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The Effects of Endothelin-1 on Cell Migration of Corneal Endothelial Cells

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THE EFFECTS OF ENDOTHELIN-1 ON CELL MIGRATION OF CORNEAL ENDOTHELIAL CELLS

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ABSTRACT

Wound healing occurs through the processes of cell proliferation (mitosis) and/or cell migration. The corneal endothelium in humans is known to be mostly non-proliferative. As a result, wounds in the corneal endothelium heal by migration. A 21-amino acid peptide called Endothelin-1 (ET-1) is studied in the Crawford research lab and is a known mitogen, i.e. increases cell proliferation. Data suggests that Endothelin-1 can stimulate cell proliferation in the corneal endothelium. This fact led to the first hypothesis that ET-1 stimulated wound closure in this cell layer. I explored this hypothesis using a bovine model of corneal endothelial cells isolated and grown in 10% calf-serum DMEM media. Half the cells were treated with ET-1 and the other half were left in media alone (control). The cells were wounded mechanically and allowed to heal for 72-hours with photo micrographs taken every 24-hour to record wound closure. The data suggested that ET-1 stimulated wound closure because the cells treated with ET-1 had a higher percent wound closure when compared to the control. This experiment was modified to test my second hypothesis, that inhibition of cell proliferation would inhibit wound closure. In this experiment, half the cells were treated with 5-Flurouracil, an inhibitor of cell proliferation. The data showed that the prescence of 5-FU decreased the percentage of wound closure, but did not eliminate it. ET-1 was still able to increase wound closure in the presence of 5-FU by 10.8% compared to the cells treated with 5-FU alone. My third hypothesis then became that ET-1 stimulates cell migration. I explored this hypothesis using a chemotaxis assay. The endothelial cells were

loaded with Calcein-AM, a fluorescent dye, and migration was examined with a Chemotx chemotaxis system. The cells were treated with serum free media (control), 0.1 nM ET-1, 1 nM ET-1, 10 nM ET-1, or serum (positive control). They were allowed to incubate for 48-hours and fluorescence of migrated cells was recorded. The data suggests that ET-1 stimulates wound healing, promotes wound closure in the absence of proliferation, and that ET-1 stimulates cell migration.

Keywords: cornea, corneal endothelium, Endothelin-1, wound healing, cell migration, cell proliferation

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CHAPTER 1

INTRODUCTION

The cornea is a dome-shaped organ located on the anterior surface of your eye that to maintain function has to remain transparent.[1] It provides the eye with 65-75% of its focusing power and shields the eye from harmful bacteria, viruses, and environmental factors. The cornea consists of five layers starting with the outermost layer the epithelium, then the Bowman's membrane, the stroma, Descement's membrane, and finally the innermost (posterior) layer the endothelium.[2] The cornea is unique in the fact that it is avascular or does not have a direct connection to the blood supply of the body.[3] Therefore, to receive nutrients and dispose of waste the cornea relies on aqueous humor located in a chamber behind the cornea and the tears.[4]

The fluid balance in the cornea is maintained by the endothelial cells. The aqueous humor naturally leaks from the anterior chamber behind the cornea to the stromal layer. If left uncompensated, the stroma would swell and the cornea would become opaque and no longer function properly. Therefore, the endothelium pumps salt and the excess fluid out of the stroma and back into the chamber. The endothelium also serves as a barrier function and protects the inside of the eye from any molecules that have diffused across the cornea. The endothelial cells are hexagonal in shape and the endothelium has a honeycomb appearance naturally.[5]

As we age, the endothelium loses this honeycomb appearance due to the fact that the endothelial cells do not proliferate. Instead, when damage to this cell layer occurs the cells heal through migration or the cells enlarge, extend, and slide over to fill the gap.[6] This becomes a problem when major damage happens or the cells become overextended, because there is not enough cell material to cover the wounds. If the endothelium has gaps it loses its function and the stroma swells becoming opaque and the patient blind.[7] The only cure to restore eyesight is a corneal transplant, which is very expensive and has issues being a long-term solution.[8]

Endothelial cells are located throughout the body and not just in the cornea. They are normally found in organ cavity linings and especially the linings of veins and arteries. The vascular endothelial cells have been found to produce a chemical called Endothelin-1 (ET-1).[9] In fact, Endothelin-1 is produced by all endothelial cells in the body. Endothelin-1 is a 21 amino-acid peptide and is the most potent vasoconstrictor known. Endothelin-1 has also been shown to induce proliferation in some tissues.[10]

In the lab, a bovine model was used. Bovine corneal endothelial cells (BCECs) are very similar to the human eye, but they have the ability to proliferate. As a result, they are easy to grow in cell culture and are an affordable method to study the cornea. The bovine cells are made more comparable to the human cornea by serum-starving before every experiment to greatly reduce their proliferative abilities.

A wound can heal by two processes known as cell proliferation (mitosis) or cell migration. Since ET-1 is a known mitogen, my first hypothesis was that ET-1

should increase the rate of wound closure. My second hypothesis was that the inhibition of cell proliferation would inhibit wound closure. Finally, my third hypothesis was that ET-1 stimulated cell migration. I further examined this hypothesis using a chemotaxis assay. This assay works by placing the cells on top of the filter, allowing them to incubate and migrate through the porous filter, and finally removing any excess cells from the top of the filter before analyzing.

CHAPTER 2

METHODS AND MATERIALS

Bovine eyes were obtained from a local abattoir and brought back to the lab on ice; so the cells could be isolated immediately. The isolation process starts by removing the excess muscles and treating the eyeballs with an anti-bacterial solution for thirty minutes. Next the cornea was removed and placed endothelium side up in an aluminum well contoured to the shape of a cornea. An enzyme solution (dispase) was placed on the endothelium and the corneas were incubated for seventy five to ninety minutes. The endothelial cells were then removed using a silicon tipped tool and placed in media. The cells were pelleted and the supernatant was poured off and the cells re-suspended in media and placed in a tissue culture flask. The endothelial cells were allowed to grow and were sub-cultured so that one 25 cm^2 flask (T-25) makes three T-25 flasks full of cells. The cells were incubated and allowed to grow again in a 37 degrees Celsius incubator.

For the first set of experiments, the cells of two T-25 flasks were then liberated from the plastic and basement membrane they have secreted using 0.25% Trypsin. Once the cells were in solution, they were centrifuged and the cells pelleted. The supernatant was discarded and the cells were re-suspended in 24 mL serum-free DMEM media. For this experiment, two 12-well flasks were used and

1mL of media containing cells and 1 mL of serum-free DMEM media was placed in each well. The cells were then allowed to grow to confluence in each well.

Before beginning each set of experiments, the cells were serum-starved, meaning they were feed a DMEM media solution containing no calf serum for 24-hours. This ensures that any proliferative ability of the bovine cells has been greatly reduced and any results observed is due to the addition of our variables and not something found in the calf-serum. The cells were then mechanically wounded with a specialized tool. The tool was made of silicon and had a blunted tip a few millimeters wide (Fig 2.1). The tool was drug down the center of the well creating a wound approximately 0.5-1 mm wide. The media was discarded and replaced with fresh media, the first plate receiving serum-free media only (control) and the second plate receiving media containing a 10 nm concentration of ET-1. Each well was photographed using an inverted phase contrast microscope at 0-hours, 24-hours, 48-hours, and 72-hours. Zero-hours is the photographs immediately after wounding and the time period of the next set of photographs is based on the amount of time that has passed since the first set of photographs (Fig 2.2). The cells were also given fresh solutions of media before the 48-hour photos to ensure plenty of Endothelin-1 was available for the cells for the remainder of the experiment. After the 72-hour photos were taken, the cells were disposed of and the four sets of photographs were analyzed using an image analysis computer program from Simagis Research and the percent wound closure was calculated.

The second hypothesis was investigated using the same proliferation assay as described above in the second set of experiments. However, the treatments varied slightly from before. Half our wounded cultures were treated with 5-

Fluorouracil (5-FU). 5-FU is a chemotherapy drug that is a known inhibitor of cell proliferation by blocking DNA replication.[11] Therefore, in our cells treated with 5-FU we should see no wound closure if all wound closure is due to cell proliferation. Each 12-well plate contained two different experimental groups. The first six wells were treated with serum-free media (control), the second six wells were treated with media containing 100 nM ET-1, the third set of six wells were treated with a media containing a 7.68 μM concentration of 5-FU, and the fourth set of six wells were treated with both concentrations of ET-1 and 5-FU. The experiment continued as described above with photo micrographs being taken every 24-hours and analyzed with Simagis Research.

For the cell migration experiment, the cells were serum-starved and the cells of one T-25 flask were liberated from the plastic and basement membrane they have secreted using 0.25% Trypsin. Once the cells were in solution, they were centrifuged and the cells pelleted. The supernatant was discarded and the cells were re-suspended in 18 mL EBSS. Next, the cells were loaded with a fluorescent dye Calcein AM (Invitrogen). 18 μL Calcien AM was added to the cell solution for a 1 μM final concentration. The cells were incubated at 37˚C for thirty minutes, while being lightly agitated. The wells of the ChemoTx chemotaxis plate (Neuroprobe) were filled with 299 μL of solution containing the respective concentrations of the chemicals the cells would be treated with in upcoming steps (Fig 2.3). A filter was placed above the wells that contained pores with an 8 μm diameter. The cell solution was removed from the incubator and the cells were pelleted using a centrifuge. The endothelial cells were then re-suspended in serum-free DMEM media with the exception of the positive control group which was re-suspended in

DMEM media containing calf serum. The re-suspended cells were then treated with nothing (negative control), 0.1 nM ET-1, 1 nM ET-1, or 10 nM ET-1. Using a multi-channel pipet, 50 μL of each treated cell solution was loaded on top of the ChemoTx filter. The plate was incubated for 48-hours and the cells were allowed to migrate through the pores of the filter. The cells remaining on top of the filter were removed after 48-hours by rinsing the top of the filter with serum-free media and wiping the excess fluid off with KIM wipes. The plate was centrifuged to allow any cells stuck on the bottom of the filter or floating in the media to be brought to the bottom of the well.[12] The plate was then placed in a Biotek plate reader and the fluorescence was recorded at an absorbance of 525 nm and an excitation wavelength of 485 nm.

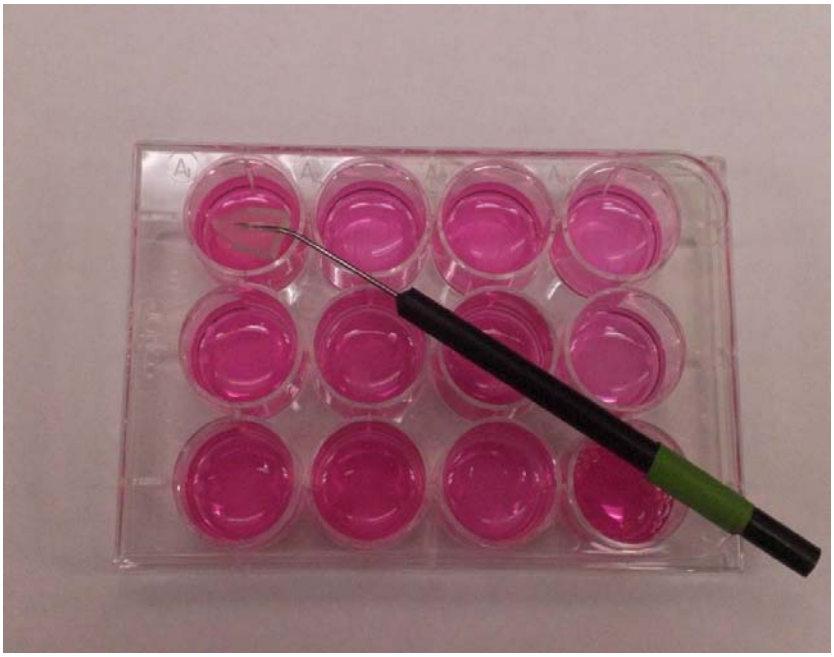

Figure 2.1: Wound Closure Assay. 12-well plate filled with DMEM media and silicon tipped wounding tool.

Figure 2.2: Photo micrographs. Photo micrographs of the cell cultures at 0-hour, 24-hours, and 48-hours.

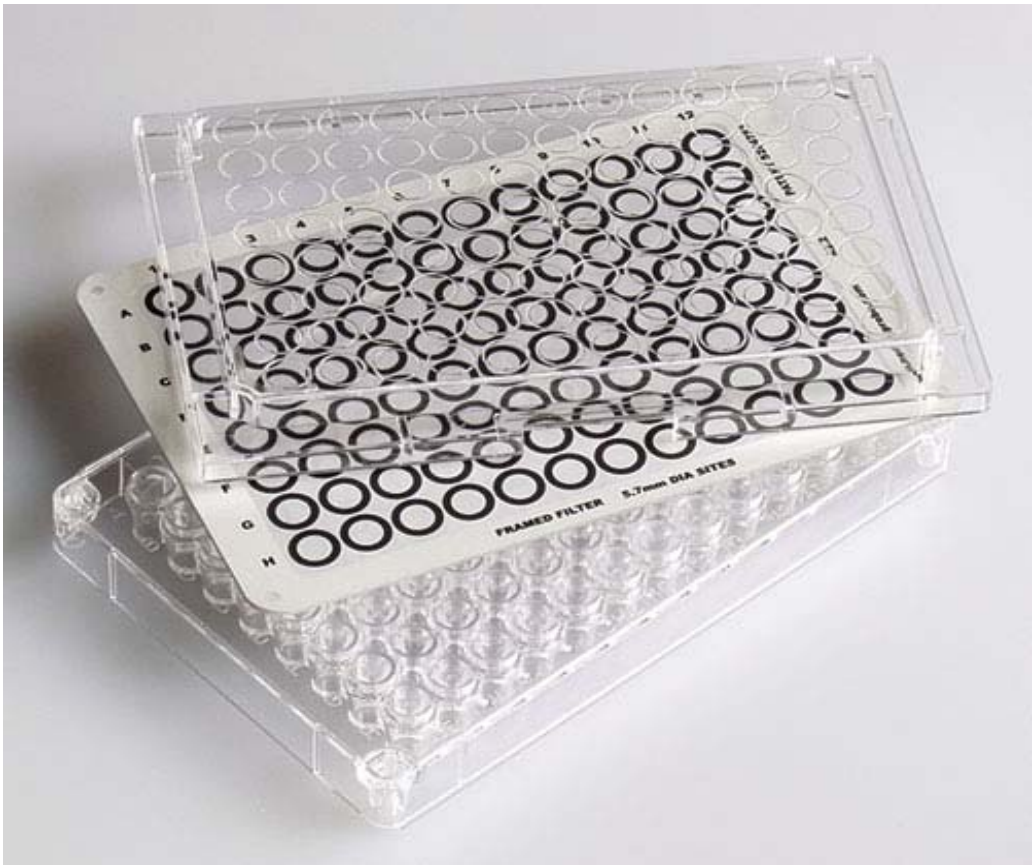

Figure 2.3: Chemo Tax chemotaxis assay. The 96-well chemotaxis plate and porous filter with pores 8µm in diameter.

CHAPTER 3

RESULTS

For the first set of experiments, we were looking at the effects of ET-1 on wound closure, but not the specific mechanism (proliferation or migration). The results were as follows (Fig 3.1): the cells treated with 10 nM ET-1 (represented by open circles) had statistically significant (t-test) more wound closure than the control group (represented by closed circles).

In the second set of experiments, we were investigating the amount of wound closure when cell proliferation was inhibited. When comparing the different treatment groups to the control, we see the highest percent of wound closure in the group treated with Endothelin-1 (Fig 3.2). The groups treated with 5-Flurouracil showed less wound healing when compared to the control, but the group treated with Endothelin-1 and 5-Flurouracil showed 10.8% percent higher wound closure when compared to the group treated with just 5-Flurouracil. If we take a closer look at the percent of wound closure at 48-hours (Fig 3.3) and compare the values using an ANOVA, followed by the Bonferroni multiple range test, we see that the values of the experimental groups when compared to the control all have a p-value less than 0.05 which makes them statistically significant. We chose to take a closer look at the

48-hour data because the percent closure in the control and the group treated with Endothelin-1 was approaching one hundred percent but had not reached one hundred percent yet. At 72-hours several of the wells had reached one hundred percent wound closure and therefore the 48-hour photos gave a better picture of the relationship between the groups

In the cell migration assay, the fluorescence of each well of cells was recorded, and the average fluorescence of each treatment was calculated (Table 1). To correct for possible differences in dye loading, the results were normalized by calculating percent change from control. When the different concentrations were compared, we saw that there was an increase in cell migration in the cells treated with ET-1 with an apparent inverse dose response (Figure 3.4). The percent change from control was statistically significant compared to the control using a one-sample t-test and the 0.1 nM and 1nM concentrations had a p-value less than 0.0001 while the 10 nM concentration had a p-value equal to 0.0003. In order to be statistically significant, a p-value less than 0.05 are needed. Also, when we compare our average fluorescence for our negative control with the positive control, we see a large increase in average fluorescence in the positive control (Table 1).

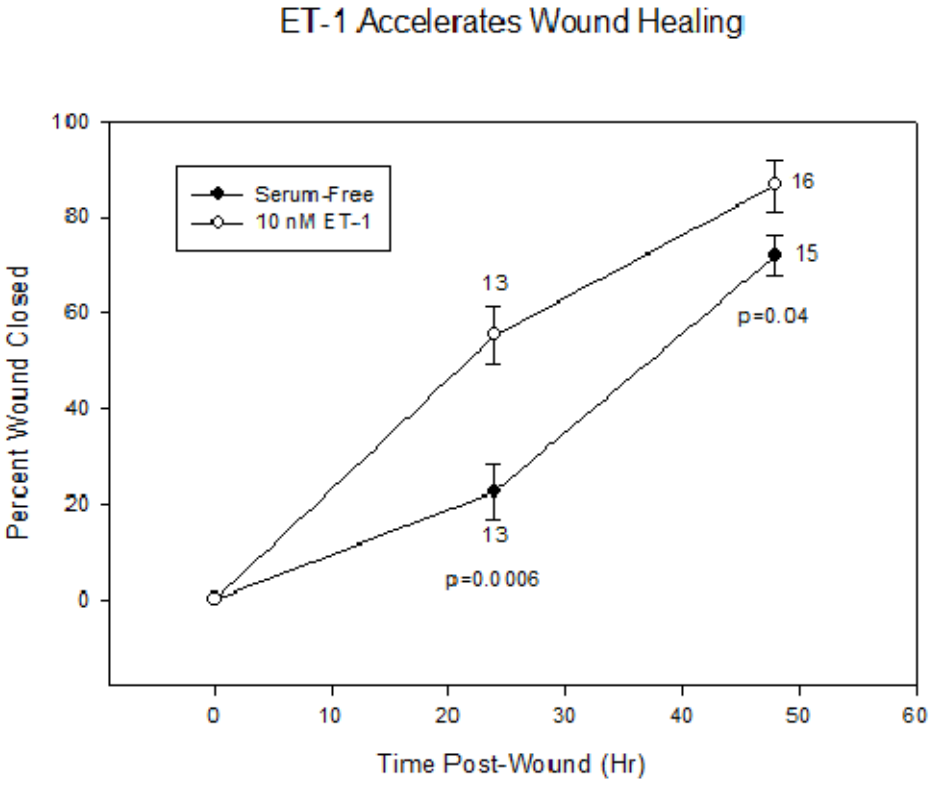

Figure 3.1: ET-1 Accelerates Wound Healing. Mechanically wounded bovine endothelial cell cultures were allowed to heal for 48-hours total with analysis of wound closure performed every 24-hour. Half the cells were treated with 10 nM ET-1 and the rest were treated with serum-free DMEM media (control).

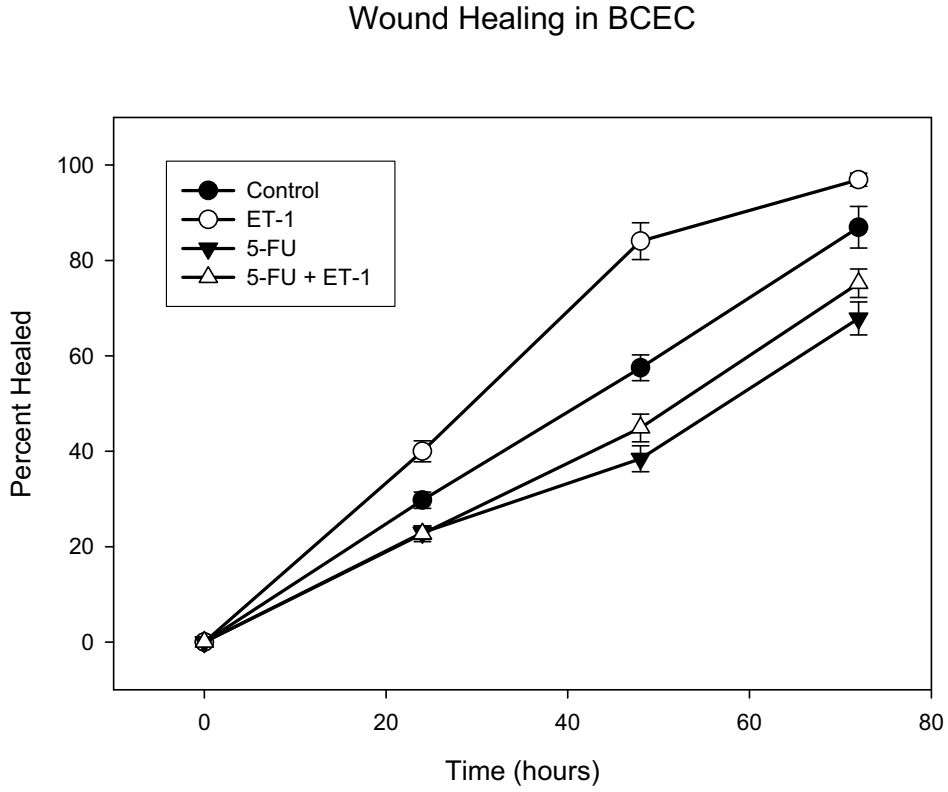

Figure 3.2: Wound Healing in BCEC. Cell cultures of BCECs were mechanically wound and allowed to heal for 72-hours total with percent healed of the wound analyzed every 24-hour. The cells were treated with serum-free DMEM media (control), ET-1, 5-FU, and ET-1+5-FU respectively.

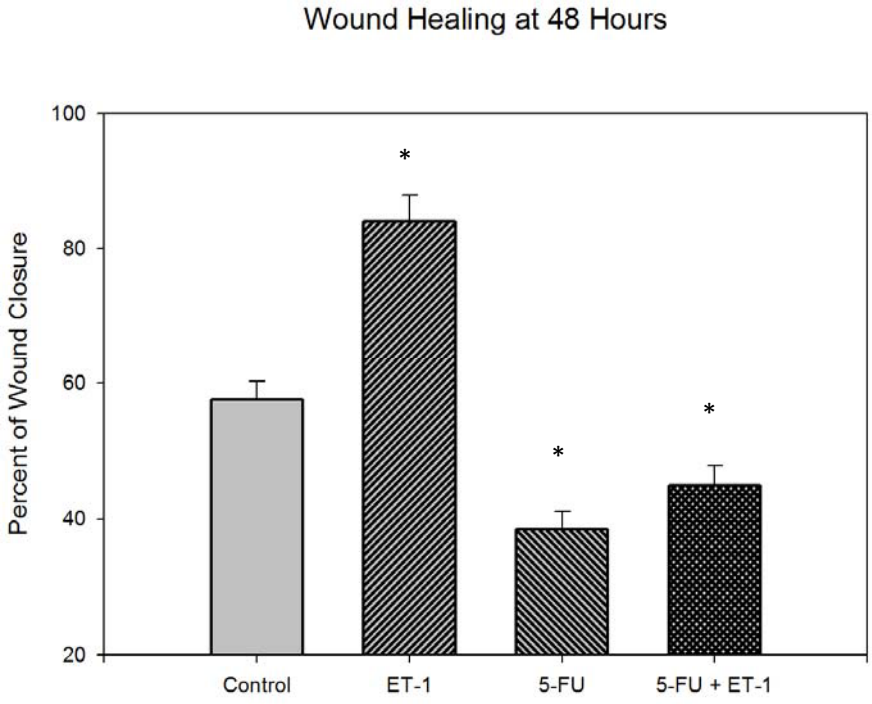

Figure 3.3: A Comparison at 48-Hours. A comparison of three experimental groups to the control in a bovine corneal endothelial cell wound healing experiment at 48-Hours. *p<0.05

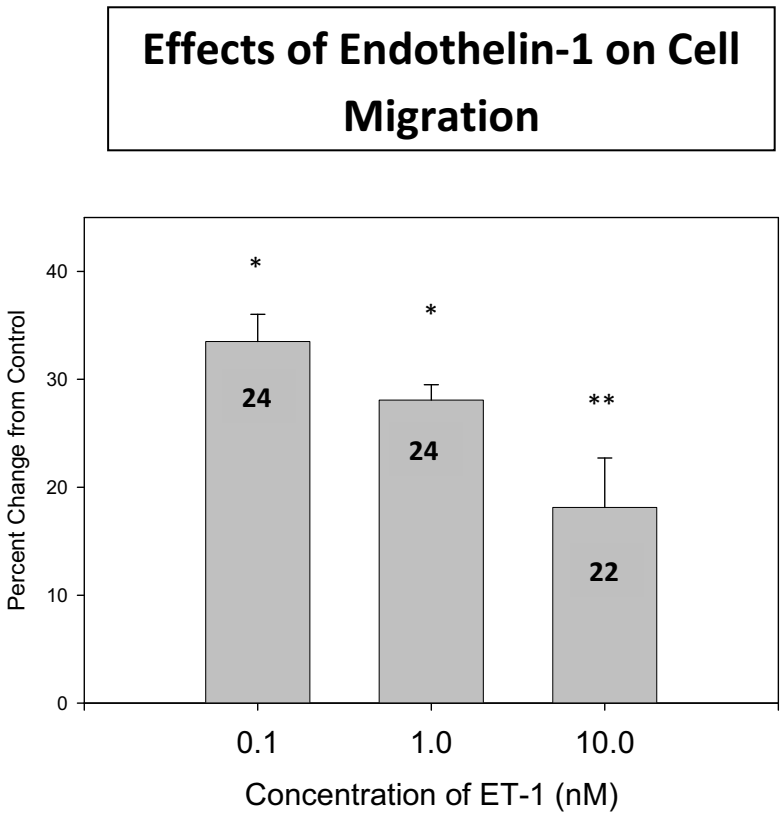

Figure 3.4: Effects of Endothelin-1 on Cell Migration. BCECs were loaded with a fluorescent dye and allowed to migrate through a porous filter for 48-hours. Average fluorescence of the cells at absorbance 485 nm was measured and recorded. The results were then normalized by calculating a percent change from the control. *p<0.0001 **p=0.003

Treatment	Average Fluorescence at 520 nm
Negative Control	9,789
0.1 nM ET-1	13,869.5
1 nM ET-1	13,315.6
10 nM ET-1	11,111.3
Pos. Control (Serum)	35,238.2

Table 1: Average Fluorescence. The total number of trials was 3 with each treatment having a total n=24.

CHAPTER 4

DISCUSSION

In the case of the first experiment, I saw an increased amount of wound closure between the group treated with ET-1 and the control. This data suggested that ET-1 was indeed causing increased wound closure in the corneal endothelial cell cultures, which led to our second hypothesis that if we blocked cell proliferation wound closure would be inhibited since ET-1 is a mitogen. This hypothesis was investigated via our second set of experiments.

In these experiments, the data suggested something slightly different than what was expected. We again observed an increase in wound closure among the group treated with ET-1 only when compared to the control. This data again helped support our first hypothesis that ET-1 stimulates wound closure. When we looked at the groups treated with 5-FU we saw a decrease in wound closure as compared to the control. Again, this was a result we expected. However, the groups treated with 5-FU did not have zero percent wound closure suggesting that the wounds may be healing through cell migration. Comparing the group treated with ET-1 and 5-FU to the group treated with just 5-FU, we see a 10.8% increase of wound closure in the

ET-1+5-FU group. The data suggests that ET-1 may be inducing cell migration, which led to our third hypothesis that ET-1 stimulates cell migration.

The third hypothesis was investigated using a chemotaxis assay. Since we saw a noticeable difference in the average fluorescence between the negative and positive controls, we can safely assume that the chemotaxis assay was a successful way to analyze cell migration in corneal endothelial cells. The increase in fluorescence among the cells treated with ET-1 can be directly correlated to an increase in cell migration. Therefore, based on the results, my third hypothesis that stated ET-1 stimulates cell migration was confirmed. Not only was a difference observed, but the percent change from control was extremely statistically significant with a p value < 0.0001 in the 0.1 and 1 nM concentrations and p-value=0.0003 in the 10 nM concentrations using a one-sample t-test.

In the data a reverse dose response on cell migration was observed as we increased concentration of ET-1. While positive dose response relationships are much more frequently observed, inverse or non-montonic dose responses are not uncommon.[13] For example, a higher concentration may activate a different endothelin receptor. Previous work in the Crawford lab has shown that BCEC express the ET_A receptor, with a Kd of 200pM.[14] Corneal endothelial cells could also possess ET_B receptors that generally have lower affinities for ET-1 and may interact with ET_A receptors to modify their function.[15] In this cell migration assay, the non-monotonic dos response may be due to ET-1 decreasing cell attachment/adhesion as part of increased migration causing decreased attachment of the cells to the filter or “float up” during migration. Another possible explanation could be that higher concentrations of ET-1 cause a change in the structure of the cell due to preparation

for increased cell proliferation that does not favor migration. Cells undergoing mitosis “round-up” and are more likely to detach from the substrate. This eventually may be investigated by sampling the media above the filter (before wiping off unmigrated cells) and determining fluorescence.

In my research with Endothelin-1 as a whole over three and half years, I have been able to conclude three things concerning this peptide. The first being that Endothelin-1 does increase the rate of wound closure/healing in the corneal endothelium. The second being that wound closure still persists in the absence of cell proliferation. Finally, the last piece of the puzzle concerning the effects of this peptide on wound healing has been observed, that not only does ET-1 stimulate cell proliferation, but also cell migration. This correlates with results found by researchers in smooth muscle that found Endothelin-1 can induce migration in pulmonary smooth muscle, but surprising not aortic smooth muscle.[12] This suggests that not only can this peptide work on endothelial cells throughout the body, but also some other types of tissues.

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