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# The Effects Of Endothelin On Embryonic Optic Lobe Cultures

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**The Effects of Endothelin on  
Embryonic Optic Lobe  
Cultures**

**By**

**Christine Herczku**


**A Thesis  
August, 1997**

**Submitted in partial fulfillment of the requirements for the degree of  
Master of Science from the Department of Biology of  
Seton Hall University**

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## Acknowledgement

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This thesis is dedicated to my parents. If it were not for their adamant interest in my education, this project could not have been possible.

I would also like to thank my friends and the Seton Hall Biology Faculty. At many times they have gone above and beyond for me and it was and will always be greatly appreciated.

**Abstract:**

Protocols for establishing tissue cultures of optic lobes from embryonic chicks were developed. The optimal age of donor embryo, the type of culture substrate, the method of dissociation for monolayer cultures and the composition of the growth medium were examined. The effects of the vasoactive peptide endothelin (ET-1) on optic lobe cultures were also evaluated. Dissociated optic lobe cultures from 8 day old chick embryos were treated with single or multiple doses of endothelin (20pM to 100nM). The progressive differentiation of the ET-1 treated monolayer cultures were compared to cultures maintained in defined medium containing essential hormones and growth factors. Although previous research involving an *in vivo* model indicated that endothelin did exhibit growth promoting characteristics, the growth promoting effects of endothelin *in vitro* could not be demonstrated. Alternatively, embryonic optic lobe explants were used. With this model system, a low dose of ET-1 (40 pM) in unsupplemented medium elicited long thin neurite outgrowth within one day in culture, and a high dose of ET-1 (200pM) was correlated with short, thick neurite outgrowth composed to untreated explants.

## Introduction:

Endothelin (ET) is a potent vasoconstrictive peptide which is derived from endothelium (Simonson et al., 1990; Macumber et al. 1990). In human mesenteric arteries, it is approximately one hundred times more potent than norepinephrine. ET also contracts non-vascular smooth muscle, including the trachea, and uterine and intestinal muscle (Katzung, 1995). ET may also play an important role in cardiovascular regulation. This is due to its participation in hypertension and other cardiovascular diseases. However, the precise role of ET in this respect still remains unknown (Katzung, 1995).

Endothelin was first isolated from cultured aortic endothelial cells (Yanagasawa, 1989). The amino acid sequences of endothelin derived from various tissues ranging from the vertebrate eye through unicellular organisms such as yeast are highly conserved evolutionarily. It consists of twenty-one amino acid residues and two disulfide bonds. The ET peptide is the result of intracellular proteolytic processing of a 203-amino-acid precursor protein called preproendothelin. Mature endothelin is derived from the residues 53-73 of the preproendothelin. The residues 53-73 are preceded by paired basic amino acids. In preproendothelin, a cleavage at this paired basic amino acid produces a 39-amino acid intermediate, called "big" endothelin (Bloch et al., 1989). From this stage on, there is a cleavage by specific endothelin converting enzymes (ECE), which will generate mature endothelin. Through the use of specific inhibitors of endothelin synthesis, it has been discovered that the formation of ET-1 from big endothelin requires

a proteolytic cleavage between Trp73 and Val74. (Huggins and Pelton, 1997)

Endothelin is widely distributed throughout the body, with high concentrations found in the lungs, kidneys, heart, hypothalamus and the spleen (Katzung, 1995). There are three different isoforms of endothelin, ET-1, ET-2, and ET-3, with ET-3 being most prominent in the brain. Interest in defining the mechanism of action of this pervasive peptide has led to studies on its receptor profile. ET receptors are found in blood vessel walls, cardiac muscle, the CNS, lungs, kidneys, adrenal gland, spleen, and intestine. Two receptor subtypes have been identified: ET-A and ET-B. ET-1 and ET-2 show similar affinity for the ET-A receptor subtype, while all three isoforms of endothelin have similar affinity for the ET-B subtype receptor (Huggins and Pelton, 1997). Both of these receptor subtypes belong to the family of G-protein coupled receptors. The binding of ET to receptors results in an increase in intracellular calcium concentrations. This increase in the calcium levels is believed to be responsible for the increase in the contractility of smooth muscle cells, therefore resulting in vasoconstriction (Katzung, 1995).

Although ET was originally thought to be primarily involved in the cardiovascular system, it is found in the highest reported densities up to 5000-fmol/mg protein in the brains of humans as well as other animals. Strangely enough, ET-1 is not found to cross the blood brain barrier; therefore, the ET receptors in the brain must respond to an ET-like ligand which can be detected by immunocytochemistry, suggesting that this ligand is synthesized within the cells of the CNS, such as neurons, rather than to the peripherally released peptide (Huggins and Pelton, 1997). In other words, the ET receptors respond to a local stimulus rather than a peripherally located one. There is localized ET binding in the brain, as trends are seen for the density to decrease from the



hindbrain to the forebrain. High receptor densities have been seen in the brainstem, cerebellum, and the hippocampus, while low ET receptor densities are seen in the hypothalamus and the cortex (Huggins and Pelton, 1997).

Although the sites of synthesis and action of ET in the brain are unclear, immunohistochemical localization of ET to the motor neuron in the human spinal cord has been demonstrated (MacCumber et al. 1990). ET receptor sites have been mapped by autoradiography and occur heterogeneously in the brain independently of the distribution of blood vessels. The identity of the cells containing these receptors have not yet been established. There is evidence that ET enhances the inositol phospholipid turnover in glia, since it stimulates glial cell growth and is synthesized within astroglia (MacCumber et al., 1990).

Neuroglial cells, the non-neuronal elements of the CNS, are literally the "nerve glue" in the brain tissue. Depending on the specific site in which they are located, these supporting cells may be 10 to 50 times more numerous than the neurons. Unlike neurons, the glial cells retain their ability to replicate themselves. (Marieb, 1992). Tissue culture studies confirm that ET receptors are located on these glial cells. They are found to express the ET-B subtype; this is evident through a study which proved that ET-3 was equally effective as ET-1 in stimulating the intracellular free calcium (MacCumber, 1990; Huggins and Pelton, 1997).

Astrocytes are another type of glial supporting cell, which contain numerous radiating projections with bulbous ends that cling to neurons and capillaries, bracing the neurons and anchoring them to the nutrient supply of the capillaries. Astrocytes also control the chemical environment around the neurons (Marieb, 1992). A homogenous

distribution of [ $^{125}$ I] ET-1 binding has been detected in astrocytomas, which are tumors mainly composed of astrocytes. This demonstrates that while astrocytes are proliferating, they express ET receptors (Huggins and Pelton, 1997 p 60).

ET receptors are also detected in a proportion of rat cerebellum neuron cultures. Because only ET-1 and ET-2, but not ET-3, were able to increase the intracellular calcium levels, it is suggested that the neurons express ET-A subtype receptors only (Huggins and Pelton, 1997).

Although ET was originally identified as a vasoconstrictive peptide, recent research has shown that it has growth promoting effects. Thus ET can stimulate the proliferation of smooth muscle fibroblasts, glomerular mesangial cells, and osteoblasts. (Takuwa et al., 1989). An additional effect of ET on development was first demonstrated (Kurihara et al., 1994) in a gene knockout experiment. It was seen that mice lacking the ET-1 gene died of respiratory failure at birth. This respiratory failure was mostly likely due to the formation of an abnormal, doughnut shaped structure, which appeared to be the result of the fusion between the hyoid and the basisphenoid bone. This abnormal structure obstructed the trachea and the esophagus by forcing them to pass through the odd-shaped structure. Such abnormalities were believed to have resulted from disturbances in cells of neural crest lineage, which are responsible for forming the craniofacial skeletal elements. These results implied that ET played a role in the development of embryos. Also, because ET was originally known for its pressor effect, which is its ability to cause and increase in blood pressure, it was unexpectedly found that a decrease in ET-1 production caused arterial blood pressure to be significantly higher in ET-1 in +/- heterozygous mice than the ET-1 +/+ wild type mice (Kurihara et al., 1994).

In a complementary study to Kurihara's work to examine the developmental role of ET, Hsu and Jeng (1994) have demonstrated that by adding ET to developing chicks, the overall sizes of the ET-treated chicks were larger, including the beak, wings, trunk, and the brain. The treated chicks did not show increased incidence of vascular abnormalities or structural malformations due to exogenous ET. ET was added on the fifth day of incubation because ET receptors are evident in the largest quantities on that day (Hsu and Jeng, 1995). Histological evidence suggested that the growth promoting effects of ET on brain development involved more rapid neuronal maturation. Hsu and Jeng demonstrated that the size of the 10-day ET-treated brain was correlated with differences in histological organization of various regions when they were compared to normal controls. Their evidence was most noticeable when examining of the cytoarchitecture of the optic lobes; however, it has yet to be determined whether ET causes the proliferation of cells during brain development (Hsu and Jeng, 1995).

ET also has the ability to promote growth in peripheral neuron tissue of chick embryos. In the peripheral ganglia, ET enhances the neurite promoting effects of phorbol esters in the dorsal root ganglion explants (Hsu et al., 1992). In this latter experiment, the effects of ET were believed to be synergistic with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) through cell signaling mechanisms involving phosphoinositol turnover (Hsu, 1989). The phosphoinositol biphosphate turnover results as a direct effect from the phospholipase C activation, which is initially linked to the coupled- $\beta$ -G-protein transmembrane receptor (Alberts et al., 1994). Phosphoinisitol biphosphate (PIP<sub>2</sub>) is converted to diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>), which are the second messengers, which then go on to perform a wide variety of actions, primarily to stimulate

calcium release (Alberts, et al., 1994).

This project described here focuses on the question, what is the effect of ET on embryonic brain cells in culture? By looking at the cellular level, the growth promoting effects of ET might be examined. For this research, the optic lobes were chosen as the target of study for several reasons. During the development of the brain in birds, the optic lobes stand apart as large structures which reach maturity very rapidly. Thus, on the 8th day of development they are seen as the most prominent structures in the chick embryo and finished their differentiation stages. Optic lobes have been used in both *in vivo* and *in vitro* studies because of their distinctive phases of growth in development (Alder et al, 1979; Pearson, 1972). Thus cell proliferation, cell migration and pattern formation of the brain region devoted to vision can be followed using optic lobe tissues.

The goal of this experiment was to develop a tissue culture system to determine precisely what the effects of ET are *in vitro* and compare that to the *in vivo* results. By establishing the tissue culture conditions which will maintain optic lobe cells, it is hoped to have a cellular model to analyze the growth promoting effects of ET.

## Materials and Methods:

**Animals:** White Leghorn eggs (Avian Services, Frenchtown, NJ) were used for all experiments. The fertile eggs were kept at room temperature for a minimum of 24 hours before the incubation period. During incubation, the eggs were periodically candled to check for live, developing embryos. Extraembryonic blood vessels visible around the fifth day of incubation were indicative of a live embryo, whereas a black ring around the circumference of the egg shell signified dissolution of the vascular supply and death. The chicks were allowed to develop until they were either six or eight days old before optic lobes were used for tissue culture.

All experimental methods from this point on took place in the laminar flow hood in order to maintain an environment that was free from contamination. On days in which the hood was used, it was turned on for 30 minutes prior to experimentation and cleaned thoroughly with 70% ethyl alcohol. When a clean environment was achieved under the hood, sterile tools or materials which were wiped down with 70% ethyl alcohol were placed under the hood.

**Microscopic Surgery:** The eggs were cracked on the animal (top) pole, part of the shell was removed and the embryo was displaced from the egg by its neck using curved forceps (Freshney, 1983). The curved forceps were hooked between the body and the head, under the neck. Then, the body was lifted carefully from the egg shell. After the embryo was retrieved from the egg, the head was dismembered from the body by simply pulling it apart. The head was placed in a plastic petri dish and kept moist using defined medium. The medium was a modified culture medium containing 50% MEM (Minimal

Essential Medium) and 50% Ham's F12 with 80ul insulin (6.25 mg/ml), 400ul transferrin (12.5 mg/ml), 2ul progesterone (1mM), 10ul putrescine (10 mM), 6.5ul sodium selenite (80 ug/ml), and 1 ml P/S (penicillin/streptomycin) (Bottenstein et al, 1980; Adler et al. 1985). This is referred to as defined media. The essential hormones and growth supplements were added from stock concentrations kept frozen at -20°C

**Microscopic Surgery:** The optic lobes are found in the dorsal position in front of the cerebellum and behind the forebrain (Pearson, 1972). Under a dissecting scope, the optic lobes and diencephalon were dissected from the 6 or 8-day- old chick embryos. Caution with fine forceps was required to peel off the meninges which surrounds the brain tissue, since the meninges contain connective tissue cells which will contaminate the brain tissue if not removed (Adler, et al., 1985). In addition, blood cells were rinsed from the brain tissue by changes of media solution in a depression slide. The optic lobes were then dissected away from the diencephalon. The tissues from 1-2 optic lobes were collected in 300-400 ul of defined media and subjected to dissociation by either enzymatic digestion or mechanical disruption.

**Dissociation:** Two methods were tested for dissociation to determine which one will yield the most viable cells.

***Mechanical:*** Optic lobes were initially minced using sterile scalpel blades. When minute cubes of tissue were produced from the mincing, the pieces were collected and then passed through syringes equipped with needles with decreasing gauges (18, 22, and 26 gauge respectively). The cell suspension was passed a total of 5 times through each needle size. With each passage, additional defined media was incorporated into the cell suspension until the cells were contained in a total of 5 ml.

*Enzymatic:* Optic lobes were initially minced using scalpel blades to produce small fragments as described as above. After this mechanical dissociation step was achieved, the tissues were contained in a total of 5 ml of 0.05% trypsin which was diluted with Hank's balanced salt solution to produce the final concentration of 0.01%. The cell suspension was then incubated for 10 minutes at 37°C. 5ml of medium containing 10% fetal calf serum was then added to inactivate the trypsin. The cell suspension was subjected to centrifugation for 5 minutes at 1000 rpm two times. After each centrifugation, the supernatant was discarded and a final 4.75 ml of Hanks Solution was used to resuspend the pellet. A cell count was then performed.

**Cell Count:** Cell viability was examined by using the dye-exclusion test. To each 18 ul of cell suspension, 2 ul of 0.1% Trypan Blue was added in an Eppendorf tube. After vortexing, a cell count was done by placing 15 ul of the cell suspension containing Trypan blue in a hemocytometer Neubauer chamber. Blue cells were indicative of non-viable cells, since the dead cells were able to uptake the blue dye due to a disruption in the cell membrane. Clear cells signified cells that were viable.

**Dilution and Seeding:** Cell suspensions were usually diluted two fold with fresh culture medium to the desired concentrations. In each control and experimental set, two drops (approximately 200ul) of the cell suspension were seeded into 6 mm units of a 96 well cluster dish precoated with laminin and incubated over night at 37°C. Six rows of 8 well units were routinely plated.

**Laminin and Collagen:** Laminin and collagen, which are two naturally occurring extra-cellular matrices, were evaluated for the ability to promote cell growth and progressive differentiation. The laminin and collagen were preplated in 96 well cluster

dishes. These are BioCoat Cellware dishes which are commercially available from Becton Dickinson/Collaborative Biomedical Products. To test the effect of different substrates on optic lobe cultures, on day zero, the same concentration of cells were seeded on both substrates. Each day, the development of the cells was noted by phase microscopy and the cultures were examined for any apparent differences based on their growth on the two substrates. Random areas of the cultures were photographed on a daily basis.

Maintenance and Incubation: On Day 1, an additional 200 ul of warmed defined culture medium was added to each well in both control and experimental groups and returned to the incubator. On Day 2, 100 ul of warmed defined culture medium was added to each well. All cultures were examined periodically under a phase contrast microscope to check for contamination and, each day, representative pictures were taken of various cultures.

Endothelin Treatment: Endothelin (stock concentration 100uM) was a generous gift from Dr. Arco Jeng of Novartis Corp., Summit, NJ. Cultures in the endothelin treated groups were treated with 40pM to 100nM ET-1. These concentrations were incorporated into warmed defined growth media. On day zero, when the cultures were seeded, the endothelin was added in with the starting concentration of cells. For the following two days, the feeding schedule followed that of the control groups, only with the ET-1 supplemented in the media.

Culture Fixation: To retain a permanent record of the cultures during their developmental period, the cultures were fixed and stained. The fixative used was either



7.5% - 10% formalin or 70% ETOH. To fix a culture, the medium was first removed and the fixative added into the well unit for 10 minutes. The fixative was removed and the culture was then rinsed with water, followed by the addition of 100-200 ul of Crystal Violet Stain. The dye was immediately removed followed by an additional rinse with running tap water. The culture was then air dried. In many cases, where select cultures were fixed on a day to day period, the lid to the culture dish was pulled back only to reveal the row of choice. Usually, 70% ETOH was used under this circumstance because it was less likely to produce toxic fumes as harsh as those of formalin, which may pose a potential danger to the remaining cultures. Staining of these fixed cultures were delayed until all wells were ready to be fixed and stained

Image Analysis: An image analysis system (Leica) was used to compare the growth effects of control vs. cultures treated with endothelin. This system was able to project the fixed and stained images under the microscope onto a computer screen. The images were saved and analyzed for morphological changes. To standardize the areas under analysis, the measured circumference (6mm) of the wells was taken and, using a protractor, a point reference in the middle of the well was made. This point reference was etched into the back bottom of the well. With the center as a reference point, there were 6 frames that were measured, 3 for the inner portion of the well and 3 for the outer edge. Each set of three included a frame positioned at 3, 9 and 12, with respect to a standard clock face (Fig. 1).

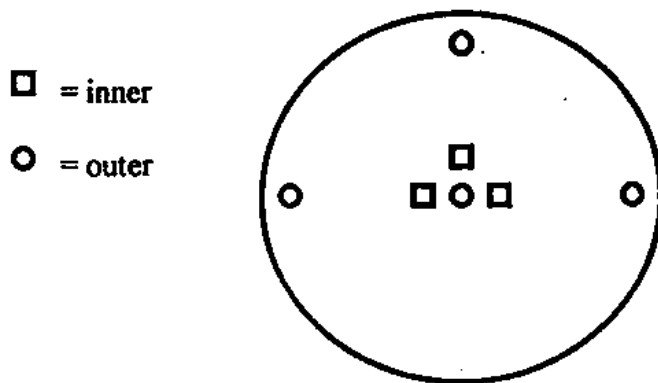


Fig. 1. A model plate of dissociated culture showing areas which were image analyzed.

Both control and endothelin-treated cultures were compared for the area occupied by clumps of cells or by lengths of the fibers. The area of the cell clumps or the fibers were converted as a percentage of the total area of the measured frame. The percentages between treated versus control cultures were statistically compared using the Student t-test.

Explants: For explantation of optic tectum, the optic lobes of embryos 8 days of age were removed under sterile conditions as previously described and placed in the Maximow depression slide with a small amount of HBSS. The meninges were removed and the brain tissue was further dissected into small pieces approximately  $1\text{mm}^3$  and explanted onto laminin coated units of 96 well cluster dishes which were pretreated with medium. Removal of the two drops of medium from the wells just before the explants pieces were placed in the center helped to wet the surface of the wells for proper positioning of the explants. After each row of wells were filled, the explants which were left to adhere to the culture substrate were fed with two drops of either control medium,

which was unsupplemented F12 or F12 which contained either 20 or 100 pmol of ET-1. Each treatment group contained at least 16 explants. The cultures were maintained at 37°C at 5% CO<sub>2</sub> for the subsequent 4 days and monitored each day by phase microscopy and photographed.

## Results:

### A. Eight day dissociated cultures:

i. Trypsin treated cells: In a trial run, the optic lobes were found to be dissociated after enzymatic digestion, since the cell suspension was cloudy, indicating individual cells were now attained. However, the solution also contained stringy residues of connective tissue. When viability test was performed, there was an extremely high percent of dead cells (100%). Therefore, this method was not feasible for further study.

ii. Mechanically dissociated cells: Passage through different gauge needles proved to be a more useful approach to disrupt the optic lobe. Single cells that were phase bright, indicative of live cells were observed. The viability range of cells was about  $200 \times 10^4$  to  $250 \times 10^4$ . Several cell samples were subjected to trypan blue due exclusion test to establish a mean cell count.

iii. Morphology of Cultured Cells: After 24 hours in culture, cells were examined with the phase contrast microscope. Cells cultured on collagen did demonstrate some growth. There were evidence of small clumps amongst the otherwise single cells, but little or no fiber formation was seen (Fig. 2) Throughout the four day culturing period, the cells were found to have a very limited degree of adhesion to the collagen substrate. When dishes were moved, the cells became dislodged. However, when cultures were seeded on laminin coated wells, a distinct progressive differentiation of single cells to clumps to fiber formation was observed. After 24 hours on laminin, cultures which were mostly composed of small clumps were seen (Figs. 3,4). After 48 hours, larger clumps of about 5-6 cells were formed and thin outgrowths or neuritic fibers

were observed to develop around the clumps (Fig. 5). After 72 hours, a more elaborate network of fibers interconnecting the cell clumps were seen (Fig. 6). After 96 hours, there was a complex network of fibers and larger clumps.

iv. Histology of cells: Cells grown on preplated laminin were not affected by the fixation procedure with either 70% ethanol or 10% formalin fixative treatment. No increase in the amount of dead or floating cells was observed when only one row was fixed which allowed the growth of the other cultures to continue. The neuronal culture in control media in the laminin substrate also did not show decreased cell density after staining with crystal violet to reveal cells.

v. Maintenance of cultures on laminin: The feeding schedule which seemed to both maintain culture and which promoted the progressive differentiation of the optic lobe cells was found to be 200ul of warmed defined optic lobe media after 24 hours with an additional 100 ul of the warmed defined media with 48 hours. After 72 hours, there was no need to add more media because it was noted that the cultures did not appear to dry up as they did if cultures were left unfed in the first and second days.

#### B. Cell density:

The most effective cell density for the progressive differentiation of optic lobe cells had a range of  $200 \times 10^4$  to  $250 \times 10^4$  cells. At this density two drops, about 200 ul, of the cell suspension was seeded onto the pre-coated culture dishes. Cultures beginning with a cell density greater than  $250 \times 10^4$  were found to grow too quickly, due to a high amount of seeded cells. In these cultures, fiber networks were well established by the second day. Also, in cultures where there was a cell count that was fewer than 200

$\times 10^4$  cells, progressive differentiation from cells to clumps to neurite formation did not take place due to a lack of cells.

### C. Treatment with endothelin:

Cells cultured treated with 40pM or 100 nM Endothelin did not demonstrate any significant difference in cell growth or cell density as compared to controls (Figs. 7-11). Treatment with fixative did not cause a significant decrease in the cell density of the plated optic lobe neuron cultures, either with or without endothelin treatment. The developmental stages of differentiation were the same in morphology in the treated groups as well as in the controls. There was no increase of dead cells seen, therefore, treatment with endothelin did not appear to enhance or decrease the numbers of dead or non-viable cells within the culture.

ET-treated cultures did not seem to have increased numbers of the non-neuronal glial cells than the control. The glial cells appeared in proportion to the neuronal cells as in the control culture.

Treatment with 200pM or 40 pM of ET did not cause a significant increase or decrease in the cell density or growth of the pre-plated optic lobe cultures. These cultures remained close to or were not significantly different than control cultures, which were also plated on laminin. After one day, and for all other following days, there was not a noticeable increase of floating or dead cells in the cultures.

1. Image analysis: The profiles of the development of the optic lobe cultures which were analyzed by the Leica Image analysis system did not show any statistically significant difference in the endothelin treated cultures versus the control

cultures. The data was not significantly different for both the growth of the clumps of cells as well as for the growth of the fibers. Table 1 shows an example of data acquired from the image analysis system.

		ET-1 Treated		Control		SIG.
		Clumps (%)	Fibers (%)	Clumps (%)	Fibers (%)	
Well:	Left	1	1	0	1	NS
	Inner Right	0	0	0	2	NS
	Top	1	3	0	5	NS
Well:	Left	7	4	2	3	NS
	Outer Right	1	5	3	2	NS
	Top	5	3	5	2	NS

Table 1. An example of a comparison of the growth pattern between ET treated and control cultures after 4 DIV (days in vitro). Areas occupied by clumps of cells or fibers are represented as a % of the total area measured.

#### D. Explants:

In our first experiment, after 24 hours, 45/48 of explanted pieces were noted to have attached to the underlying substrate. This is confirmed when the cluster dishes were removed on the stage of the phase microscopes and the attached explants did not move nor were found to be floating. Three explants, one from each group were not attached and did not show further development. In all groups, the periphery of the explants showed outgrowth of cells which anchored the explants. On the second day of incubation, the endothelin treated groups showed a clear distinction in being more advanced in neurite development than the control group. In control cultures, 27% of the cultures showed short outgrowths of fibers from the explant. In treated explants, low concentrations of endothelin promoted 82.5% of the cultures with neurite outgrowth while higher concentrations of the vasoactive peptide promoted 77% of the cultures to

develop outgrowths. By the third day, more differences were noted between the cultures. While the control cultures remained fairly undifferentiated with little fiber development (Fig. 12), the endothelin treated cultures showed differences in their neurite outgrowth. Low concentrations of endothelin promoted the formation of long thin fibers (Fig. 13) while the outgrowths from cultures treated with high concentrations of ET-1 remained short, but thick (Fig. 14). A repeat of this experiment with tissue from 6 day embryo showed similar results in that ET-treated cultures showed earlier neurite development in more explants than those treated with F12 medium alone. In this experiment, 27% of controls showed neurite outgrowth and 56% of ET-1 (low concentration) and 53% (high concentration) treated explants developed fibers by the first day in vitro.



Fig. 2: Dissociated 8 day optic lobe cell culture on collagen after 1 day incubation. Small, but sparse cell clumps are seen.



Fig. 3: Dissociated 8 day optic lobe cell culture on laminin on day 0. Single cells are seen.

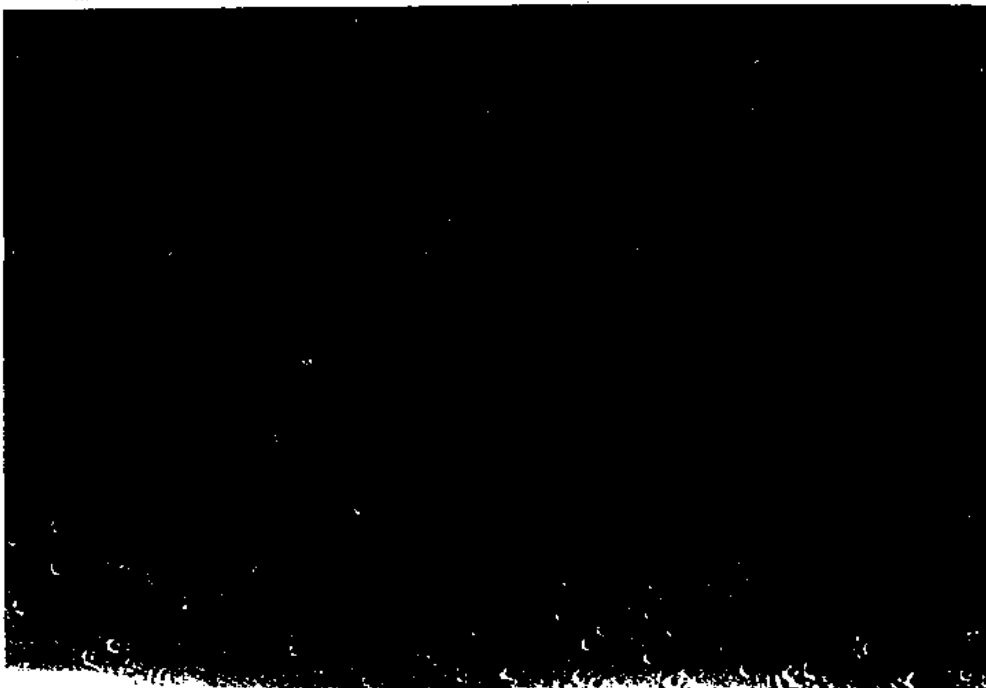


Fig. 4: Dissociated 8 day optic lobe cell culture on laminin after 1 day. Small clumps have begun to form.



Fig. 5: Dissociated 8 day optic lobe cell culture on laminin on day 2. Larger clumps have developed and the outgrowths or neuritic fibers are beginning to develop.



Fig. 6: Dissociated 8 day optic lobe cell culture on laminin after 3 days. A more elaborate network of fibers interconnecting the cell clumps are seen.

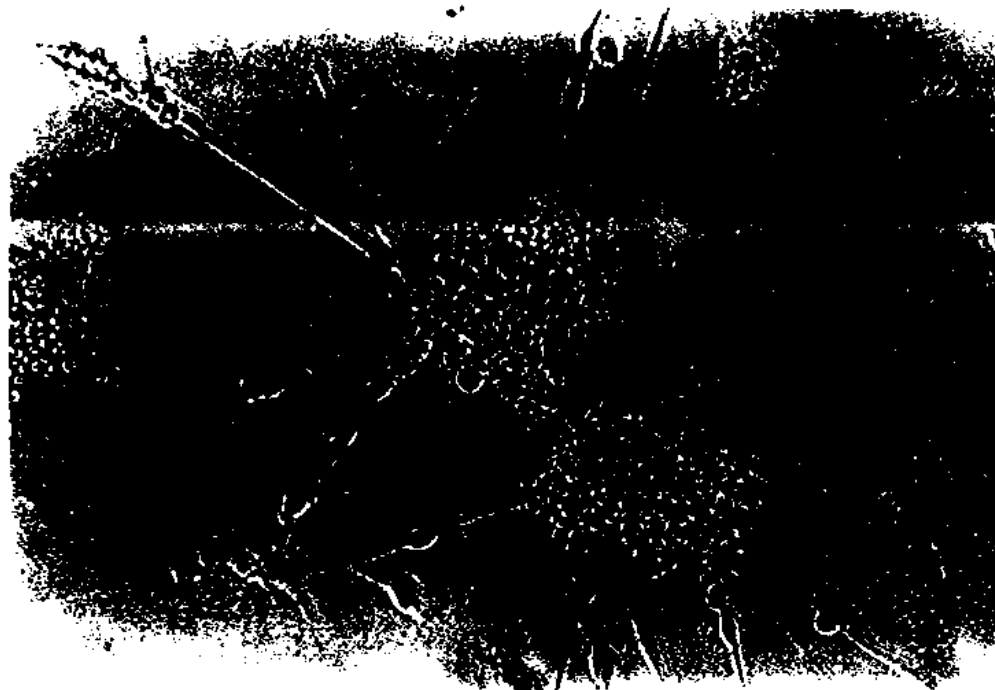


Fig. 7: Dissociated 8 day optic lobe cell culture on laminin treated with ET (45 nM) at time of seeding. Single cells are seen.



Fig. 8: Dissociated 8 day optic lobe cell culture on laminin treated with ET (45 nM) after 1 day. Small clumps have begun to form.



Fig. 9: Dissociated 8 day optic lobe cell culture on laminin treated with ET (45 nM) on day 2. Larger clumps, as well as neuritic outgrowths have begun to develop.



Fig. 10: Dissociated 8 day optic lobe cell culture on laminin treated with ET (45 nM) after 3 days. More fibers interconnecting larger clumps of cells are seen.

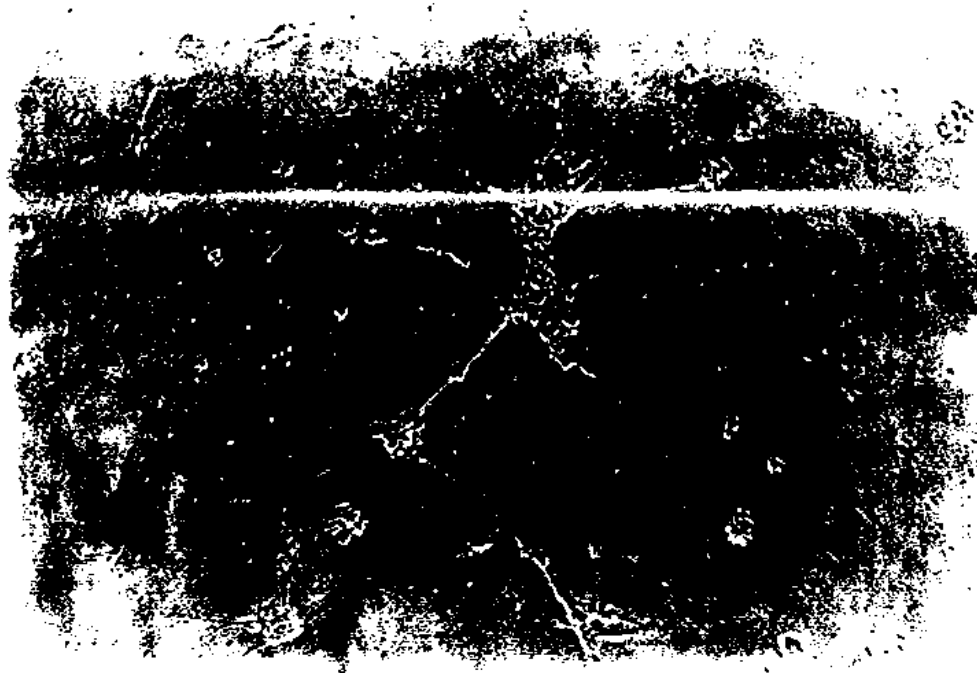
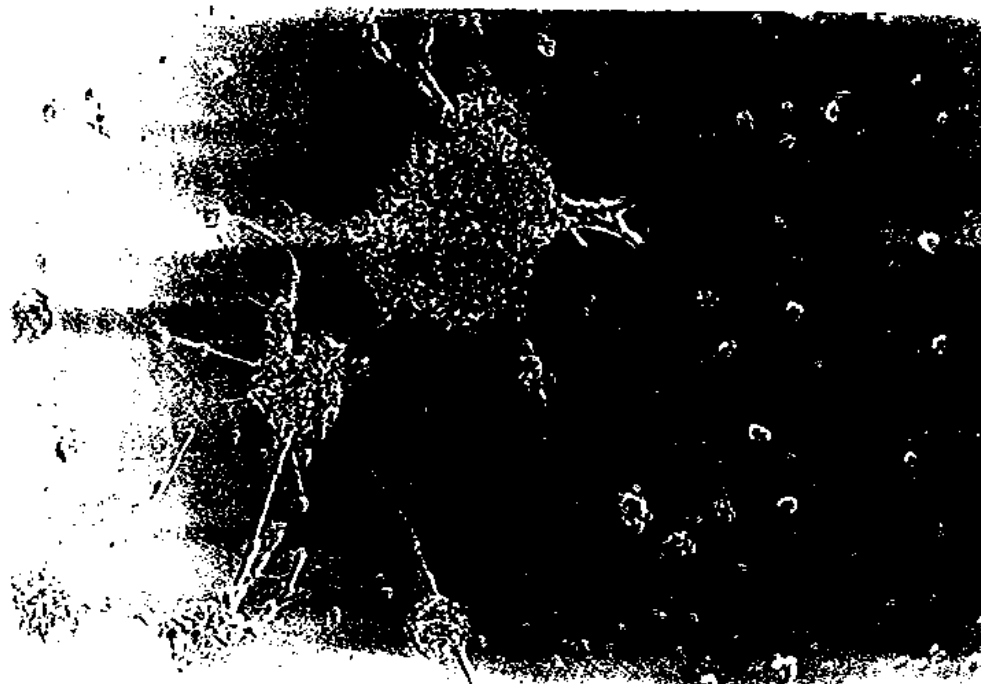
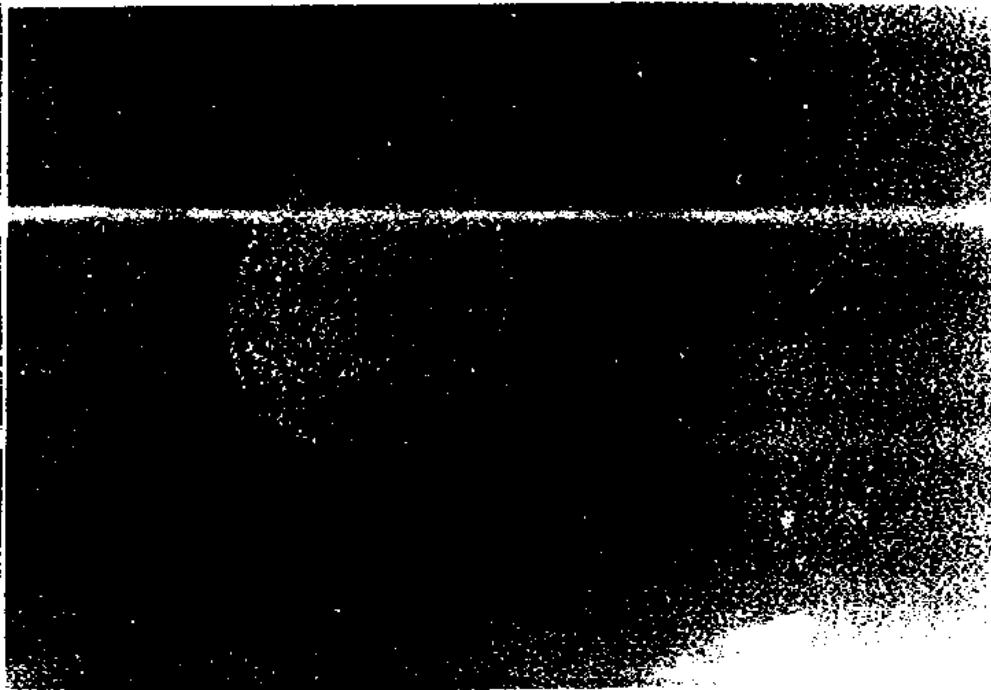


Fig. 11: Dissociated 8 day optic lobe cell culture on laminin treated with ET (45 nM) after 4 days. A complex network of fibers along with larger clumps are seen.



**Fig. 12: Control 6 day optic lobe explant culture after 3 days. There is little fiber development seen and fairly undifferentiated.**

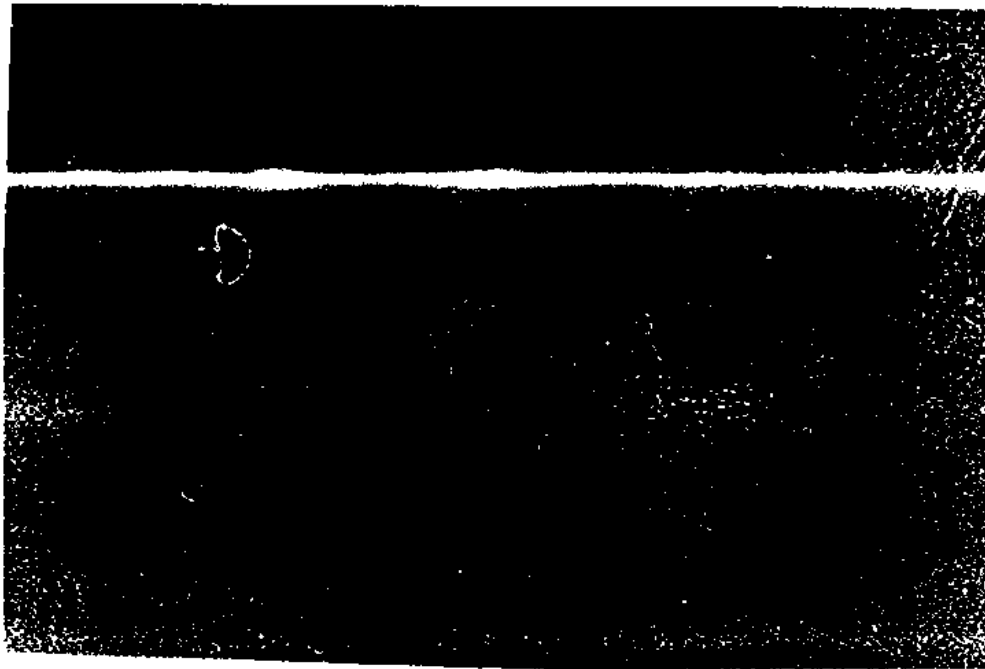


**Fig. 13: Low ET concentration (40pM) treated 6 day old explant culture on day 3. The formation of long, thin fibers are seen.**



Fig. 14: High ET (200pM) concentration treated 6 day old explant culture on day 3.

Short, thick fiber growth is seen.



#### Discussion:

Since its discovery in 1988, endothelin has been the focus of many recent studies. As it was first isolated from cultured endothelial cells, it was known to have very potent vasoconstrictive properties (Takuwa, 1989; Simonson, 1990; MacCumber, 1990). As research continued, it was found to have diverse roles including contraction of non-vascular smooth muscle, cardiovascular regulation and proliferation of smooth muscle and other cells (Simonson, 1990).

Not until Kurihara's gene knockout study was endothelin's developmental role discovered (Kurihara, 1994). This study proved to be the landmark discovery leading to an increased interest in endothelin's role in development. By disrupting the ET-1 gene in mouse embryonic stem cells, mice that were deficient of endothelin died of respiratory failure at birth. These mice also exhibited morphological abnormalities of the pharyngeal-arch-derived craniofacial tissues and organs. These abnormalities may have been attributed to a disruption in the neural crest development, such as migration and glial/neuron trophic interactions (Kurihara, 1994).

In the complementary study performed by Hsu and Jeng (1995), a further developmental role of endothelin was demonstrated. When ET-1 was added to developing Leghorn chick embryos, the overall sizes of the head, trunk, beak or wings were increased by 5-14%. The lengths or widths of the telencephalic or optic lobes and the diencephalon in the embryonic brains were also increased by 4-14%. How endothelin can affect growth and development of the brain is not known and is the focus of Dr. Hsu's studies (Hsu and Jeng, 1995).



As part of the encompassing endothelin project, the aim of this research was to demonstrate endothelin's developmental role at the cellular level. While Hsu and Jeng's *in vivo* animal model gave very interesting results in the growth promoting effects of endothelin, the physiological role of endothelin at the cellular level could not be specified; hence we wanted to create an *in vitro* model that could elucidate endothelin's effect. Although there are many areas of the brain to be studied, the optic lobe itself gave a good starting point (Adler, 1979 & 1982). There is an abundance of literature on the culture of the optic lobe, and because of its location on the superior portion of the brain, it was easily accessible.

One of the first aspects of the research was to find the optimal age of the embryo. From the literature (Adler, 1979, 1982, & 1985; Pearson, 1972), we determined that eight-day-old optic lobes were used to study differentiation. The media which would allow optimal growth of the cultures also had to be determined. Because it is known that endothelin binds to FCS (fetal calf serum) (Jeng et al., 1990), we opted to use a defined medium. We found that medium containing progesterone; putrescine, sodium selenite, insulin and transferrin was optimal (Adler, 1979, 1982, & 1985).

Dissociation of the tissue to achieve single cells was attempted in two forms. The enzymatic method using trypsin did not work for our purposes, as our data indicated that there were no viable cells remaining following trypsinization. We believe that the cells were too sensitive to this method and were therefore damaged as a result. Also, it is known that trypsin is responsible for the metabolism of endothelin (Huggins & Pelton, 1997). If all of the trypsin could not be inactivated, then the effects of endothelin may have been masked.

Mechanically dissociating the cultures using surgical blades as well as passing the tissue through needles proved to be the method of choice since this protocol that was not as harsh on the cells as enzymatic dissociation. This method allowed us to start with a good cell count, in addition, the cells that were observed at time of plating to be mostly in the single cell form (see Fig. 3).

The substrate on which the cultures were grown also proved to be very critical for the experiments. The two types of substrate that were tested were laminin and collagen. Laminin and collagen are both naturally occurring extra cellular matrices critical to cell migration and other cell/substrate interactions (Halfter et al., 1983; Adler et al., 1979 & 1985). After several culture series, it was evident to us that the cultures were not conducive to growth on the collagen substrate (see Fig. 2). There was little or no differentiation, and poor adhesion to the substrate. The poor adhesion to the substrate was particularly evident when fixation and staining caused the cells to be dislodged from the wells.

On the other hand, we were able to demonstrate, on the laminin substrate, the progressive differentiation of the cells (see Figs. 3-6). On day zero, single cells were seeded. During the next three to four days, we were able to show the formation of small clumps, to larger clumps, to the larger clumps with the beginning of fiber formation, then larger clumps with an extensive network of fibers. These changes suggest that within the culture, there was mitotic activity as well as differentiative events occurring. Upon fixation, the cells had very good adhesion to the substrate and there was little to no loss of cells. The protocol we developed whereby we could fix and stain selective rows of cells also did not have any apparent adverse effect on the living cultures. Using 70% ethanol

rather than formalin during this procedure was less harsh to the living cells. We also noted that allowing the culture to airate under the laminar flow hood at the time of fixation did not increase the number of dead cells nor did it appear to inhibit the further growth of cells remaining in culture.

This laminin culture model served as the template for all of the endothelin experimentation (Adler et al., 1985). Before any of the endothelin trials could begin, we had to make sure that the amount of cells seeded was consistent from experiment to experiment. The cell density that we found to work optimally on the laminin ranged from  $200 \times 10^4$  to  $250 \times 10^4$  cells.

When we began testing the endothelin on the cultures, we were not able to show any significant differences in the treated cultures as compared to the control groups (see Figs. 7-11). Even with a wide range of endothelin concentrations, from 20 pM to 100 nM, we were not able to demonstrate a significantly different culture. Since we knew from Hsu and Jeng's *in vivo* animal model that endothelin did indeed exhibit growth-promoting characteristics, we surmised that there had to be an alternate way to demonstrate endothelin's developmental role *in vitro*. We believe that we were not able to demonstrate any differences with endothelin on the dissociated cell cultures because it was probably due to the supplements in the defined media that were able to mask the effects of endothelin.

Therefore, we tried an alternate protocol using explants of embryonic brain. These explants were placed on the laminin substrate and in media that was free of any of the growth factors and hormones. These explants were a good choice because they maintained the integrity of the tissue, they adhered to the substrate with good affinity, and

also allowed us to utilize younger embryos. Explants were also supported by medium which was free of any of the factors; hence, we also did not need to be concerned with any of the growth factors competing with or masking the effects of the endothelin.

In low dose treated (40 pM) endothelin-treated cultures, we were able to consistently see that there were long, thin fibers extending from the tissue (see Fig. 13). The endothelin high dose (200 pM) exhibited, or was correlated with, shorter, thicker fibers extending from the cells (see Fig. 14). This finding is consistent with previous studies by Hsu and Jeng (1995), which showed that ganglia explants which developed short thick neurite bundles were treated with high concentrations of TPA and ET.

The biphasic effect of ET-1 on neurite outgrowth of optic lobe explants is reminiscent of the effects of phorbol ester on dorsal root ganglia (Hsu et al, 1984). This potent tumor promoter was found to be a most effective inducer of neurite development in explanted embryonic ganglia. However, while low doses of 12-O-tetradecanoylphorbol 13 acetate (TPA) induced the development of radial outgrowths which were long and thin, high concentrations of TPA produced short thick fascicles. The presumed mechanism for TPA effects is likely through the formation of neurite-neurite interaction mediated by the neural cell adhesion molecule (NCAM), since an antibody to NCAM altered the morphological appearance of the outgrowths of neurites from explants treated with high concentrations of TPA (Hsu, 1989). Whether the same cellular mechanism is underlying what we observed with ET-1 can only be speculated. That TPA and ET both affect the phospholipid cascade of signal transduction is one common basis that exists between these neurite-promoting agents. In addition, both TPA and ET are effective without other supplemental agents in vitro. Unlike TPA, which is

effective in medium with or without the growth hormones and supplements, we were not able to demonstrate ET effects in the complete medium necessary to maintain dissociated cultures, yet the growth promoting effects of ET on explants was very clearly and quickly seen in unsupplemented F12 medium.

In conclusion, these experiments showed that under the most restricted tissue culture conditions (laminin and unsupplemented media containing ET) the vasoactive peptide appear to have neurite promoting effects. However, in dissociated cell cultures, the growth promoting effects of ET could not be demonstrated.

**Summary:**

In summary, the goal to develop an *in vitro* protocol for embryonic chick optic lobe cultures were partly accomplished because the following optimal conditions have been established:

- That eight day embryonic optic lobes will show growth and differentiation within four days in culture from single cells to cell aggregates with nerve fiber networks.
- Mechanical dissociation by passing through decreasing needle gauges provided single cells with high viability.
- Laminin substrate is critical for optic lobe culture growth and differentiation.
- Endothelin effects on optic lobe differentiation can only be observed in explants in medium without hormones and growth supplements.

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