alon, Y., Carni, P. Schlessinger, J., and A. Raz (1985) The expression of ki- ras oncogene in tumor cell variants exhibiting different metastatic capabilities. *International Journal of Cancer*, 35: 227-232.

Larcher, F., Aldaz, C. M., Slaga, T. J., and C. J. Conti (1988) Multidrug resistance gene in mouse epidermal tumors in vivo and in vitro. *AACR Proceedings*, 29: 160.

LeBeau, M., and J. Rowley (1986) Chromosomal abnormalities in leukemia and lymphoma: clinical and biological significance, in: H. Harris and K. Hirschhorn (eds.), *Advances in human genetics*, vol. 15, Plenum Press, pp. 1-54.

Leibovitch, S. A., Leibovitch, M. P., Gullier, H. J., and J. Harel (1986) Differential expression of proto- oncogenes related to transformation and cancer progression in rat myoblasts. *Cancer Research*, 46: 4097-4103.

Levan, A., Manolov, G., and P. Clifford (1968) Chromosomes of a human neuroblastoma: a new case with accessory minute chromosomes. *JNCI*, 41: 1377-1387.

Libbus, B. L., and J. E. Craighead (1988) Chromosomal translocations with specific breakpoints in asbestos- induced mesotheliomas. *Cancer Research*, 48: 6455-6461.

Liebovitz, A. (1986) Development of tumor cell lines. *Cancer Genetics and Cytogenetics*, 19: 11-19.

Maniatis, T., Fritsch, E. F., and J. Sambrook (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory.

Meltzer, P., Coccia, M., Kinzler, K., Vogelstein, B., and J. Trent (1987) Renaturation gel analysis of amplified DNA sequences. *Cancer Genetics and Cytogenetics*, 28: 32.

Mitelman, F., and S. Heim (1986) Chromosomal segments of critical significance in human neoplasia, in: Genetic toxicology of environmental chemicals, part A: basic principles and mechanisms of action, Alan R. Liss, pp. 583-589.

McCormick, F., Clark, B. F. C., LaCour, T. F. M., Kjeldgaard, M., Nørskov-Lauritsen, L., and J. Nyborg (1985) A model for the tertiary structure of p21, the product of the ras oncogene. Science, 230: 78-82.

McGrath, J. P., Capon, D. J., Goeddel, D. V., and A. D. Levinson (1984) Comparative biochemical properties of normal and activated human ras p21 protein. Nature, 310: 644-649.

Mc Morrow, L. E., Newcomb, E. W., and A. Pellicer (1987) Chromosome changes in x-ray and NMU treated C57BL/6J mice at different stages of tumor development. Journal of Cellular Biochemistry, suppl. 11D: 100.

Neyfakh, A. A., Dmitrevskaya, T. V., and A. S. Serpinskaya (1988) The membrane transport system responsible for multidrug resistance is operating in non-resistant cells. Experimental Cell Research, 178: 513-517.

Nesbitt, M., and U. Francke (1973) A system of nomenclature for banding patterns of mouse chromosomes. Chromosoma, 41: 145-158.

Nishimura, J., Huang, J. S., and T. F. Deuel (1982) Platelet derived growth factor stimulates tyrosine-specific protein kinase activity in Swiss mouse 3T3 cell membranes. PNAS, 79: 4303-4307.

Nowell, P., and D. A. Hungerford (1960) A minute chromosome in human chronic granulocytic leukemia. Science, 132: 1197.

Oshimura, M., Koi, M., Ozawa, N., Sugawara, O., Lamb, W., and J. C. Barrett (1988) Role of chromosome loss in ras/myc induced Syrian hamster tumors. Cancer Research, 48: 1623-1632.

Ott, G. (1970) Fremdkorpersarkome. Exp. Med. Pathol. Klin., 32: 1.

Parada, L., Tabin, C., Shih, C., and R. A. Weinberg (1982) Human EJ bladder carcinoma oncogene is homolog of Harvey sarcoma virus ras gene. Nature, 297: 474-478.

Passanti, C., Davies, B., Ford, M., and M. Fried (1987) Structure of an inverted duplication formed as a first step in a gene amplification event: implications for a model of gene amplification. EMBOJ, 6: 1697-1703.

Pastan, I., and M. Gottesman (1987) Multiple drug resistance in human cancer. The New England Journal of Medicine, 316: 1388-1393.

Pohjanpelto, P., Alitalo, K., Holtta, E., Janne, O., and S. Knuutila (1985) Amplification of ornithine decarboxylase gene in response to polyamine deprivation in chinese hamster ovary cells. *Journal of Biological Chemistry*, 260: 8532-8537.

Popescu, N. C., Chahinian, A. P., and J. A. DiPaolo (1988) Nonrandom chromosome alterations in human malignant mesothelioma. *Cancer Research*, 48: 142-147.

Quinn, L. A., Moore, G. E., Morgan, R. T., and L. K. Woods (1979) Cell lines from human colon carcinoma with unusual cell products, double minutes and homogeneously staining regions. *Cancer Research*, 39: 4914-4924.

Rachko, D., and K. G. Brand (1983) Chromosomal aberrations in foreign body tumorigenesis of mice. *Proceedings of the Society for Experimental Biology and Medicine*, 172: 382-388.

Reddy, L., and P. J. Fialkow (1984) Probable clonal development of foreign body- induced murine sarcomas. *JNCI*, 72: 467-470.

Roberts, J. M., Buck, L. B., and R. Axel (1983) A structure for amplified DNA. *Cell*, 33: 53-63.

Rowley, J. D. (1983) A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence Giemsa staining. *Nature*, 243: 290-293.

Salamanca, F., and S. Armendores (1974) C- bands in human metaphase chromosomes by barium hydroxide. *Ann. Genetics*, 17: 135-136.

Sandberg, A., and C. Turc-Carel (1987) The cytogenetics of solid tumors. *Cancer*, 59: 387-395.

Sandberg, A., Turc-Carel, C., and R. Gemmill (1988) Chromosomes in solid tumors and beyond. *Cancer Research*, 48: 1049-1059.

Sawey, M. J., Hood, A. T., Burns, F. J., and S. J. Garte (1987) Activation of c- myc and c-k- ras oncogenes in primary rat tumors induced by ionizing radiation. *Mol. Cell. Biol.* 7: 932-935.

Scheiness, B., and M. Gardinier (1984) Expression of protooncogene (protomyb) in hemopoietic tissues of mice. *Molecular and Cell. Biology*, 4: 1206-1212.

Schimke, R. T. (1982) *Gene Amplification*. Cold Spring Harbor Lab., Cold Spring Harbor, N. Y.

Schimke, R. T., Brown, P. C., Kaufman, R. J., McGrogan, M., and D. L. Slate (1980) Chromosomal and extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. Cold Spring Harbor Symposium, Quantative Biology, 45: 785-797.

Schwab, M., Alitalo, K., Varmus, H., Bishop, M., and D. George (1983) A cellular oncogene (c- ki- ras) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. Nature, 303: 497-501.

Schwartz, D. C., and C. R. Cantor (1984) Separation of yeast chromosome- sized DNAs by pulsed field gradient gel electrophoresis. Cell, 37: 67-75.

Scotto, K. W., Biedler, J. L., and P. W. Melera (1986) Amplification and expression of genes associated with multidrug resistance in mammalian cells. Science, 232: 751-755.

Seabright, M. (1971) Rapid banding technique for human chromosomes. Lancet, ii: 971-971.

Selden, J. R., Emanuel, B. S., Wang, E., Cannizzaro, L., Palumbo, A., Erikson, J., Nowell, P., Rovera, G., and C. Croce (1983) Amplified c- myc and c- abl genes are on the same marker chromosome in K562 leukemia cells. PNAS, 80: 7289-7292.

Shih, T. Y., Papageorge, A. G., Stokes, P. E., Weeks, M. O., and E. M. Skolnick (1980) Guanine nucleotide binding and auto phosphorylating activities associated with the p21 src protein of Harvey murine sarcoma virus. Nature, 287: 686-691.

Silver, J., Whitney, J. B., Kozak, C., Hollis, G., and I. Kirsch (1985) ErbB is linked to the alpha- globin locus on mouse chromosome 11. Molecular and Cellular Biology, 5: 1784-1786.

Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98: 503-517.

Stam, K., Heistercamp, M., Grosveld, G., deKlien, A., Nema, R. S., Coleman, M., Dosik, H., and J. Groffen (1985) Evidence of a new chimeric bcr/ c- abl mRNA in patients with chronic myelocytic leukemia and the Philadelphia chromosome. New England Journal of Medicine, 313: 1429-1433.

Sukumar, S., Notario, V., Martin-Zanca, D., and M. Barbacid (1983) Induction of mammary carcinoma in rats by nitroso-methylurea involves malignant activation of H-ras 1 locus by single point mutations. Nature, 306: 658-661.

Tabin, C., Bradley, S., Bargmann, C., Weinberg, R., Papageorge, A., Scolnick, E., Dhar, R., Lowy, D., and E. Chang (1982) Mechanism of activation of a human oncogene. *Nature*, 300: 143-148.

Taparowsky, E., Shimizu, K., Goldfarb, M., and M. Wigler (1983) Structure and activation of the human N- ras gene. *Cell*, 34: 581-586.

Taub, R., Moulding, C., Battey, J., Murphy, W., Vasicek, T., Lenoir, G., and P. Leder (1984) Activation and somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. *Cell*, 36: 339-348.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M., Pastan, I., and M. C. Willingham (1987) Cellular localization of the multidrug resistance gene product p- glycoprotein in normal human tissues. *PNAS*, 84: 7735-7738.

Threadgill, D. W., and J. E. Womack (1988) Regional localization of mouse abl and mos proto- oncogenes by in situ hybridization. *Genomics*, 3: 82-86.

Udea, K., Caradelli, C., Gottesman, M., and I. Pastan (1987) Expression of a full- length cDNA for the human MDR1 gene confers resistance to colchicine, doxorubicin, and vinblastine. *PNAS*, 84:3004-3008.

Ullrich, A., Coussens, L., Hayflick, J. S., Dullit, J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Liebermann, T., Schlessinger, J., Downyard, J., Mayes, E. L., Whittle, N., Waterfield, M. D., and P. Seeburg (1984) Human epiderman growth factor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, 309: 418-425.

VanDerBlik, A. M., Baas, F., Houte DeLange, T., Kooiman. P. M., VanderVelde- Koerts, T., and P. Borst (1987) The human MDR 3 gene encodes a novel p- glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver. *EMBO J*, 6: 3325-3331.

Von Hoff, D. G., Needham- VanDevanter, B. R., Yucel, J., Windle, B. E., and G. M. Wahl (1988) Amplified human myc oncogenes localized to replicating submicroscopic circular DNA molecules. *PNAS*, 85: 4804-4808.

Vousden, K. H., and D. J. Marshall (1984) Three different activated ras genes in mouse tumors; evidence for oncogene amplification during progression of a mouse lymphoma. *EMBOJ*, 3: 913-917.

Wahl, G. M., deSaint Vincent, B. R., and M. L. DeRose (1984) Effect of chromosomal position on amplification of transfected genes in animal cells. *Nature*, 307: 516-520.

Willingham, M. C., Richert, N. D., Cornwell, M. M., Tsuruo, T., Hamada, H., Gottesman, M., and I. Pastan (1987) Immunocytochemical localization of p170 at the plasma membrane of multidrug resistant human cells. *The Journal of Histochemistry and Cytochemistry*, 35: 1451-1456.

Woods, G., Lund, L. A., Naik, M., Ling, V., and A. Ochi (1988) Resistance of multidrug resistant lines to natural killer- like mediated cytotoxicity. *FASEB Journal*, 2: 2791-2796.

Zabel, B., Fournier, R., Lalley, P., Naylor, S., and A. Y. Sakaguchi (1984) Cellular homologs of the avian erythroblastosis virus erbA and erbB genes are syntenic in mouse but asyntenic in man. *PNAS*, 81: 4874-4878.

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