

THE UNIVERSITY OF TENNESSEE
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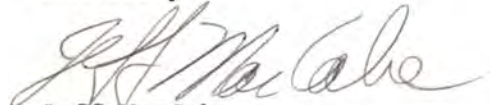


Dr. Bruce Wheeler
Tennessee Scholars Program
1113 McClung Plaza and Tower

Dear Dr. Wheeler:

This Monday, April 23, 1990, Bobby Blaylock gave a public presentation of his research for the Tennessee Scholars Program. This successfully completed his work on this research project. His research, which involved the electron microscopy of embryonic tissues treated with "fibroblast growth factor," was an important contribution to the research in my laboratory. His contribution is recognized by his coauthorship of a manuscript we recently sent to the "Journal of Experimental Zoology." I have enclosed a copy of this manuscript for your information. Bobby did an excellent job on his research and is a credit to the Tennessee Scholars Program.

Sincerely,



Jeff MacCabe

College of
Liberal Arts
Department of
Zoology

*Submitted to
The Journal of
Experimental
Zoology*

Fibroblast Growth Factor and Culture in Monolayer Rescue
Mesoderm Cells Destined to Die in The Developing Avian Wing

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5 figures

Reviewers Comments and Proofs to:

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Running head line: FGF Inhibits Programmed Mesodermal Cell Death

Abstract

In an effort to elucidate control mechanisms for developmentally programmed cell death, conditions were sought which rescue the cells destined to die. Three areas of mesodermal cell death in the chick wing were examined, the posterior necrotic zone (PNZ), the opaque patch (OP) and apical mesoderm. The PNZ and OP are areas of normally programmed cell death whereas the apical mesoderm undergoes cell death only after the overlying apical ectodermal ridge is excised. Cell death in vitro was quantitated using the chromium-release assay. While these tissues undergo apparently normal cell death in organ culture, in monolayer culture almost all are rescued. In addition, the cells are rescued by the addition of fibroblast growth factor to organ cultures. Since fibroblast growth factor is present in decreasing amounts in the limb at this stage of development, normal cell death may occur upon growth factor withdrawal.

In spite of extensive research, the answer to how developmentally programmed cell death is controlled remains elusive (see Hurle, '88 for review). Work on mesodermal cell death in the avian limb has continued more-or-less unabated since Saunders called our attention to these interesting regions of apparently morphogenetic cell death (Saunders et al., '62; Saunders and Fallon, '67). In their early work this group found that one of these regions, the posterior nectrotic zone (PNZ), undergoes cell death on schedule after being excised and explanted to organ culture. This has provided a useful experimental system with which to study developmentally programmed cell death.

Programmed cell death occurs within the embryo at very precise stages of development. Generally, not all of the cells die within an area of cell death. Thus two critical questions emerge when considering the control of programmed cell death. One is how some cells die while their immediate neighbors remain viable. The second deals with how the stage specificity or timing of cell death is controlled. We have approached the question of temporal control by looking for conditions that will inhibit the onset of specific areas of cell death. We examined three areas of cell death in the developing chick wing, the posterior nectrotic zone, the opaque patch (OP) and apical mesoderm under the apical ectodermal ridge. The PNZ and OP are normally programmed to undergo cell death beginning at about stage 23 (Hamburger and Hamilton, '51). The apical mesoderm does not normally undergo cell death but can be induced to do so by the surgical removal of the apical ectodermal ridge (Cairns, '75; Rowe et al., '82). Here we report two conditions that rescue cells destined to die: culture in monolayer and exposure to fibroblast growth factor (FGF) in organ culture.

MATERIALS AND METHODS

Culture of Cells and Tissues:

Fertile eggs from white Leghorn chickens were obtained from the University of Tennessee, Department of Animal Science and incubated at 37.5-38.0°C for three days. The eggs were windowed and returned to the incubator until the embryos reached stage 21. Tissue excised from the PNZ measured approximately .3x.2x.2 mm; from the prospective OP approximately .3x.3x.3 mm and from the apex approximately .4x.2x.2 mm. These tissues were either cultured intact as an organ culture or trypsinized in .01% trypsin for 15 minutes at 37.5°C, then cultured either intact, intact without ectoderm or they were disaggregated with 3-3 second bursts on a vortex-genie (Scientific Industries, Inc., Springfield, MA) and cultured in monolayer. Each culture consisted of cells or tissues from 4-6 wing buds in 200 μ l of minimum essential medium with 10% calf serum (GIBCO, Grand Island, NY). The cells or tissues were cultured in 6.4 mm diameter wells of a multi-well plate in a 5% CO₂ atmosphere at 37.5°C.

Chromium Release Assay:

The chromium release assay was used to quantitate cell death. This assay has been used for a number of years to monitor cell death in monolayer cultures, largely in studies of cell-mediated cytotoxicity (Brunner et al., '76). We have shown it can also be used in organ culture where tissues are small and loosely organized as in embryonic mesenchyme (MacCabe and Brewton, '84). The assay is based upon the ability of cells to take up ⁵¹Cr rapidly while only very slowly releasing it unless cells are damaged or die. The release of ⁵¹Cr to the medium thus can be used to quantitate cell death.

Preliminary experiments indicated it takes limb mesoderm three hours to recover their normal ⁵¹Cr release rates after trypsinization. In initial

experiments cells were cultured for 3 hours after trypsin treatment, then labelled for 3 hrs with ^{51}Cr (250-500 mCi/mg Cr, 0.1 mCi/ml, Amersham, Arlington Height, IL). However we found the same results with a continuous 6 hr uptake beginning as soon as the cells were plated. Most of the experiments were thus done in this manner. So experiments would be as comparable as possible, the tissues not trypsinized (in organ culture) were also given a six hour ^{51}Cr uptake period. The cells or tissues were then rinsed six times in medium without ^{51}Cr by removing and replacing the 200 μl of medium. The addition of FGF (bovine pituitary, Collaborative Research, Bedford, MA) to some cultures was done at this time. The cultures were incubated an additional 15 hours (^{51}Cr release period), after which 100 μl of the medium was counted by liquid scintillation to determine the ^{51}Cr released. The remaining medium was discarded, 200 μl of water added and then frozen and thawed three times to lyse the cells. A 100 μl sample of the lysate was counted to determine the ^{51}Cr remaining in the cells at the end of the release period. From this data the percent chromium release was calculated. Each experiment was repeated at least five times. The results were compared using an unpaired two tailed t-test and expressed as the average percent chromium release $\pm$ the standard error.

Histology:

Some tissues were removed from culture wells after 21 hrs of culture (corresponding to 6 hrs of ^{51}Cr uptake + 15 hrs of release), fixed in 3% gluteraldehyde, rinsed overnight in 0.2 M phosphate buffer and postfixied for one hour in phosphate buffered 2% osmium tetroxide. They were then dehydrated, embedded in Epon LX112 (Ladd Research Industries, Inc., Burlington, VT) and sectioned for electron microscopy. The sections were poststained in uranyl acetate and lead citrate and examined with a Zeiss EM952A transmission microscope.

RESULTS

Opaque patch, posterior necrotic zone or apical tissues were excised approximately 6 hours before the expected onset of cell death (and in some cases disaggregated), labelled with ^{51}Cr , rinsed extensively, then allowed to incubate for an additional 15 hours. The percent chromium release during this 15 hour period was used to quantitate cell death.

Tissue from the OP region in organ culture released 55-60% of their ^{51}Cr over the 15 hour release period (Fig. 1,a). During this period of time considerable cell death occurred as seen by histological sections of tissues fixed at the end of this culture period (Fig. 2,a). Previous studies show a much lower ^{51}Cr release rate (20-30%) for wing mesoderm not undergoing programmed cell death (MacCabe and Brewton, '84). Unlike the PNZ, where much of the cellular debris is found within phagocytes (Saunders and Fallon, '67), much of the debris in the OP is found outside phagocytes, at least at the stage we examined histologically (see also Dawd and Hinchliffe, '71). This may result from the higher rate of cell death in the OP as seen by the nearly 60% ^{51}Cr release in OP cultures compared to about 40% in PNZ cultures (These studies).

Cells from the OP that have been disaggregated and plated as a monolayer show a ^{51}Cr release drastically lower than the intact tissues (Fig. 1,c), suggesting an inhibition of cell death occurs under these culture conditions. If OP tissue is trypsinized but not disaggregated, no inhibition of cell death occurs (Fig. 1,b). Thus it is the monolayer culture condition and not the trypsinization that inhibits cell death. In some of these cultures the ectoderm was removed and discarded, but the mesoderm left intact. Ectoderm removal had no effect on the results and thus the data in Figure 1,b include both groups.

Previous studies have implicated peptide growth factors as modulators of some kinds of cell death. We examined the effect of fibroblast growth factor on cell death in both intact OP tissue (Fig. 1,d) and monolayer OP cells (Fig. 1,e). Chromium release figures of just over 30% in both instances, suggest a considerable inhibition of cell death in the intact OP tissue ($p=.0001$), but only a slight if any inhibition in OP monolayer cells where cell death is already considerably inhibited ($p=.0457$). In addition, histological sections of FGF treated and untreated OP tissues show a considerable difference in the amount of cellular debris that results from cell death (Fig. 2a,b). It thus appears that both FGF and monolayer culture conditions inhibit cell death in OP mesoderm. A dose-response curve for the inhibition of cell death by FGF shows the 100 ng/ml dose used in most of our experiments gave about 75% of the maximum response (Fig. 3). The lowest dose used (2 ng/ml) gave no detectable response.

To see if the effects of monolayer culture and FGF are unique to the opaque patch region of programmed cell death, we also looked at the effect of these conditions on the PNZ. Unlike the opaque patch, PNZ cells in monolayer showed no inhibition of cell death when compared to intact PNZ tissue (Fig. 4,a and b). Fibroblast growth factor, on the other hand, affected PNZ tissue in organ culture the same way it did the OP, i.e. it drastically inhibited cell death (Fig. 4,c). However FGF did not inhibit the cell death which occurred under monolayer culture conditions (Fig. 4,d).

We also examined the effects of monolayer culture and FGF on cell death in apical mesoderm, an area which does not undergo developmentally programmed cell death, but where cell death can be induced by apical ectodermal ridge excision. Intact apical tissue does not undergo significant cell death in organ culture unless the ridge is excised prior to culture (Fig. 5,a,b). As with the OP,

apical mesoderm is rescued from death by culture in monolayer (Fig. 5,c). In addition under organ culture conditions FGF also rescued cells destined to die because of apical ridge excision (Fig. 5,d).

DISCUSSION

Hamburger ('34) and Hamburger and Levi-Montalcini ('49) demonstrated nerve hypoplasia due to cell death and a lack of growth upon extirpation of the chick embryo limb bud. These studies not only began the search for Nerve Growth Factor (NGF), but demonstrated a possible dual role for such factors, their stimulation of cell division and their necessity for the survival of neurons. A demonstration of the requirement for (NGF) for neuron survival was shown by injecting NGF antiserum into animals, resulting in neuron destruction (Cohen, '60; Levi-Montalcini and Bouker, '60). More recently several workers have shown an effect of FGF on the survival of neural and neural crest-derived cells (Walicke et al., '86; Morrison et al., '86; Sievers et al., '87; Ferrari et al., '89; Kalcheim, '89). We have shown in this study that FGF rescues chick limb mesoderm cells developmentally programmed to die. In both the OP and PNZ, cells die in organ culture, but many are rescued by 100 ng/ml FGF. This is a higher level of FGF than found to be effective for neurons, though we have used a highly purified source of basic fibroblast growth factor to be effective at a considerably lower concentration. It is tempting to speculate that decreasing concentrations of FGF in vivo may have a role in the timing of developmentally programmed cell death in the avian limb. Munaim et al. ('88) have found FGF in the limb in decreasing amounts over the period of time both the PNZ and OP are undergoing cell death and Aono and Ide ('88) showed limb mesoderm responds to the growth promoting effects of FGF. In addition, the PNZ, OP and anterior necrotic zone (ANZ) are being left behind by the apical ectodermal ridge at the apex of the limb as outgrowth proceeds. The ridge is a known mitogen for underlying mesoderm (Reiter and Solursh, '82) and could produce or result in the production of FGF (Munaim et al., '88). A possible relationship between a mitogen and cell death is suggested by the death that occurs in apical mesoderm

when the ectodermal ridge is removed (Cairns, '75; Rowe et al., '82). In our own studies we have shown these cells can also be rescued in organ culture by FGF.

In addition to FGF, culture in monolayer also rescues cells of the OP and the limb apex. Cells of the PNZ however, undergo considerable cell death in monolayer as well as in organ culture. At first glance this suggests these cells are not rescued by monolayer culture conditions. However, two things suggest that in the PNZ cell death in monolayer is different than that in organ culture. First, there is more cell death in monolayer than in organ culture (the difference is small but statistically significant, $p=.0613$). Second, FGF does not rescue monolayer PNZ cells from death. Thus there is a possibility that in the PNZ monolayers FGF rescues the cells programmed to die but a second type of cell death occurs which is not inhibited by FGF. Possibly cells from the PNZ have a more difficult time adjusting to these culture conditions.

Why mesoderm cells of the OP and apex are rescued in monolayer culture is not clear. Since primary cultures frequently go through a burst of cell division when cultured at less than confluent densities, we suspected growth factors may be produced by the monolayers. However we could find no convincing evidence that monolayers affected cell death in organ cultures when we cocultured monolayers and intact pieces of OP tissue (data not shown). The drastic alteration in extracellular matrix in going from tissue to monolayer cultures may be involved in the rescue since the matrix may be a mediator of the stimulation of cell division by FGF (Nakagawa et al., '89; Damon et al., '89).

We have found one other procedure that affects mesodermal extracellular matrix and rescues cells programmed to die. In vivo removal of the ectoderm over the PNZ just prior to the onset of cell death results in an almost

complete inhibition of programmed cell death (Brewton and MacCabe, '88) (for unknown reasons this does not occur in vitro). Several studies suggest the ectoderm may influence the matrix composition in underlying mesoderm (Kosher and Savage, '81; Solursh et al., '81; Kosher et al., '82; Solursh et al., '84; Zanetti and Solursh, '86). Taken together, these lines of evidence suggest the apical ridge may control cell death in the mesenchyme, possibly mediated by the extracellular matrix and FGF.

Hurle and Ganan ('86) have suggested a role for the ectoderm in the interdigital cell death that occurs later in limb development. Here, as in the PNZ, the removal of overlying ectoderm in vivo results in an inhibition of cell death. This lack of death in the interdigital mesenchyme is followed by the appearance of cartilage nodules in the interdigital spaces. We saw no evidence of differentiation in cultures where cell death was inhibited by FGF, but the relatively short culture time in our experiments probably would not have allowed significant chondrogenesis. Our current experiments are focusing on the relationship between FGF's ability to stimulate cell division and its ability to rescue cells destined to die. Preliminary evidence suggests that under the culture conditions we used (MEM + 10% bovine serum) there is little or no stimulation of cell division by FGF in these tissues. In addition, cells are rescued in the presence of 10^{-5} M methotrexate, an inhibitor of cell division by virtue of its inhibition of dihydrofolate reductase and thus of thymidylate synthesis. Apparently, FGF needs not stimulate cell division in order to rescue cells. Similar results were reported by Scher et al. ('82) for the inhibition of confluence induced 3T3 cell death by platelet derived growth factor. Future experiments will examine the basis for the independence of cell rescue and cell division in response to growth factors.

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Figure Legends

Figure 1. Cell death in the opaque patch region as measured by ^{51}Cr released over a 15 hour culture period. Higher rates of cell death are indicated by higher levels of ^{51}Cr release. (a) Intact blocks of untreated tissue in organ culture and (b) tissue trypsinized but not disaggregated both show a high rate of cell death. (c) Tissue disaggregated and cultured as a monolayer, on the other hand, show considerable inhibition of cell death. (d) The addition of 100 ng/ml FGF to organ cultured OP tissue also inhibits cell death. (e) Fibroblast growth factor has only a slight effect on monolayer cells, presumably because little cell death occurs in monolayer.

Figure 2. Histological sections of OP tissue after a 15 hour culture period shows considerable debris (arrows) in untreated cultures (a) but little in cultures with 500 ng/ml FGF (b) (5400 X).

Figure 3. A dose-response curve for the rescue (^{51}Cr -release) of OP cells in organ culture. The lowest dose used (2 ng/ml) had no effect and the maximum rescue was obtained at a dose of 500 ng/ml.

Figure 4. Cell death in the PNZ measured by ^{51}Cr -release. (a) Intact PNZ in organ culture loses about 40% of its chromium. (b) When PNZ mesoderm is disaggregated and plated as a monolayer considerable cell death still occurs. (c) The addition of 100 ng/ml FGF considerably inhibits cell death in intact, organ cultured tissues. (d) Monolayers of PNZ cells are not rescued by FGF however.

Figure 5. Cell death in apical wing mesoderm measured by ^{51}Cr -release. (a) Little or no cell death occurs in organ cultures of apical tissue which has its overlying apical ridge. (b) When the ridge is excised prior to culture,

however, considerable cell death occurs. (c) When the apical mesoderm is disaggregated (and the ridge discarded) and cultured as a monolayer, cell death is inhibited compared to intact ridge-less tissues. (d) The addition of 100 ng/ml FGF to intact ridge-less apical tissue rescues the cells that would die due to ridge removal.

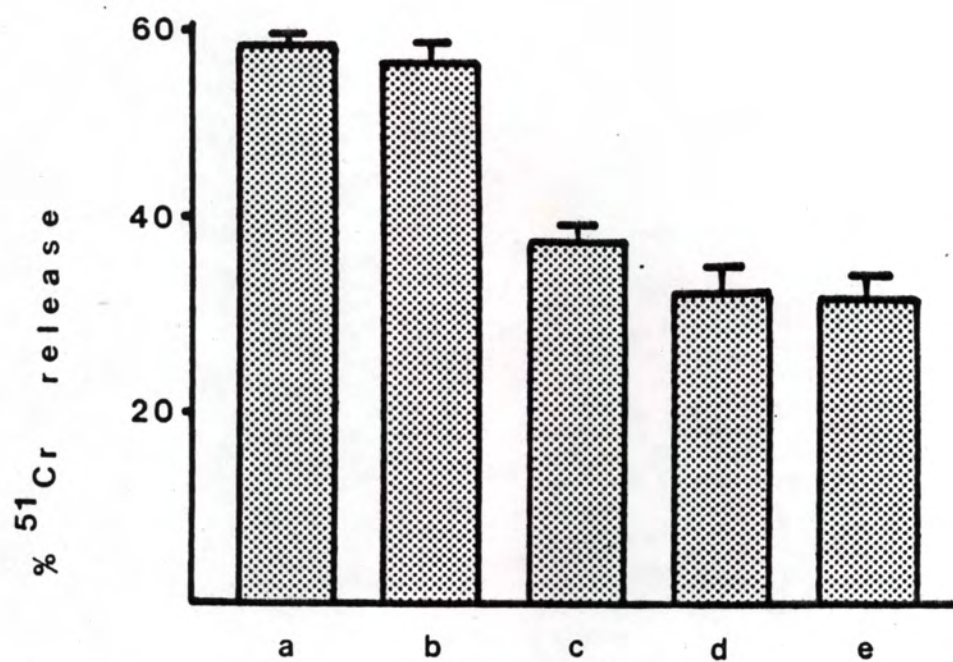


Figure 1



a



b

Figure 2

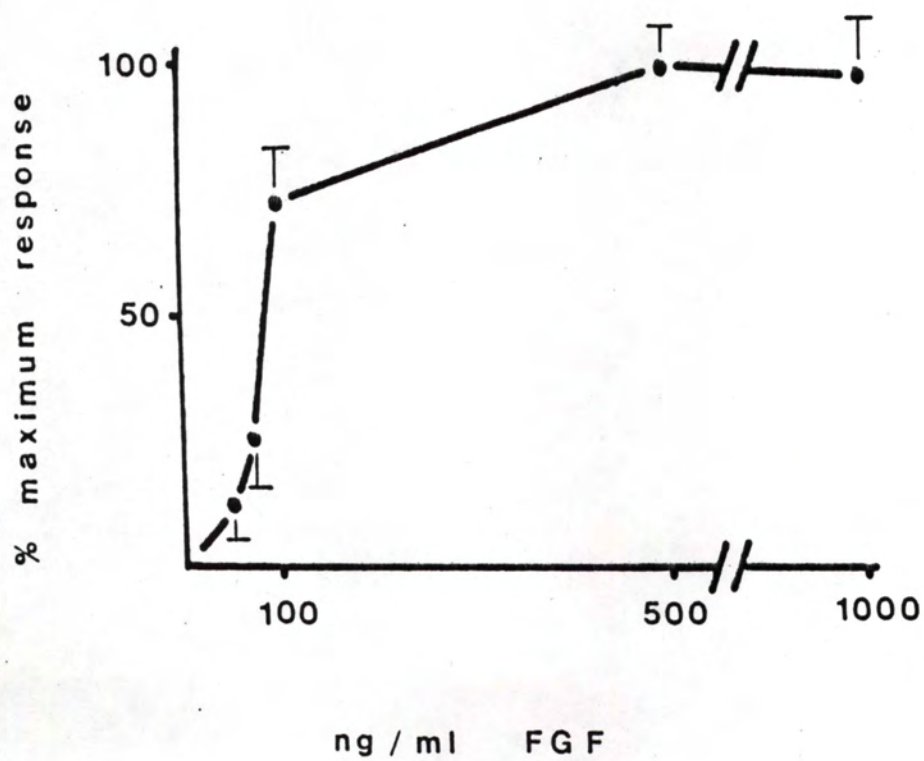


Figure 3

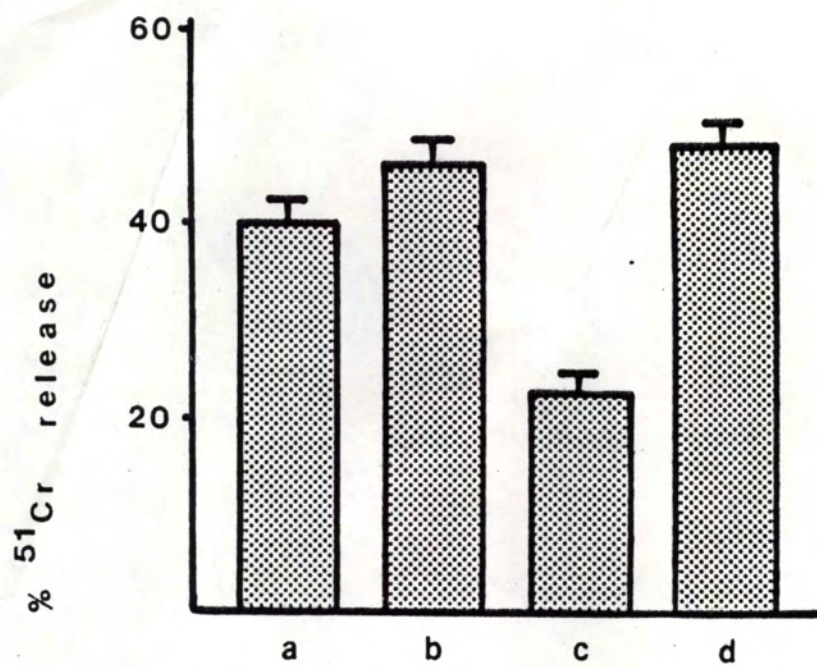


Figure 4

SUMMARY OF MY WORK ON THE PROJECT
FIBROBLAST GROWTH FACTOR AND CULTURE IN MONOLAYER RESCUE
MESODERM CELLS DESTINED TO DIE IN THE DEVELOPING AVIAN WING

Robert L. Blaylock, Jr.
April 30, 1990
Senior Tennessee Scholar in
Biology

I participated in two activities which enabled me to contribute to Dr. MacCabe's ongoing research on cell death. These two activities were taking the electron microscopy course and auditing a graduate seminar on growth factors. The notebook of electron micrographs submitted is representative of my creative work in electron microscopic photography during the class. This class focused on techniques. The seminar focused on the cutting edge of receptor-ligand recognition, signal transduction, and cellular response research as it relates to growth factors.

The primary goal of research in Dr. MacCabe's lab is to elucidate control mechanisms for developmentally programmed cell death (MacCabe, et al p. 2). I looked at the ability of fibroblast growth factor (FGF) to rescue cells destined to die in the chick embryo wing bud. This was accomplished by looking at embedded sections of tissue at low magnifications (approx. 1800x) and by looking for qualitative signs of cell death (primarily loose cellular debris) and for healthy cells. By comparing a control tissue whose cells were allowed to die on schedule with a tissue which had been incubated with FGF, we were able to confirm that FGF did play a significant role in rescuing cells normally programmed to die. A large amount of the time was taken up taking different magnification shots (including light microscopy), examining different tissues, interpreting the pictures from each run and making adjustments, and doing multiple reps to insure that our results were valid. Since the histology work I did played primarily (though not

wholly) a confirmatory role for the relative data from the Chromium 51 release assay, the final data (micrographs) are few. All that was needed was one micrograph of the control tissue and one of the treated tissue of the dozens I took, to visually confirm the other results.

Two contributions that were unique to the histological data are worth mentioning. It was determined that the opaque patch (OP) region of cell death has much more cell debris not engulfed by surrounding cells than the posterior necrotic zone (PNZ). This confirms the higher ^{51}Cr release data. The other contribution was that considerable cell death remained even after addition of FGF to the tissues which led to a refiguring of the dosage which gave maximum response and eventually to using a more purified source of FGF.

In an attempt to begin to understand why OP cells are rescued by plating in a monolayer while PNZ cells are not as well as to expand my exposure to different techniques and instrumentation, cells in monolayer were examined with the scanning electron microscope (SEM). While the results were inconclusive, the micrographs obtained show nicely the surface features of cells undergoing programmed cell death.

What I learned while doing my Senior TS project.

This senior project performed as the primary privilege and the primary responsibility of participation in the Tennessee Scholars program has been of much personal benefit. Since this is the capstone experience of my undergraduate career which has as one of its main objectives my exposure to and exploration of the whole realm of academia and since I am a member of the pioneer group of Tennessee Scholars, I consider the following presentation of my personal assessment of the value of undertaking this project of considerable importance. Perhaps the greatest benefit of this undertaking has been the opportunity to spend time both formally and informally getting to know professors. The clues I have received from these people about the possibilities and limitations of a career in biological research and the philosophies of life which work best in this field and which are partially shaped by inclusion in this field have been of much value in helping me to make decisions about my future life plans and to appreciate more fully the field of biology and its advancers. I shall likely cherish the slow, firm, quiet, and painstakingly thorough approach to not only research but to life that Dr. Patrica Walne gave me. The common sense and skepticism of Dr. Jeff MacCabe and Dick Williams provide equally important lessons. The kind facade of impatience of Dr. Kennedy speaks eloquently of the pressures placed on the modern researcher. Many other personalities from the graduate students in the growth factor seminar I sat in on to my long time advisor Dr. Mary Ann Handel each have added to my education with both specific biological

information and knowledge of what the world of modern research is all about. Taken together, I have been impressed with their tireless efforts, their undying curiosity, their respect for me as a person and with their overall humanness.

About the field itself I have learned a bit about how and when science and art interact, the respect for and knowledge of history necessary to add new insights into its fabric, and the mindboggling degree to which research efforts are interdependent. Technology has garnered new respect from me as I have sat in awe trying to make sense of the hidden mysteries of the cell revealed by the electron microscope. I have learned to use my hands more skillfully through embedding, trimming, sectioning, and handling small pieces of tissue. My self confidence has risen as I have been trusted to investigate independently and as I have carried through on a weeklong sequence of events which actually contributed something to an ongoing piece of research. For all these experiences and insights, many of which likely lie in the future as I reflect on my experiences, I thank The University of Tennessee all the people mentioned above, particularly Dr. MacCabe, Dr. Susan Becker, and Dr. Bruce Wheeler.

Sincerely,
Bobby Blaylock