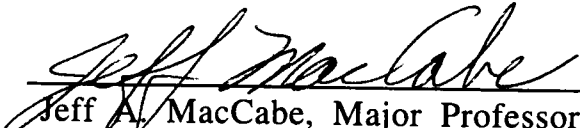
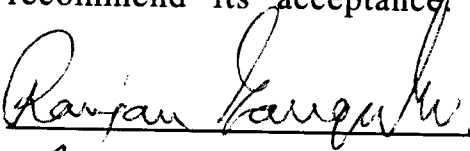
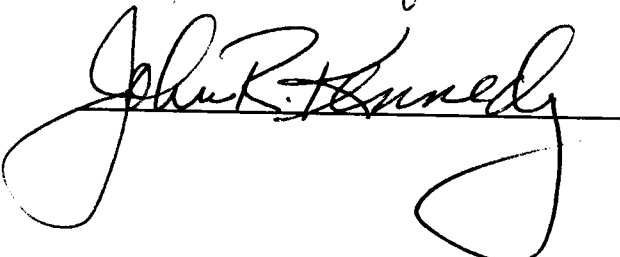


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APOPTOSIS IN THE CHICK EMBRYO WING BUD AND THE
PERMANENCE OF FGF-2 RESCUE

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Janice K. Noveroske

May, 1995

DEDICATION

This thesis is dedicated to
Dr. Robert L. Noveroske and Mrs. Elaine M. Noveroske
who are the most spectacular parents
a person could ever have.

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ABSTRACT

Two regions of programmed cell death that occur in the mesoderm of developing chick wing buds were studied *in vitro*. The permanence of FGF-2 rescue in the opaque patch (OP) and posterior necrotic zone (PNZ) was examined using the chromium release assay. While rescue in the OP was found to be permanent, rescue in the PNZ only delayed death while FGF-2 was present in the culture medium. Also, the OP and PNZ were examined for the presence of internucleosomal DNA degradation and for rescue by protein synthesis inhibition, two defining characteristics of apoptosis. Agarose gel electrophoresis showed that DNA from OP and PNZ tissue was cleaved into nucleosome size pieces and this cleavage was prevented by the presence of cycloheximide. Both the OP and PNZ showed rescue by protein synthesis inhibition with cycloheximide, as determined by the chromium release assay and examination of electron micrographs. This research suggests that *in vitro* FGF-2 rescue is permanent in the OP but is merely a delay of cell death in the PNZ, and that death in both of these regions is prevented by inhibition of protein synthesis with cycloheximide. It also shows that death in the OP and PNZ exhibits internucleosomal DNA fragmentation, biochemically characterizing this death as apoptosis.

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CHAPTER 1

INTRODUCTION

Programmed cell death or "apoptosis" (Wyllie, 1980) is an active process of cell degeneration which occurs naturally during embryo development, metamorphosis, and tumor regression. It can also be induced in certain cell types by various agents such as, quinone compounds, glucocorticoids, radiation, and serum deprivation (Trauth et al., 1989; Rodriguez-Tarduchy and Lopez-Rivas, 1989; Schulze-Osthoff et al., 1994). In development, this cell death shapes growing structures, aids in tissue and organ differentiation and is responsible for loss of vestigial structures. (Glucksmann, 1951; Hurle, 1988). Programmed cell death in adult tissues is involved in epithelia cell turnover in the skin and gut, removal of T lymphocytes in the thymus, regression in some hormone-dependent tissues (Wyllie, 1980; Ellis et al., 1991) and is also suspected in the extensive cell death seen in a variety of malignant tumors (Walker et al., 1988). Apoptosis is a physiological form of cell death associated with pronounced cellular morphology changes including shrinkage, chromatin condensation and margination, and eventually the formation of protuberances on the cell surface which subsequently separate, forming apoptotic vesicles (Arends et al., 1990; Raff, 1992). Organelle integrity is initially maintained (Garcia-Martinez et al.,

1993). Often, circumstances are such that dying cells are quickly phagocytosed by adjacent cells and therefore, the morphology of late features of apoptosis are not always observable (Garcia-Martinez and Climent, 1985). The most distinctive biochemical characteristic of apoptotic death is the fragmentation of DNA into nucleosome size pieces (Garcia-Martinez et al., 1993; Owens et al., 1991) due to the activation of intracellular endonucleases (Wyllie, 1980; Compton, 1992). Apoptosis is distinguishable from many forms of pathological cell death (necrosis) which usually occurs as a result of irreversible physical injury from external agents or environmental conditions (Garcia-Martinez et al., 1993; White, 1993). Necrosis is morphologically characterized by pronounced swelling of the nucleus and organelles and eventual rupture of the cytoplasmic and nuclear membranes leading to disintegration of the cell (Garcia-Martinez et al., 1993).

Whether apoptosis is induced by incompatible growth signals, the absence of survival signals, unbalanced growth, or insufficient growth signals, the control mechanism is a multi step, tightly regulated process with growth factors and cytokines playing an important regulatory role (White, 1993). Lysosome activation (Hurle and Hinchliffe, 1978) and the cytoskeleton (Hurle et al., 1981; Duvall et al., 1985; Walker et al., 1988) are also associated with embryonic cell death but their roles during degeneration are not yet established (Garcia-Martinez, 1993). Whatever the control, apoptosis is an extremely important regulatory event.

Failure to suppress it is associated with degenerative diseases, and failure to induce it is associated with cancer and autoimmune diseases (White, 1993). Either can result in abnormal development.

It is generally thought that apoptosis is an active process requiring specific gene expression. Specific genes involved in control of cell death in adult and embryonic systems are being identified, but the biochemical basis of apoptosis has not been determined (Schwartz et al., 1990; Ellis et al., 1991). *In vivo* neuronal cell death during invertebrate development (Fahrbach and Truman, 1988) and *in vitro* death of vertebrate neurons (Martin et al., 1988) have been shown to require active protein and RNA synthesis, a characteristic common in cells dying by apoptosis. Genes required for apoptotic execution, engulfment and degradation in nematode *C. elegans* have also been identified (Ellis et al., 1991; Yuan and Horvitz, 1992). Both activation and inhibition of cell death are required in *C. elegans* development and the mechanisms are believed to be analogous to cell death in other systems (White, 1993).

Saunders was the first to report the presence of cell death regions in the early limb buds of developing avian embryos (Saunders et al., 1962; Saunders and Fallon, 1967). Since then, four regions of concentrated mesodermal cell death have been described: the anterior necrotic zone (ANZ), posterior necrotic zone (PNZ), interdigital necrotic zone (INZ), and the opaque patch (OP)

(Hinchliffe, 1982). Death in the OP and PNZ has been shown histologically and biochemically to begin at approximately stage 23 of chick development (Hamburger and Hamilton, 1951; MacCabe et al., 1991). Later in avian development, as in many amniotes, cell death also occurs in the interdigital tissue, and is believed to be responsible for sculpting digits in the early hand and foot plate (Saunders, 1966; Saunders and Fallon, 1967; Pautou, 1975). In the case of these interdigital necrotic zones, cell death has a clear morphological role, functioning to completely eliminate the tissue between digits in species with free digits, and to only partially remove it in avian species with webbed feet (Saunders and Fallon, 1967; Kieny et al., 1976). In the other regions (the ANZ, PNZ and OP) the role of cell death is less clear (MacCabe and Brewton, 1984), but there, timing and distribution are believed to be related to establishment of skeletal elements (Hinchliffe, 1982). The PNZ is the most thoroughly studied of these regions and has been found to occur on schedule when prospective PNZ tissue is excised and placed in organ culture or transplanted to the somites of a host embryo (Fallon and Saunders, 1968). More recently, OP cell death has also been studied in organ culture (MacCabe et al., 1991). Since it has been shown that changing the culture environment of prospective dying cells can inhibit the death program (Hurle and Ganan, 1986; Brewton and MacCabe, 1988; Hurle et al., 1991; MacCabe et al., 1991), the ability to examine the OP and PNZ in culture provides

an excellent system in which to study the characteristics and controls of developmental cell death.

Peptide growth factors are involved in embryo growth, differentiation and morphogenesis, as well as regeneration, wound healing and cancer in adult tissues (Glucksmann, 1951; Hurle, 1988; Walker et al., 1988; Fiddes et al., 1991). They are also involved in cell death control as seen by growth factor promoted survival of neural crest cells (Kalcheim, 1989), and triggering of death upon growth factor removal from extending neurons (Levi-Montalcini and Bouker, 1960). In an effort to determine the regulation of programmed cell death in the OP and PNZ, growth factors have been used to create conditions that will prevent this death. The presence of basic fibroblast growth factor (FGF-2) is one such condition that will rescue cells of these regions in organ culture (MacCabe et al., 1991; MacCabe, 1993). One member of a large family of FGF proteins involved in cell proliferation, differentiation and survival, FGF-2 is believed to play an important role in early developmental events of the chick limb. During limb formation, the distal margin of the early bud consists of a thickened region of ectoderm termed the apical ectodermal ridge (AER). This ridge is necessary for limb outgrowth (Saunders, 1948; Rowe et al., 1982) and as the AER moves away from the body of the embryo, limb mesoderm cells differentiate (Stark and Searls, 1973; Summerbell, 1974). It is thought that FGF-2 may be produced by the AER as a mechanism for promoting limb

outgrowth (Munaim et al., 1988). Significant quantities of FGF-2 (Munaim 1988; Seed et al., 1988) and FGF receptors (Orr-Urtreg et al., 1991; Peters et al., 1992) have been found in the limb bud, and Fallon et al. (1994) have shown that FGF-2 can substitute for the AER if it is removed from the limb. Since FGF-2 is present in the limb bud at the time of OP and PNZ programmed cell death, it is likely that it may have a role in the control of death in these regions.

Previous work has determined that the presence of FGF-2 in OP and PNZ organ cultures rescues cells from the death program (MacCabe et al., 1991). Studies have also shown that dying cells of the OP and PNZ follow the degenerative morphological features characteristic of apoptosis (Kerr et al., 1972; Wyllie, 1980; MacCabe and Brewton, 1984; MacCabe et al., 1991). However, so far there has been no biochemical evidence showing that this death is indeed apoptosis.

The following work was done to determine whether FGF-2 mediated cell rescue in the OP and PNZ of the developing limb is permanent, in the sense that rescue continues after removal of exogenous FGF-2, or whether death is simply delayed while the growth factor is present. Also, we have examined whether these regions of death have the biochemical characteristics of apoptosis, namely rescue by protein synthesis inhibition, and internucleosomal DNA fragmentation.

CHAPTER 2

MATERIALS AND METHODS

Tissue Cultures

Fertile, White Leghorn strain chicken eggs were obtained from the Department of Animal Science, University of Tennessee. Eggs were incubated for three days at 37.5°C. Then 2 ml of albumen was removed and a window was cut in the shell and resealed with parafilm. Eggs were returned to the incubator for 8 to 14 hours until the embryos reached stage 21 (Hamburger and Hamilton, 1951). Prospective OP and PNZ regions were excised from stage 21 wing buds using fine tungsten filaments, transferred to cold phosphate-buffered saline (PBS) until cutting was completed and then cultured at 37°C with 5% CO₂ atmosphere in minimal essential medium (MEM) containing 10% calf serum (CS) (GIBCO, Grand Island, NY).

Chromium Release Assay

The Chromium release assay (Brunner et al., 1968, 1976) was used to quantitate cell death (see appendix). This assay uses ⁵¹Cr uptake by live cells and release by dying ones to determine relative differences in cell survival between experimental and control cultures (MacCabe and Brewton, 1984). Five excised OP or PNZ regions were cultured in 6.4mm microtest wells with 200μl

MEM with 10% CS and 20 μ l sodium chromate (^{51}Cr) in isotonic saline (1mCi/ml, 250-500mCi/mg ^{51}Cr , Amersham Radiochemicals, Arlington Heights IL). Cultures were incubated for six hours to allow for ^{51}Cr uptake and tissue attachment, then rinsed six times with 195 μ l MEM with 10% CS to remove extracellular chromium. After an additional 2.5, 5, 7.5, 10, or 15 hours of incubation in the presence or absence of human recombinant FGF-2 (200ng/ml) (UBI, Lake Placid, NY) or cycloheximide (9 μ g/ml) (Sigma, St Louis, MO), the medium was removed, added to 3ml of scintillation fluid (Fisher Scientific, Fair Lawn, NJ) and counted by a Beckman LS 8100 scintillation counter. The remaining tissue was lysed in 200 μ l 1% Triton-X 100 (Sigma, St Louis, MO) and the resulting lysate was added to 3ml of scintillation fluid and counted by liquid scintillation. These two counts were used to determine the total chromium uptake and the percent chromium released ($\text{cpm released} / \text{cpm released} + \text{cpm lysate} \times 100$). Experiments were repeated a minimum of five times and results were compared using an unpaired two-tailed t-test using 0.05 as the significance level.

Electron Microscopy

Intact OP or PNZ tissues were cultured in 3ml of MEM with 10% CS for six hours and then in the presence or absence of cycloheximide (9 μ g/ml) for an additional 15 hours (corresponding to the 15 hour ^{51}Cr release incubation). Tissue was fixed in 3%

glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 1 hour, and rinsed twice for 15 minutes and then overnight in 0.1M phosphate buffer. Tissue was post fixed for 1 hour in 0.1M phosphate-buffered 2% osmium tetroxide, pH 7.2. Samples were dehydrated by incubation for 5 minutes each in 50, 70, and 95% ethanol, and then three times for 5 minutes each in 100% ethanol. Tissue was then treated to two 15 minute changes in propylene oxide followed with an overnight infiltration in a 1:1 propylene oxide, epon mixture. Samples were embedded in EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA) and placed in a 60°C oven overnight. Tissues were sectioned by ultramicrotome, post-stained in saturated uranyl acetate with 50% ethanol for 30 minutes and then 0.3% lead citrate (pH 12.0) for 2 minutes. Sections were examined on a Hitachi H-600 transmission electron microscope.

DNA Electrophoresis

Intact OP and PNZ regions were either frozen at -20°C in PBS immediately after excision, or cultured in 3ml of MEM with 10% CS with or without cycloheximide (9µg/ml) for 21 hours (corresponding to the six hour uptake and 15 hour incubation of the chromium release assay). The tissue was rinsed in PBS, spun down by centrifugation and frozen in PBS at -20°C. Tissue was collected in this manner until 200 pieces were obtained. At that time, tissue was homogenized in nuclear isolation buffer (49mM

Tris, 24.9mM KCl, 5.0mM MgOAc, 3.1mM Sucrose, pH 7.6) and resuspended in 150µl DNA extraction buffer (DEB, 15.0mM NaCl, 100mM Tris, 1mM EDTA, pH 8.0). To the extraction buffer, 7.5µl of NaClO₄ and 7.5µl of 10% sodium dodecyl sulfate (SDS, Mallinckrodt, Paris, KY) were added. The mixture was allowed to equilibrate at room temperature for 5 minutes after each addition. The homogenate was then extracted twice with DEB-saturated phenol and once with chloroform (Sigma). To the final aqueous phase, 0.1 volume sodium acetate, pH 7.5, and 2 volumes of 95% ethanol were added and left at -20°C overnight to precipitate DNA. The DNA was pelleted by centrifuging at 12,000rpm for 30 minutes, rinsed with 80% ethanol, dried and dissolved in 20µl 1X TE (10mM Tris, 1mM EDTA, pH 8.0). Each 20µl DNA sample was treated with 1µl each of Ribonuclease T-1 (stock 1370units/µl, GIBCO BRL, Gaithersburg, MD) and Ribonuclease A (stock 5.1mg/ml, USB Cleaveland, OH) for 30 minutes in a 37°C water bath. After adding 4µl microliters of 6X bromophenol blue loading dye (USB), samples were run along with DNA standards (36-2645bp range, Promega Co., Madison, WI) on a 1.5% low melting/gelling temperature agarose gel (USB) for 2.5 hours at 72 volts using a Buchler Instruments power source. Both gel and TBE (2.5mM NaOH, 89.2mM Tris-borate, 89.0mM boric acid, 1.99mM EDTA) running buffer contained 0.5µg/ml ethidium bromide (USB). Each experiment was performed two times.

Amino Acid Uptake As a Measure of Protein Synthesis

Twenty OP or PNZ regions were excised and cultured in 6.4mm microtest wells with 200 μ l MEM containing 10% CS. After a six hour incubation, cultures were rinsed once with MEM containing 10% CS and replaced with medium with or without cycloheximide (9 μ g/ml) for a one hour incubation. [^{3} H]]leucine(1mCi/ml, Amersham) was added (9.9 μ Ci/ml) and cultures were incubated for 14 hours (for a total cycloheximide incubation time of 15 hours). Cultures were rinsed twice with PBS, and incubated 20 minutes in 10 μ l of 0.25% trypsin (Sigma) at 37°C. One hundred ninety microliters of MEM with 10% CS were added and tissues were disaggregated by forcing through fine pipets and vortexing. For each 200 μ l suspension, a 20 μ l sample was used to count cells on a hemocytometer and the remaining cell suspension was placed on glass fiber filters (Gelman instrument Co., Ann Arbor, MI) and dried overnight. Filters were washed 3 times in 5% trichloroacetic acid (TCA, Fisher Scientific) and once in 95% ethanol for 10 minutes each. Dry filters were submerged in 4ml of scintillation fluid and counted by liquid scintillation. Uptake experiments were performed two times for each region.

CHAPTER 3

RESULTS

FGF-2 Rescue Permanence in the OP

In order to determine the permanence of FGF-2 rescue in the OP, it was first necessary to determine the earliest time after FGF-2 administration that rescue could be detected. The chromium release assay was utilized for these experiments. After a six hour ^{51}Cr uptake, cultures were incubated for 2.5, 5, 7.5 or 10 hours in medium containing FGF-2. The percent chromium release for individual experiments are shown in Tables 1, 2, 3 and 4. A graph of the mean chromium release for each experiment is shown in Figure 1. Greater ^{51}Cr release represents greater cell death. There was no significant difference in chromium release between experimental and control cultures 2.5 hours after FGF-2 administration. On average, OP cultures incubated with FGF-2 released 16.51% of their chromium, while control cultures released an average of 18.52%. Significant cell rescue by FGF-2, however, is seen in the OP by 5 hours of incubation. Experimental cultures had an average chromium release of 19.43%, while controls averaged 26.71% release. By 7.5 hours the mean chromium release was 23.21% for FGF-2 containing cultures and 45.05% for controls. Even greater rescue is seen in the OP by

Table 1

**The Percent Chromium Release in the OP
After 2.5hrs with FGF-2***

Experiment	Control	+FGF
1	10.42	9.11
2	14.29	18.03
3	10.77	7.14
4	12.59	9.52
5	31.13	9.38
6	7.89	15.39
7	13.72	20.49
8	26.92	13.37
9	39.77	37.75
10	17.73	24.96
Mean $\pm$ SD	18.52 $\pm$ 10.52	16.51 $\pm$ 9.39

*Cell death in the opaque patch region as measured by ^{51}Cr release over a 2.5hr culture period. Control tissue was incubated in medium with 10% calf serum, and experimental tissue was incubated in medium with 10% calf serum and 200ng/ml FGF-2. The difference in the means is not significant (P value 0.6726).

Table 2

**The Percent Chromium Release in the OP
After 5hrs with FGF-2***

Experiment	Control	+FGF
1	23.00	11.25
2	30.39	19.79
3	29.96	22.61
4	20.93	23.97
5	29.25	19.54
Mean $\pm$ SD	26.71 $\pm$ 4.41	19.43 $\pm$ 4.94

*Cell death in the opaque patch region as measured by ^{51}Cr release over a 5hr culture period. Control tissue was incubated in medium with 10% calf serum, and experimental tissue was incubated in medium with 10% calf serum and 200ng/ml FGF-2. The difference in the means was significant (P value 0.0396).

Table 3

**The Percent Chromium Release in the OP
After 7.5hrs with FGF-2***

Experiment	Control	+FGF
1	40.00	31.03
2	67.77	23.82
3	37.47	22.35
4	46.71	17.24
5	33.28	21.62
Mean $\pm$ SD	45.05 $\pm$ 13.61	23.21 $\pm$ 5.01

*Cell death in the opaque patch region as measured by ^{51}Cr release over a 7.5hr culture period. Control tissue was incubated in medium with 10% calf serum, and experimental tissue was incubated in medium with 10% calf serum and 200ng/ml FGF-2. The difference in the means was significant (P value 0.0098).

Table 4

**The Percent Chromium Release in the OP
After 10hrs with FGF-2***

Experiment	Control	+FGF
1	51.11	24.07
2	66.74	44.05
3	60.87	37.64
4	54.24	31.98
5	63.13	30.80
Mean $\pm$ SD	59.22 $\pm$ 2.87	33.71 $\pm$ 3.37

*Cell death in the opaque patch region as measured by ^{51}Cr release over a 10hr culture period. Control tissue was incubated in medium with 10% calf serum, and experimental tissue was incubated in medium with 10% calf serum and 200ng/ml FGF-2. The difference in the means was significant (P value 0.0004).

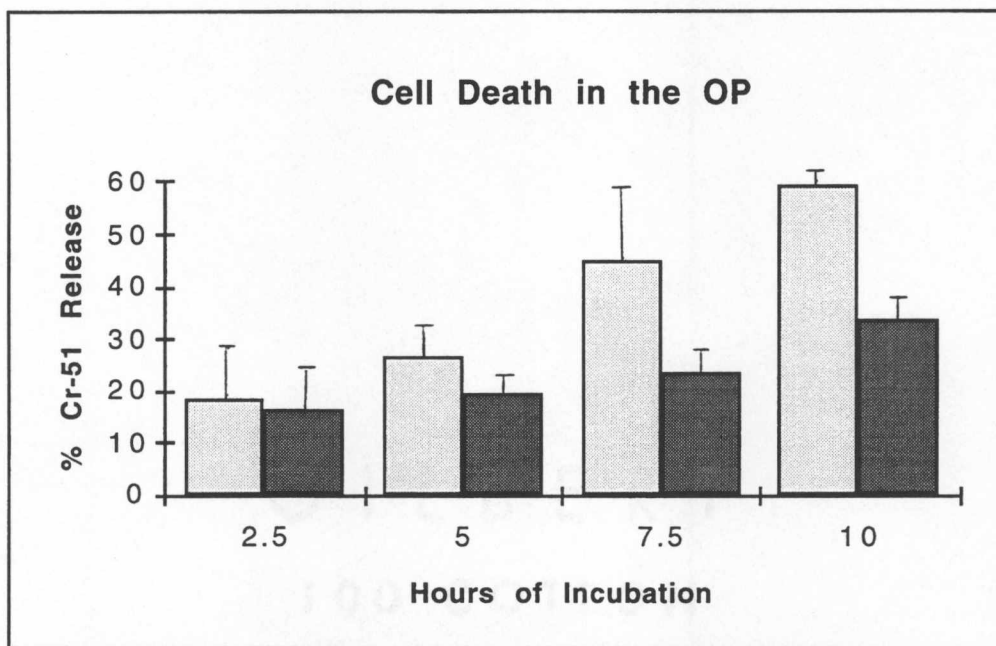


Figure 1 Cell death in the opaque patch region as measured by ⁵¹Cr release. Control tissue (shaded) was incubated in medium with 10% calf serum for the indicated amounts of time. Experimental tissue (solid) was incubated in medium with 10% calf serum and 200ng/ml FGF-2.

10 hours of FGF-2 incubation (33.71% release for experimental cultures and 59.22% release for control cultures).

Since considerable FGF-2 rescue is detectable by 7.5 hours, this time was used as the FGF-2 exposure time to determine the permanence of rescue. Therefore, to determine FGF-2 rescue permanence in the OP with chromium release, organ cultures were incubated for 7.5 hours (after ^{51}Cr uptake) in medium containing FGF-2. Just following this incubation, a scintillation count was performed on the culture medium. Tissues were then incubated for an additional 7.5 hours (for a total incubation time of 15 hours) in FGF-2 free medium after which, the amounts of ^{51}Cr in the medium and tissues were determined.

Individual release values and means for the first and second 7.5 hour incubations of OP cultures are shown in Table 5. Controls were never exposed to FGF-2, and experimental cultures received FGF-2 only for the first 7.5 hour incubation. The mean chromium release for the first 7.5 hour incubation showed that as expected, significant rescue had occurred in FGF-2 containing cultures. Experimental cultures had a mean chromium release of 23.21% while the mean for controls was 45.05%. For the second 7.5 hour incubation, release for experimental cultures which had previously contained FGF-2 had an 18.64% mean while controls had a mean of 19.41% (not a significant difference). The releases from this second incubation indicate that once FGF-2 is removed from OP cultures, the amount of cell death in experimental

Table 5

The Percent Chromium Release in the OP During and After FGF-2 Exposure*

Experiment	0-7.5hrs Control	0-7.5hrs +FGF	7.5-15hrs Control	7.5-15hrs Post-FGF
1	40.00	31.03	21.63	20.87
2	67.77	23.82	19.17	17.00
3	37.47	22.35	19.41	21.10
4	46.71	17.24	16.07	16.13
5	33.28	21.62	20.75	18.12
Mean $\pm$ SD	45.05 $\pm$ 13.61	23.21 $\pm$ 5.01	19.41 $\pm$ 2.12	18.64 $\pm$ 2.25

*Cell death in the opaque patch during and after FGF-2 exposure as measured by ^{51}Cr release. For the 0-7.5hr incubation, control tissue was in medium with 10% calf serum and experimental tissue was in medium with 10% calf serum and 200ng/ml FGF-2. For the second incubation, 7.5-15hrs, control and experimental tissue were in medium with 10% calf serum. The difference in the means for the first incubation was significant (P value 0.0098). The difference in the means for the second incubation was not significant (P value 0.5977). The release values for the first 7.5hrs are a repeat of those seen in Table 3.

cultures is equal to that of controls which never contained FGF-2. This data suggests that OP cells are permanently rescued by FGF-2.

FGF-2 Rescue Permanence in the PNZ

Previous reports showed that death in the PNZ begins approximately 3 hours after excision at stage 21 and is completed by 12 hours of incubation (MacCabe and Brewton, 1984). Therefore, the same timing that was used for the OP could be used to determine whether FGF-2 could permanently rescue the PNZ. Organ cultures of the PNZ were incubated for 7.5 hours (after Cr-51 uptake) in media containing FGF-2. After this incubation, a scintillation count was performed on the culture medium and organ cultures were incubated for an additional 7.5 hours (for a total incubation time of 15 hours) in FGF-2 free medium. Scintillation counts were then performed on the surrounding medium and cell lysate.

Individual release values and means for the first and second 7.5 hour incubations of PNZ cultures are shown in Table 6. A significant difference was seen in the means for the first 7.5 hours (Figure 2). Controls had a mean of 36.31% chromium release while experimental cultures had a 18.38% mean. For the second 7.5 hour incubation, release values for experimental cultures which had previously contained FGF-2 had a 26.29% mean while controls had a mean of 15.98%. Therefore, after FGF-2 is removed from PNZ cultures, chromium release is higher than in controls

Table 6

The Percent Chromium Release in the PNZ During and After FGF-2 Exposure*

Experiment	0-7.5hrs Control	0-7.5hrs +FGF	7.5-15hrs Control	7.5-15hrs Post-FGF
1	47.02	19.87	15.52	27.02
2	41.03	20.62	14.49	30.77
3	30.81	17.47	18.64	28.28
4	35.17	26.15	8.35	22.83
5	33.84	18.14	19.69	28.58
6	35.34	14.93	15.32	20.18
7	30.95	11.50	19.87	26.37
Mean $\pm$ SD	36.31 $\pm$ 5.84	18.38 $\pm$ 4.61	15.98 $\pm$ 4.02	26.29 $\pm$ 3.63

*Cell death in the posterior necrotic zone during and after FGF-2 exposure as measured by ^{51}Cr release. For the 0-7.5hr incubation, control tissue was in medium with 10% calf serum and experimental tissue was in medium with 10% calf serum and 200ng/ml FGF-2. For the second incubation, 7.5-15hrs, control and experimental tissue were in medium with 10% calf serum. The difference in the means for the first incubation was significant (P value 0.0001) as well as in the second incubation (P value 0.0003).

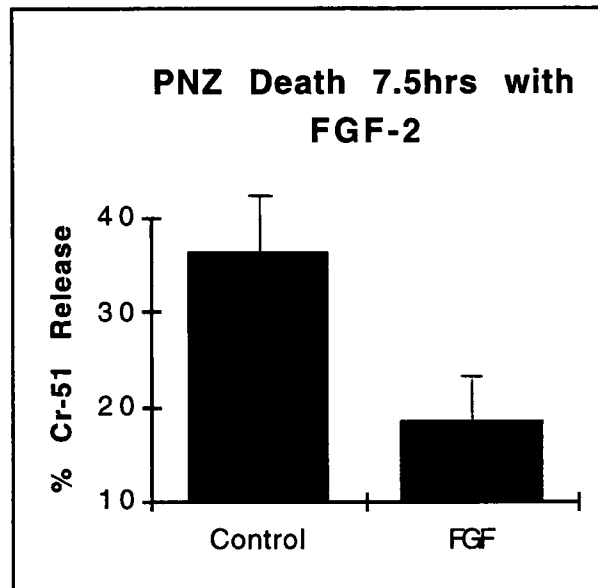


Figure 2 - Cell death in the posterior necrotic zone as measured by ^{51}Cr release over a 7.5hr culture period. Control was incubated in medium with 10% calf serum. Experimental tissue was incubated in medium with 10% calf serum and 200ng/ml FGF-2.

which never contained FGF-2. These results indicate that more cell death is occurring in cultures that were exposed to FGF-2 than those that were never exposed to the growth factor, thus suggesting FGF-2 delays cell death in the PNZ rather than rescuing permanently. Once FGF-2 is removed a spurt of compensatory cell death occurs.

Comparison of OP and PNZ Rescue Permanence

The percent chromium release in the OP and PNZ for the 7.5 hours after FGF-2 exposure are shown in Figure 3 (values were taken from Tables 5 and 6). This graph illustrates the difference in the post-FGF-2 behavior of OP and PNZ cultures. In the OP, ^{51}Cr release for control cultures is equal that of post-FGF-2 cultures, while in the PNZ release is higher in post-FGF-2 cultures than in controls. These results suggest that OP cells that were rescued prior to this incubation remained rescued in contrast to the PNZ which exhibits a spurt of compensatory cell death after FGF-2 removal.

The total chromium release values over the entire 15 hours of the FGF-2 permanency experiments for the OP and PNZ are shown in Figure 4 (values are the sum of the means from both 7.5 hour incubations in Tables 5 and 6). This graph shows that for both the OP (P value 0.0088) and PNZ (P value 0.0269), there was significant rescue even though FGF-2 was only present for the first half of the experiment. This was expected in the OP since no

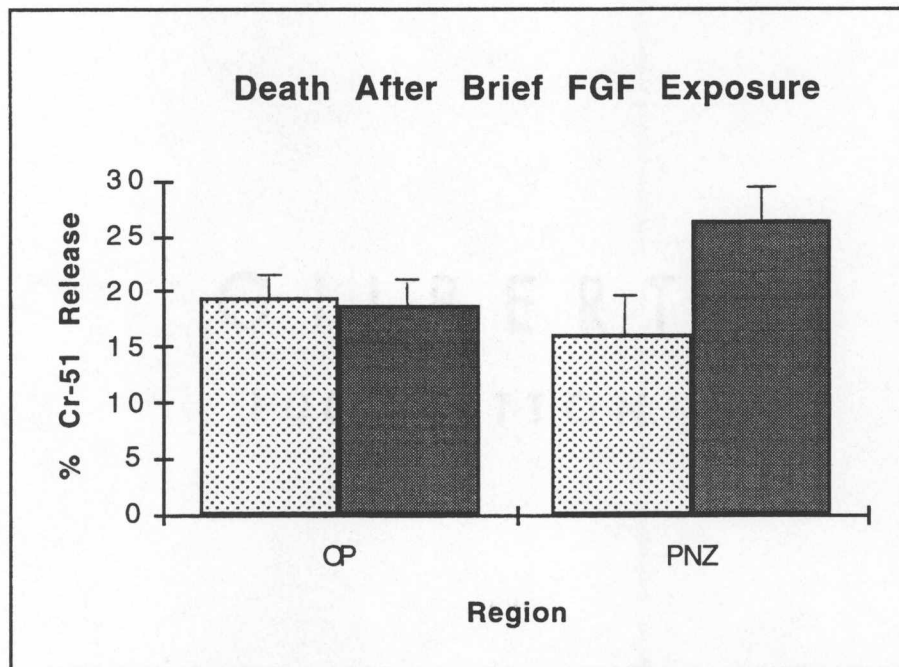


Figure 3 - Cell death in the opaque patch and posterior necrotic zone after a brief (7.5hr) exposure to FGF-2 as measured by ⁵¹Cr release. Prior to this incubation, control cultures were incubated in medium with 10% calf serum and experimental cultures in medium with 10% calf serum and 200ng/ml FGF-2. For this incubation, both control (shaded) and experimental cultures (solid) were in medium with 10% calf serum for 7.5hrs.

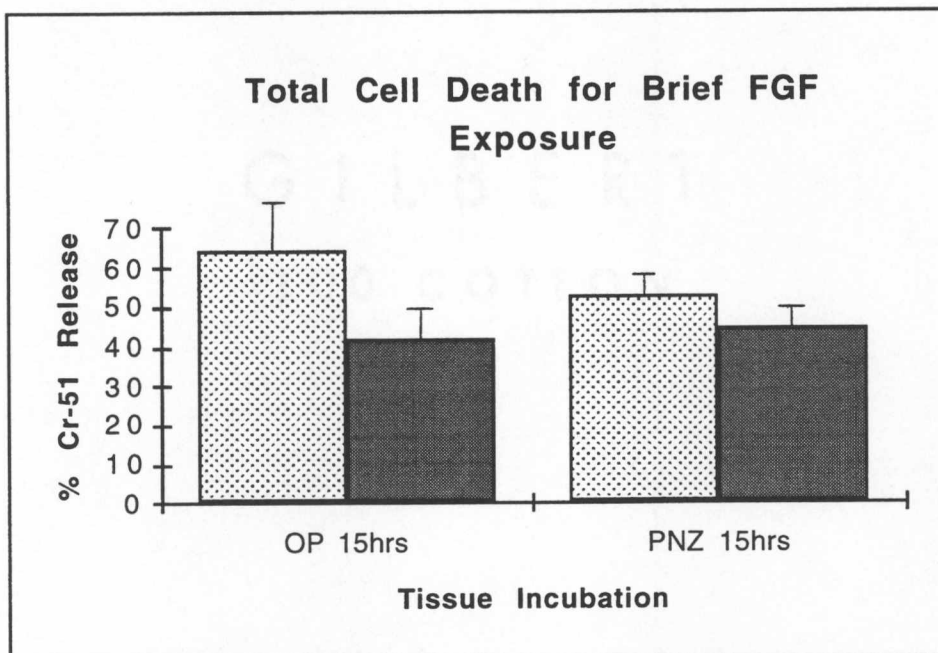


Figure 4 - Total Cell death in the opaque patch and posterior necrotic zone as measured by ^{51}Cr release over two 7.5hr culture periods. Control tissue was incubated in medium with 10% calf serum for the entire 15hrs. Experimental tissue was incubated in medium with 10% calf serum and 200ng/ml FGF-2 for the first 7.5hrs and in medium with 10% calf serum for the second 7.5hrs.

compensatory cell death occurred after FGF-2 removal. However, in the PNZ, which did exhibit compensatory cell death after FGF-2 removal, there was still significant rescue overall. Therefore, the "extra" cell death after FGF-2 removal doesn't completely compensate for the rescue during the first 7.5 hours. It is apparent in this graph that there is a smaller difference in the chromium release between experimental and control cultures in the PNZ than in the OP. We attribute this to rescued OP cells remaining in a rescued state after FGF-2 removal and rescued PNZ cells dying after FGF-2 removal.

Protein Synthesis Inhibition in the OP and PNZ

To determine the effect of inhibition of protein synthesis on OP and PNZ cell death, the chromium release assay was utilized in conjunction with cycloheximide. After a six hour ^{51}Cr uptake, cultures were rinsed and incubated for an additional 15 hours in medium containing cycloheximide. In addition, cycloheximide treated OP and PNZ organ cultures were examined by transmission electron microscopy for morphological signs of cell death and an [^3H]leucine uptake study was performed to confirm that cycloheximide was preventing protein synthesis.

The mean release for cycloheximide treated cultures was 36.78% and the mean for controls was 64.10% (Table 7). The difference in these means indicates that cycloheximide is significantly inhibiting cell death (Figure 5). Likewise, results for

Table 7

**The Percent Chromium Release in the OP After 15hrs
with Cycloheximide***

Experiment	Control	+Cycloheximide
1	64.19	27.03
2	68.98	44.15
3	63.77	41.96
4	65.93	45.91
5	53.79	39.23
6	67.98	22.40
Mean $\pm$ SD	64.10 $\pm$ 5.45	36.78 $\pm$ 9.72

*Cell death in the opaque patch region as measured by ^{51}Cr release over a 15hr culture period. Control tissue was incubated in medium with 10% calf serum, and experimental tissue was incubated in medium with 10% calf serum and 9 $\mu\text{g}/\text{ml}$ cycloheximide. The difference in the means is significant (P value 0.0001).

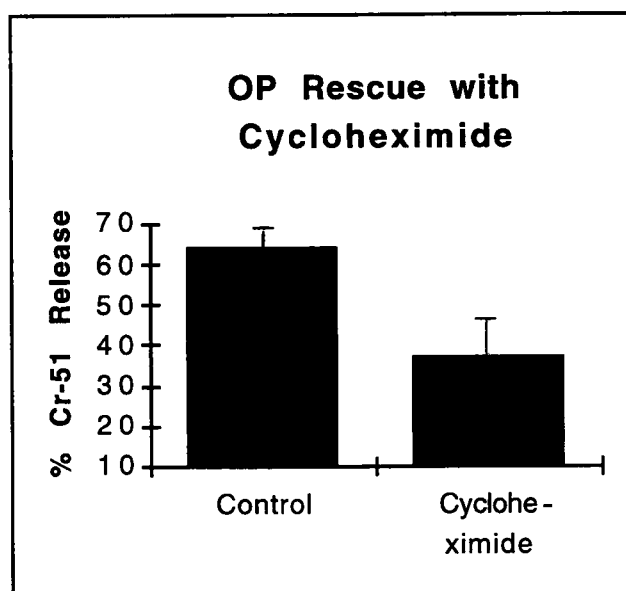


Figure 5 - Cell death in the opaque patch region as measured by ^{51}Cr release over a 15hr culture period. Control tissue was incubated in medium with 10% calf serum. Experimental tissue was incubated in medium with 10% calf serum and $9\mu\text{g/ml}$ cycloheximide.

treated PNZ cultures show significant cell rescue by cycloheximide (Table 8 and Figure 6). Chromium release for cycloheximide treated PNZ cultures averaged 31.00% and controls averaged 59.06%. These chromium release experiments show, therefore, that both the OP and PNZ exhibit rescue with protein synthesis inhibition, a landmark characteristic of apoptotic cell death.

Electron microscopy of untreated and cycloheximide treated OP (Figures 7 and 8) and PNZ cultures (Figures 9 and 10) show that in both regions, control tissues contain the abundant cell debris and macrophages with phagocytosed material associated with cell death in these regions. Cycloheximide treated tissues show the same characteristics as those rescued by FGF-2 in previous studies (MacCabe et al., 1991). Membranes are intact, nuclei and mitochondria appear normal, and there is little or no sign of cellular debris. These micrographs show that inhibiting protein synthesis in the OP and PNZ with cycloheximide prevents the morphological characteristics of cell death.

Cycloheximide was effective at preventing protein synthesis in OP and PNZ cultures (Table 9). Amino acid uptake experiments determined the cpm/cell in control tissue and used these values as 100% incorporation to determine the percent of [^3H]leucine incorporation in cycloheximide treated cultures. In two separate experiments, [^3H]leucine incorporation in the OP was 11.73 and 5.38% that of control tissue. Likewise, protein synthesis in the PNZ was inhibited by cycloheximide. Experiments for this region

Table 8

**The Percent Chromium Release in the PNZ After 15hrs
with Cycloheximide***

Experiment	Control	+Cycloheximide
1	63.86	33.21
2	52.34	28.67
3	56.34	34.40
4	62.14	21.66
5	44.42	37.75
6	65.22	37.01
7	69.11	24.30
Mean $\pm$ SD	59.06 $\pm$ 8.55	31.00 $\pm$ 6.27

*Cell death in the posterior necrotic zone as measured by ^{51}Cr release over a 15hr culture period. Control tissue was incubated in medium with 10% calf serum, and experimental tissue was incubated in medium with 10% calf serum and 9 $\mu\text{g}/\text{ml}$ cycloheximide. The difference in the means is significant (P value 0.0001).

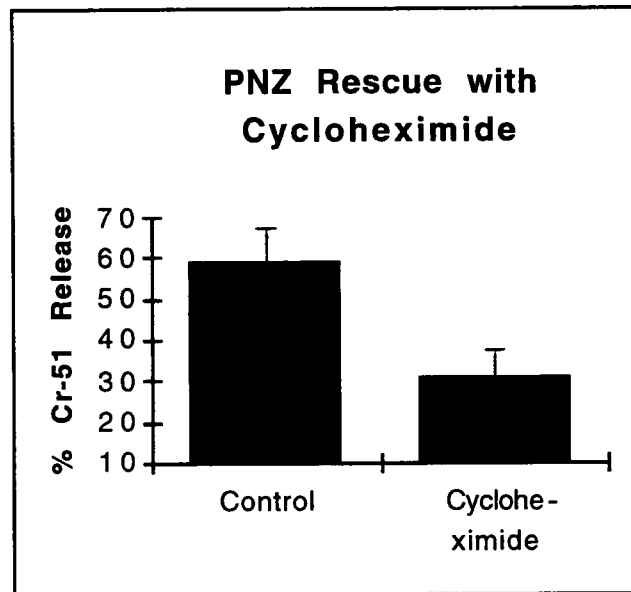


Figure 6 - Cell death in the posterior necrotic zone as measured by ^{51}Cr release over a 15hr culture period. Control tissue was incubated in medium with 10% calf serum. Experimental tissue was incubated in medium with 10% calf serum and $9\mu\text{g/ml}$ cycloheximide.

Figure 7 - Cell Death in the OP. Histological sections of normal cell death in OP tissue after a 15hr culture period in medium with 10% calf serum. Considerable cell debris (arrows) is present. X= 4,500.

Figure 8 - Cell Rescue in the OP. Histological sections of OP tissue after a 15hr culture period in medium with 10% calf serum and cycloheximide (9 μ g/ml). Cells are intact with little visible debris. X= 4,500.

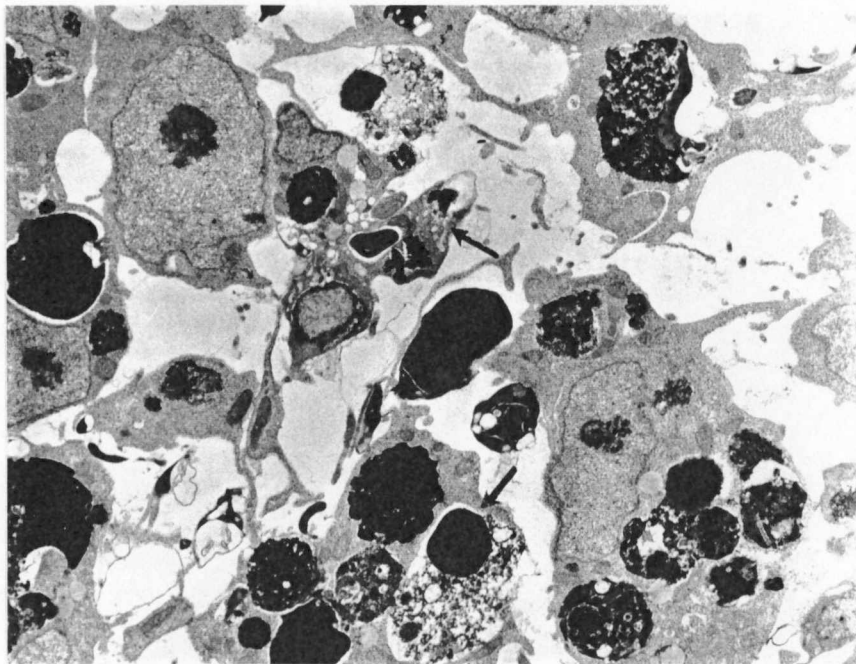


Figure 7

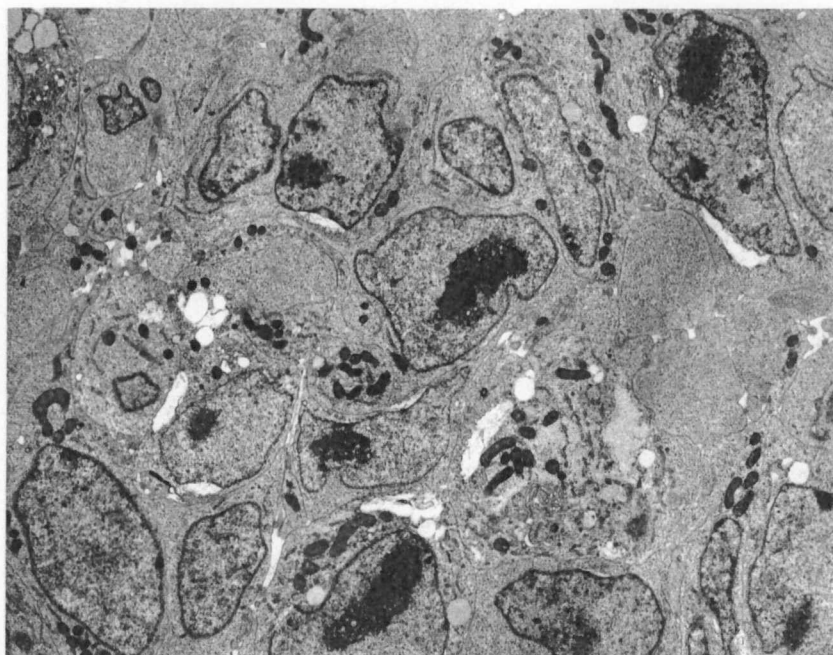


Figure 8

Figure 9 - Cell Death in the PNZ. Histological sections of normal cell death in PNZ tissue after a 15hr culture period in medium with 10% calf serum. Considerable cell debris (arrows) is present. X= 4,500.

Figure 10 - Cell Rescue in the PNZ. Histological sections of PNZ tissue after a 15hr culture period in medium with 10% calf serum and cycloheximide (9 μ g/ml). Cells are intact with little visible debris. X= 4,500.

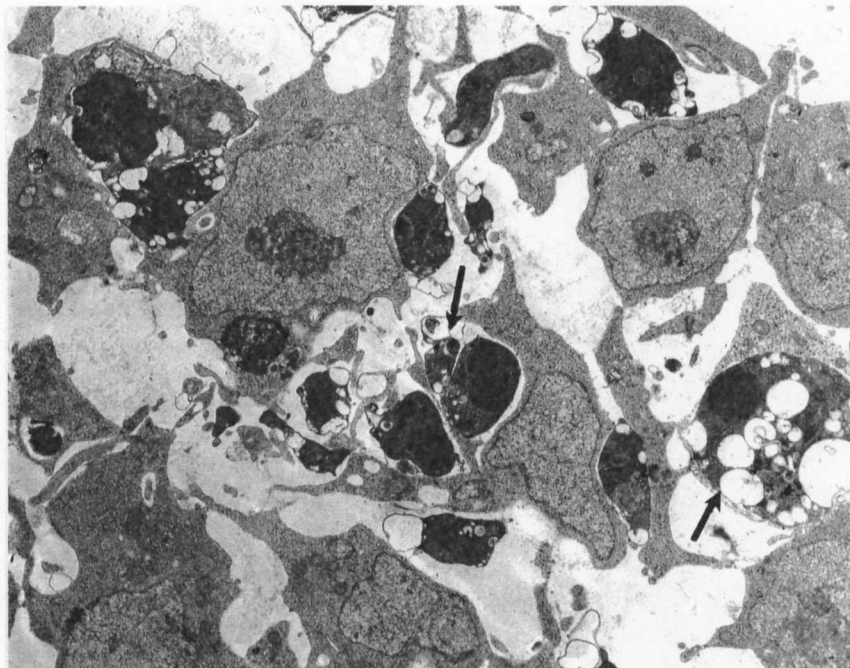


Figure 9

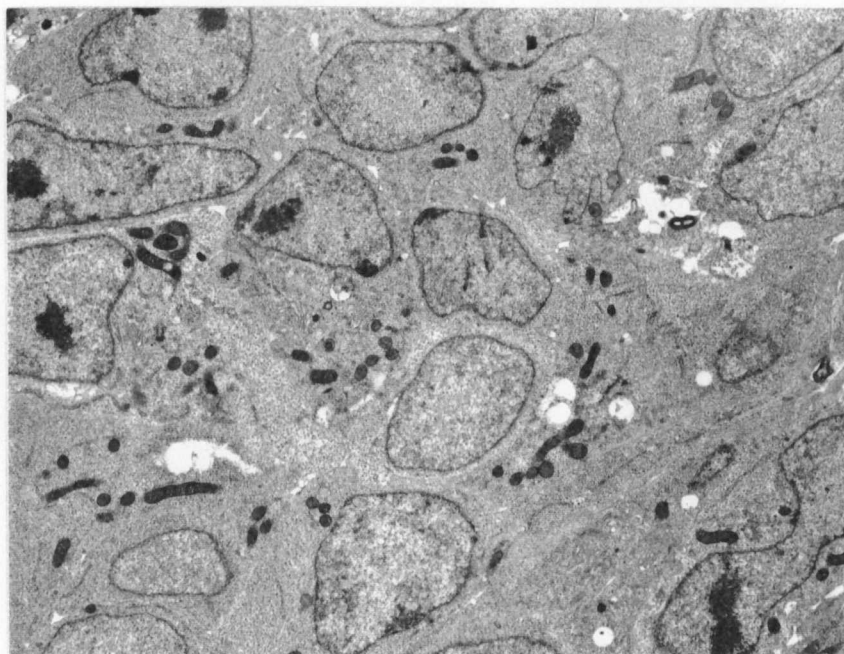


Figure 10

Table 9

Inhibition Of Protein Synthesis By Cycloheximide*

<u>Percent [³H]Leucine Incorporation</u>		
Experiment	OP	PNZ
1	11.73	5.42
2	5.38	4.35

*Cultures were incubated in medium with 10% CS and 9.9 μ Ci/ml [³H]leucine, with or without 9 μ g/ml of cycloheximide. Cells were disaggregated and counted and the cpm/cell determined by liquid scintillation. The percent of [³H]leucine incorporation in cycloheximide treated cultures was determined using controls as 100%.

showed that [^3H]leucine incorporation in treated cultures was 5.42 and 4.35% that of controls.

Internucleosomal DNA Degradation

To determine if cell death *in vitro* shows internucleosomal DNA degradation, DNA from OP and PNZ organ cultures incubated *in vitro* for 21 hours, was isolated by phenol-chloroform extraction and separated by agarose gel electrophoresis. Similar experiments, incubating OP and PNZ tissue with or without cycloheximide, were used to determine if inhibition of protein synthesis prevents nucleosome size degradation.

The result of the DNA fragmentation study in the OP is shown in Figure 11. Lane 1 contains DNA standards, lane 2 shows that DNA from the OP is cleaved up into nucleosome size DNA bands of 152, 318, 498, 636, and 937bp. Lane 3 is control DNA isolated from stage 21 OP tissue without any *in vitro* incubation. Figure 12 shows that PNZ DNA is cleaved up into nucleosome size pieces. Standards are in lane 1, lane 2 is stage 21 PNZ control DNA isolated from tissue without incubation, and lane 3 shows PNZ DNA with bands at 130, 254, 318, 488, and 623bp. These experiments show, that cell death occurring in the OP and PNZ *in vitro* does show apoptosis characteristic, internucleosomal DNA degradation.

Cycloheximide was shown to prevent internucleosomal cleavage in PNZ and OP cultures in Figures 12 and 13. Figure 13,

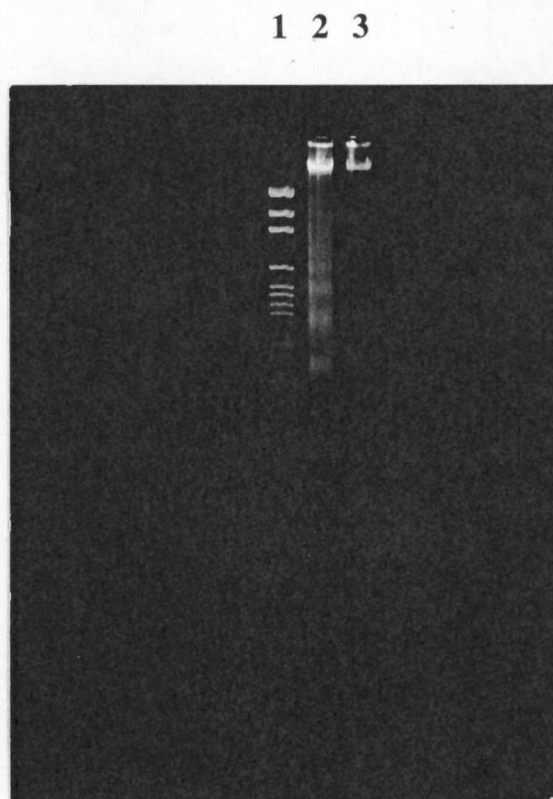


Figure 11- DNA electrophoresis of OP tissue. DNA standards are in lane 1 (36-2645bp range). Lane 2 contains DNA from tissue incubated for 15hrs in medium with 10% calf serum. Lane 3 is control DNA from tissue that did not receive any incubation.

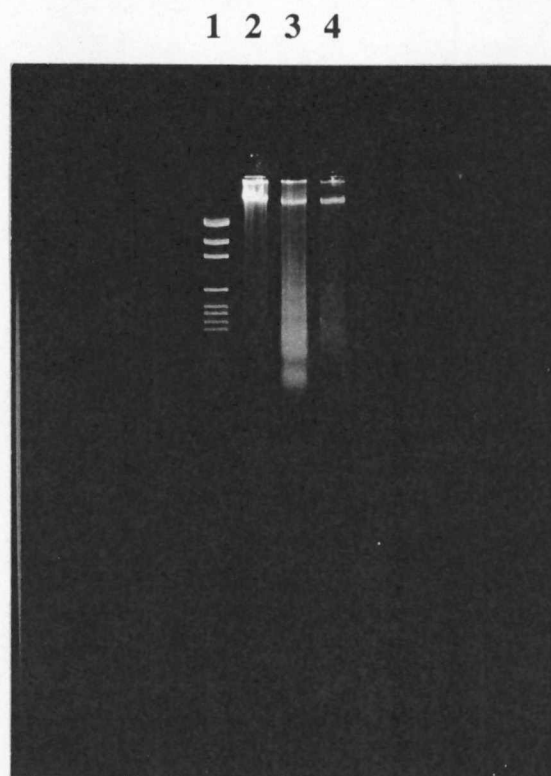


Figure 12- DNA electrophoresis of PNZ tissue. DNA standards are in lane 1 (36-2645bp range). Lane 2 is control DNA from tissue that did not receive any incubation. Lane 3 contains DNA from tissue incubated for 15hrs in medium with 10% calf serum. Lane 4 is DNA from tissue incubated in medium with 10% calf serum and 9 μ g/ml cycloheximide.

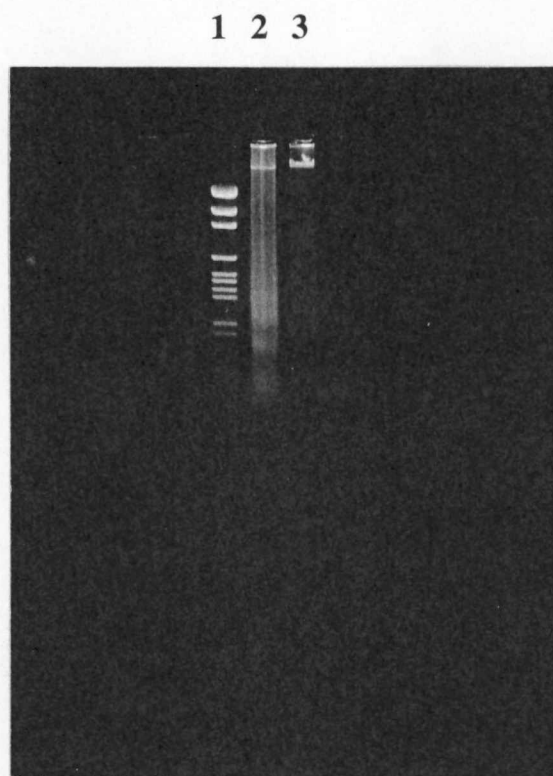


Figure 13- DNA electrophoresis OP tissue treated with cycloheximide. DNA standards are in lane 1 (36-2645bp range). Lane 2 is control DNA from tissue incubated for 15hrs in medium with 10% calf serum. Lane 3 is DNA from tissue incubated in medium with 10% calf serum and 9 μ g/ml cycloheximide.

lane 3 shows the cycloheximide treated OP DNA running as a single high molecular weight band with no evidence of the DNA ladder pattern seen in DNA isolated from untreated OP (lane 2). DNA Standards are in lane 1. Likewise, figure 12, lane 4 shows the single, high molecular weight band of DNA isolated from PNZ tissue which had been incubated with cycloheximide. DNA from untreated PNZ cultures is shown in lane 3.

CHAPTER 4

DISCUSSION

FGF-2 Permanence

Among their many other roles, growth factors have been found to play an important role in cell survival. Neuronal cells undergo death if they fail to contact target tissues which contain the appropriate growth factors (Oppenheim, 1991), and hemopoietic precursor cells undergo death upon removal of colony stimulating factors (Williams et al., 1990). Actinomycin D induced death in the human breast cancer cell line MDA-231 can be prevented with epidermal growth factor or insulin-like growth factor-1 (Geier et al., 1993), while interleukin-2 prevents glucocorticoid induced cell death of T lymphocytes (Nieto and Loopez-Rivas, 1989) and anti-CD3-induced death of human medullary thymocytes (Nieto et al., 1990). FGF-2 can prevent death of density-arrested quiescent mouse fibroblasts (Tamm et al., 1991).

It is well established that the chick limb bud apical ectodermal ridge (AER) is responsible for limb elongation (Saunders, 1948; Summerbell, 1974; Rowe et al., 1982). Based on its distribution within the limb and its mitogenic properties, FGF-2 is considered the prime candidate for the control of limb outgrowth (Savage et al., 1993). However, there does not appear

to be an anterior-posterior gradient of FGF-2 to explain the anterior-posterior asymmetry of limb outgrowth. An anterior-posterior gradient of division response specific for FGF-2 has been described in stage 22 limb buds (Ano and Ide, 1988). Exogenous FGF-2 stimulated division occurs in anterior mesenchyme, no significant division is seen in the posterior region, and an intermediate response occurs in the central limb. A response gradient is also seen when FGF-2 is virally expressed in the limb (Riley et al., 1993). In the anterior region of stage 20-22 chick limb buds, it results in duplication of anterior skeletal elements while viral FGF-2 expression in the posterior end had no effect. Intermediate regions had intermediate level responses. Research indicates, therefore, that an anterior-posterior gradient of FGF-2 response occurs.

Previous reports using the chromium release assay and examination of histological sections have established that programmed cell death in OP and PNZ organ cultures can be prevented by incubation with FGF-2 (MacCabe et al., 1991). We wanted to determine if FGF-2 rescue was permanent or if rescue is reversed upon growth factor removal from the culture environment. To this end, cells were given a brief exposure to FGF-2, then cell death was monitored after FGF-2 withdrawal. At least three outcomes were possible. Cell death after growth factor removal could decrease compared to controls (cultures not exposed to FGF-2), suggesting continued action of FGF-2 in the

tissue after its removal from the medium. This is possible since heparin sulfate proteoglycans (HSPGs) are known to sequester FGF-2 (Bernfield and Hooper, 1991). Alternatively, after FGF-2 removal cell death could be the same as controls, suggesting rescue is permanent, but long term effects due to FGF-2 retention do not occur. Finally, cell death could increase over controls suggesting compensatory cell death occurring after FGF-2 withdrawal. This would suggest cell death is not permanent, but is only delayed by the brief exposure to FGF-2. My results suggest two of these three possible scenarios occur, depending on the source of the cells. Cells from the OP displayed cell death levels equal to controls after FGF-2 removal. These results are consistent with OP cells staying rescued but with no additional cells being rescued after FGF-2 removal. The data indicate, therefore, that FGF-2 is either not stored by OP tissue *in vitro* or is stored but unavailable to cells, and also that continuous exposure to FGF-2 is not required for OP rescue.

For the PNZ, results from the post-FGF-2 incubation yielded very different results from the OP. Once FGF-2 is removed from PNZ organ cultures, a significant increase in cell death is seen when compared to controls. The data indicate that cells initially rescued by FGF-2 die once the growth factor is removed. Also, since no additional rescue was seen in experimental cultures over controls, FGF-2 does not appear to be stored or available in PNZ tissue once it is removed from the medium.

Even though the data suggest that PNZ cells, initially rescued, die upon growth factor removal, the difference in chromium release between experimental and control cultures was not enough to account for the death of all initially rescued cells. Extending experimental incubation times to determine if all would eventually die was not feasible with this assay. It should be noted as well that these experiments do not prove that the cells dying during the post-FGF-2 incubation are those which were initially rescued. An alternative explanation would require that mesodermal cells not programmed to die become dependent on FGF-2 during the initial incubation and then proceeded to die upon its removal in the second incubation. Though this is possible and can not be ruled out by these experiments, there was no indication of it occurring in cells from the OP region.

Based on the results of a brief exposure to FGF-2, it does not appear that the OP or PNZ *in vitro* have the ability to store FGF-2. This growth factor has a strong affinity for heparin and heparinlike molecules (Lobb et al., 1986; Burgess and Maciag 1989) and in many cell types, heparin sulfate proteoglycans (HSPGs) are found in the extracellular matrix and on cell surfaces where they act as low affinity receptors for FGF-2 (Bernfield and Hooper, 1991). When FGF-2 is added to cultured cells, they may bind high-affinity receptors (Moscatelli, 1987) as well as HSPGs which may function as a slow release reservoir, facilitating long-term responses to brief exposures as well as serving to protect

FGF-2 from protease digestion (Moscatelli et al., 1991). Studies on bovine capillary endothelial cells (BCE) have shown that HSPGs may be crucial to FGF-2 activity (Moscatelli et al., 1991). When these cells were briefly exposed to FGF-2, they responded for an extended amount of time as if they were being continuously exposed to the growth factor, suggesting that FGF-2 remained in culture after a change in medium. Further evidence showed that BCE cells exposed to heparinase to remove HSPG storage sites, did not exhibit a prolonged FGF-2 response after growth factor removal. Likewise, the ability of Swiss 3T3 cell to proliferate, or MM14 myoblasts to differentiate after exposure to FGF-2 was significantly decreased when these cultures were treated to remove functional HSPGs (Rapraeger et al., 1991).

Based on the inability of the OP and PNZ to maintain a FGF-2 response once growth factor is removed, my data suggest that these regions may lack cell matrix HSPGs which store FGF-2. This idea is further backed by preliminary results which show that treating OP organ cultures with heparinase has no effect on FGF-2 cell rescue, indicating that HSPGs, if present, do not function in FGF-2 storage (MacCabe, unpublished). Whether the lack of ability to store FGF-2 could be a part of the *in vivo* control of cell death in these regions remains to be determined. *In vivo* expression of syndecan, an integral membrane HSPG, in mouse development has been found which suggests its involvement in anterior-posterior patterning (Solursh et al., 1990). Gastrulating

mouse embryos show an asymmetric distribution of syndecan (weakest expression in the dorsal and anterior mesodermal structures and strongest in the posterior and lateral structures). In the mouse limb, detection by monoclonal antibodies has located syndecan throughout the bud at its earliest stages in both mesoderm and ectoderm. Distribution then decreases in the AER and central core and by the thirteenth day of limb development, syndecan is lost in areas which are determined to undergo chondrogenesis and myogenesis. As yet, no protein pattern has been found in the chick limb that corresponds to the FGF-2 response (Riley et al., 1993). The fact that FGF-2 is apparently not stored by either region but they still have different responses after growth factor removal suggests that FGF-2 storage is not responsible for differences in FGF-2 rescue of the OP and PNZ. Significant differences have also been noted between the OP and PNZ in a previous report where OP cells were found to be rescued when cultured in monolayer, whereas PNZ cells would die in monolayer (MacCabe et al., 1991).

Though little work has been done on growth factor rescue permanency in other systems, the permanency of FGF-2s division effects has been reported on both primary cultures of newborn rat astroglial cells (Korr et al., 1992) and adult mouse astroglial cells *in vivo* (Korr, 1986). In both systems FGF-2 caused a re-entry into the cell cycle but with newborn rat cells re-entry was for a single cell division, whereas in adult rat cells re-entry was

permanent. In both instances, exposure to FGF-2 was continuous. It may be that in the chick limb, a differential response to FGF-2 removal may reflect a difference in the way these areas of cell death are controlled.

Protein Synthesis Inhibition

Apoptosis is seen as an active, self induced process, in which transcription and translation are important components (Martin and Cotter, 1994). Numerous studies on different cell types under various conditions have shown that apoptotic death can be prevented by inhibiting protein and RNA synthesis. Epithelial cell death in the palate of fetal rats (Pratt and Greene, 1976), and induced death of immune cells in adult rats by glucocorticoids (Compton and Cidlowski, 1987) have been shown to require macromolecule synthesis. Death of chick neurons (Oppenheim et al., 1990) and epidermal growth factor deprived serum-free mouse embryo cells (Rawson et al., 1991) have both been shown to be rescued by protein synthesis inhibition with cycloheximide. Cycloheximide, a compound which prevents protein synthesis by blocking ribosomal peptidyl transferase activity (Baliga et al., 1969; Munro et al., 1968), has been used on various cell types to determine if cell death requires active protein synthesis. The purpose of the experiments reported here is to determine if programmed cell death in OP and PNZ organ cultures also requires protein synthesis.

Chromium release values showed that after 15 hours of incubation with cycloheximide, significant inhibition of cell death was observed in both the OP and the PNZ. Treated OP organ cultures showed a 27.32% decrease in chromium release relative to controls and treated PNZ cultures showed a 28.06% decrease. Amino acid uptake studies confirmed that cycloheximide was preventing protein synthesis. Protein synthesis inhibition averaged 91% in the OP, and 95% in the PNZ cultures.

To confirm that lower chromium release in cycloheximide treated cultures is due to rescued cells, thin sections of OP and PNZ cultures were examined by electron microscopy. These micrographs confirm that cycloheximide prevented cell death in these regions. Treated OP and PNZ sections have the morphological characteristics of intact mesodermal tissue while control cultures show the abundant cell debris and macrophages with engulfed material associated with cell death in mesodermal limb tissue (MacCabe et al., 1991). Collectively, these experiments show that inhibiting protein synthesis in OP and PNZ organ cultures, prevents programmed cell death in these regions. Chromium release results for the OP show that the percent difference in release, attributed to cycloheximide rescue, is nearly identical to that observed when OP cultures are incubated with FGF-2 for the same amount of time (MacCabe et al., 1991). This indicates that these two methods of cell rescue are equally and possibly completely effective at preventing cell death in this

region. In the PNZ, however, rescue by cycloheximide is greater than that previously reported by FGF-2 (MacCabe et al., 1991).

Though current research is looking more and more to the cytoplasm for factors playing a role in programmed cell death (Hopkin, 1995), the idea that apoptosis requires activation of death genes is still well accepted. In fact in some systems, identification of proteins suspected to have a role in the death program have begun to be identified. Radiation induced death of rat thymocytes, which can be prevented by RNA and protein synthesis inhibition (Cohen and Duke, 1984; Sellins and Cohen, 1987), has been used to find two, death specific mRNAs, RP-8 and RP-2. The RP-8 sequence is a good candidate for a DNA regulatory protein, and RP-2 sequence suggests it is an integral membrane protein. Transcripts of both of these proteins increased while other maintenance proteins were decreasing as the cells began to undergo death. In the nematode *C. elegans*, genes involved in the different stages of programmed cell death, onset (Yuan and Horvitz, 1992), engulfment and degradation (Ellis et al., 1991), have also been identified.

Some reports have shown that protein synthesis inhibition can cause apoptosis. When human leukemic HL-60 cells are exposed to cycloheximide, they undergo death with apoptotic characteristics including chromatin condensation and DNA fragmentation (Martin et al., 1990). This suggests that programmed cell death in some cell types may be controlled by

continuous translation of genes which suppress apoptosis and when their synthesis is prevented, cell death is triggered.

Internucleosomal DNA Degradation

Morphological evidence of death in the OP and PNZ suggests it is apoptosis (Kerr et al., 1972; Wyllie et al., 1980; MacCabe and Brewton, 1984; MacCabe et al., 1991). To further characterize these regions as such, we wanted to determine whether they exhibit internucleosomal DNA fragmentation, the most distinctive biochemical characteristic of apoptotic cell death. The pathway leading to DNA fragmentation is unknown but current ideas suggest that it may include activation of new nucleases, or reorganization of chromatin which makes DNA available to digestion by preexisting endonucleases (Arends et al., 1990; Tomei et al., 1993; White, 1993). Little work has been done on DNA fragmentation during chick development due to the difficulty in recovering significant quantities of tissue from regions of programmed cell death. These studies revealed that *in vitro* cell death in the OP and PNZ does exhibit internucleosomal DNA fragmentation. This is consistent with the activation of endonucleases within a cell, actively cleaving DNA between nucleotides. This DNA cleavage is prevented by the addition of the protein synthesis inhibitor cycloheximide in both the OP and PNZ.

DNA ladder patterns associated with programmed cell death have been detected in the INZs in chick and mouse limb buds

(Garcia-Martinez et al., 1993; Tone et al., 1994) and, chick retina (Ilschner and Waring, 1992). Since internucleosomal DNA fragmentation is considered a hallmark characteristic of apoptosis, much effort has been placed on determining whether it plays a causative role in cell death. As of yet, the role of DNA cleavage and the identity of the endonuclease which causes it have not been determined. Though it may be system specific, current research indicates that DNA degradation is not the initiator of cell death. In the INZ of the embryonic chick leg bud, DNA fragmentation occurs at the same time as the morphological features of apoptosis and nonspecified DNA fragmentation from phagocytic digestion of dying cells (Garcia-Martinez et al., 1993), indicating that changes which activate phagocytosis of dying cells are occurring before DNA fragmentation by endonuclease. In some cases, apoptotic death can occur without DNA fragmentation (Cohen et al., 1992; Falcieri et al., 1993; Oberhammer et al., 1993; Schwartz et al., 1993), as in the case of L929-APO-1 cells, a transfected murine fibrosarcoma cell line, which when pre-incubated with the endonuclease inhibitor ATA, undergo otherwise characteristic APO-1-mediated death (Schulze-Osthoff et al., 1994). In fact, in the case of L929-APO-1 cells, apoptotic death showing characteristic cell shrinkage and membrane blebbing can be initiated in cells which have been enucleated (Schulze-Osthoff et al., 1994).

In conclusion, the research presented here shows that FGF-2 rescue in the OP is permanent while in the PNZ, FGF-2 merely delays cell death. Also, cell death in these regions exhibits internucleosomal DNA fragmentation and this death is prevented by protein synthesis inhibition.

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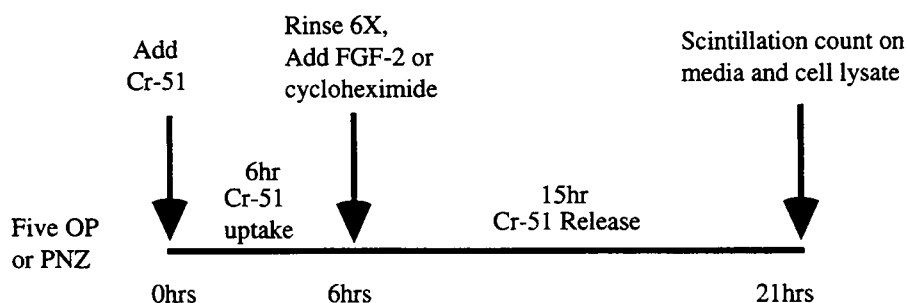
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Appendix

Chromium Release Assay

Chromium-51 is a radioactive element taken up by live cells and released by dead and dying ones. It can be used to determine relative differences in cell survival between treated and untreated cultures. This assay was used to obtain the data on OP and PNZ cell rescue with FGF-2 and cycloheximide. For these experiments, five OP or PNZ regions were incubated for six hours in media containing ^{51}Cr . Cultures were rinsed free of extra-cellular chromium and incubated in the presence or absence of FGF-2 or cycloheximide. During this incubation, dying cells release ^{51}Cr into the surrounding media while live cells retain it. At the end of the incubation, a scintillation count is performed on the surrounding media as well as the cell lysate. These two counts together comprise 100% of the chromium taken up by the tissue, a value that allows calculation of the percent chromium released from dying cells. The difference in the chromium release percents between treated and control cultures is indicative of amount of cell rescue that has taken.



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