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P27 Expression in Endothelin-1 Stimulated Mitogenesis

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P27 Expression in Endothelin-1 Stimulated Mitogenesis

A thesis

Presented to

The graduate faculty of the Department of Biology

Western Kentucky University

In partial fulfillment

Of the requirements for the degree of

Master of Science in Biology

Joseph Kirui

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APPROVAL

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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This work is dedicated to my dear parents, Mr. and Mrs. Andrew Siele, my Wife, Mercy, and our little son Jacob who have been a source of encouragement and support of my graduate work.
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ABSTRACT

Bovine corneal endothelial cells (BCECs) have been shown in our lab to synthesize and secrete endothelin-1 (ET-1). The endothelin-1 binds ETA receptors, this elevates intracellular calcium, stimulate DNA replication and cell division. This investigation explores ET-1’s role as a mitogenic factor in corneal endothelial cells and addresses whether mitogenic activity is mediated through the cyclin dependent kinase inhibitor p27. BCEC were isolated from bovine eyes and grown in DMEM-10% serum in a 5% CO₂, 37°C incubator. Confluent cultures of BCEC were incubated for 24 hours with 100nM Endothelin-1, 1μM BQ123 and BQ123 +100nM Endothelin-1 and some left untreated. Total cellular protein was isolated and the concentration quantified by the Peterson modification of the Lowry protein assay. Protein samples and prestained molecular weight standards were separated by 4-15% gradient SDS-PAGE and transferred overnight to PVDF Hyperbond membrane by western blot. The relative abundance of p27 was detected using a polyclonal antibody for p27 (1:100) and HRP conjugated secondary antibody (1:500) and alkaline phosphatase conjugated secondary antibody (goat-anti rabbit). The membrane was then washed thoroughly with PBST and treated with enhanced chemiluminescence (ECL) or enhanced chemifluorescence (ECF). The data show a marked decrease in cellular p27 in cells treated with 100nM endothelin-1. ET-1 appears to have mitogenic properties in BCEC by downregulating the expression of p27. Stimulation of cell proliferation may be mediated by suppressing the expression of p27, a cyclin dependent kinase inhibitor.
CHAPTER 1

Introduction

The Cornea

The cornea is a transparent dome shaped window that covers the anterior of the eye. It is a highly organized group of cells and proteins that is smooth and clear but strong and durable. The cornea receives its nourishment from tears and aqueous humor that fills the chamber behind it. The cornea must remain transparent to refract light properly. To see well, all of the cornea must be free of any particles, which prevent light from reaching retina (1). The corneal organ is comprised of five layers, each having a distinct function. The outermost layer is the stratified epithelium which comprises approximately ten percent of the tissue’s thickness. Its main function is to block the passage of microorganisms and nonliving particles into the eye. It also provides a smooth surface for absorption and distribution of oxygen and cell nutrients to the rest of the cornea. The epithelium also acts as a sensory tissue; it is filled with thousands of tiny nerve endings that make the cornea extremely sensitive when rubbed or scratched. The epithelium possesses a basement membrane which serves as foundation on which the epithelial cells anchor and organize themselves. Lying below this membrane is a transparent sheet of tissue known as Bowman’s layer, which is composed of collagen (strong layered protein fibers). If the Bowman’s layer is injured, it can form a scar as it heals, and this scarring can result in vision loss.
Beneath the Bowman’s layer is the stroma, which comprises about 90% of cornea’s thickness. It consists of water (about 78%) and collagen (about 16%); its main function is to give the cornea strength, elasticity and form. Collagen’s unique shape, arrangement and spacing are essential in producing light-conducting transparency. Under the stroma is Descemet’s membrane, which is a thin but strong sheet of tissue that serves as a protective barrier against infection and injuries. The Descemet’s membrane is made by the endothelial cells that lie below; these endothelial cells, collectively called an endothelium, form the innermost layer of cornea.
**Corneal Endothelium**

The corneal endothelium is a single layer of cells located at posterior of the cornea. The chief function of the corneal endothelium is to maintain corneal transparency (2). As fluids leak slowly from the aqueous humor into the stroma, the endothelium pumps this excess fluid out of the stroma (1, 4). Without the pumping action, the stroma would swell with water, and become hazy and eventually opaque. A healthy eye maintains a perfect balance between the fluids moving in and out of the cornea. In the early stages of corneal development, the endothelial cells actively divide to form the endothelium (3). When these cells mature they establish the gap and tight junctions, which results in the cessation of proliferation. The endothelial density decreases with age (4), and this loss is further accelerated by disease (5). The capacity of the corneal endothelium to regenerate is severely limited in man and other primates (3, 4). Thus corneal wound repair is achieved primarily by cell enlargement and by redistributing existing cells (4). It is not clear why corneal endothelium exhibits limited capacity to proliferate, but molecular events governing the cell cycle may play a role (4, 6). Recent findings on the expression of cell cycle associated proteins in donor human corneas suggest that human corneal endothelial have not exited the cell cycle but are arrested in G-1 phase, suggesting these cells could possess proliferative potential (6).
**The Cell Cycle**

The cell cycle is a series of events in a eukaryotic cell between one cell division and the next. It consists of four phases: the G1 phase, the S phase the G2 phase (together these phases are called interphase) and the M phase. The M phase is composed of two tightly coupled processes: Mitosis and cytokinesis. In mitosis the cell’s chromosomes are replicated and divided between the two daughter cells. In cytokinesis the cell cytoplasm physically divides. Cells that have temporarily stopped dividing are said to be in a state of quiescence (also called G0 phase), while cells that have permanently stopped dividing due to age or accumulated DNA damage are said to be senescent. The cell cycle coordinates events needed for the growth of all eukaryotic cells. Events such as DNA replication (S-phase) and cell division (M-phase) occur in the right temporal sequence and proceed in an orderly fashion (7). In addition, the cell cycle receives and integrates signals from diverse growth regulatory pathways, ensuring that the cell grows only in the presence of the appropriate signals and in the right environment (8). There are two key classes of regulatory molecules that determine cell’s progress through the cell cycle: cyclins and cyclin dependent kinase.
**Important regulatory proteins in the cell cycle**

In mammalian cell division, the progression through the first gap phase (G1) of the cell cycle and the initiation of DNA synthesis (S phase), of which are mitogen-dependent, are cooperatively regulated by several classes of cyclins and cyclin dependent kinases (CDKs) (8). Cyclins control the progression of cells through the phases of the cell cycle by forming complexes with cyclin dependent kinases (CDKs). The CDKs are a family of heterodimeric serine/threonine protein kinases, each consisting of a catalytic CDK subunit and an activating cyclin subunit. In most eukaryotes, there are different CDKs that control the different stages of the cell cycle. They coordinate the cell cycle by acting as on and off switches (7). When a CDK is on, the cell cycle progresses through the stage that particular CDK controls. When a CDK is off, cell cycle stops when it reaches the stage controlled by that particular CDK (7, 8).

Regulation of cyclin-CDKs complexes occurs at multiple levels, including the assembly of cyclin and CDK. Cyclins are the regulatory subunits of these complexes, and the enzymatic activity of CDK is dependent on physical interaction with one of the cyclin proteins. In addition, CDK activity can be negatively regulated by a group of proteins collectively termed CDK inhibitors (CDKIs) (5). The CDKIs are a newly recognized family of proteins (7) and are well characterized for their role as negative regulators of the G-1 phase of the cell cycle (9). CDKIs that govern these events have been assigned to one of two families based on their structures and CDK targets. The first family includes the INK4 proteins (inhibitors of CDK4). The inhibitors work by binding to
CDK4 and CDK6 (8); they bind isolated CDK and prevent its association with the cyclin (7).

The second family of inhibitors, CIP/KIP, inhibits a broad array of cyclin-CDK complexes (8) hence they are designated as universal CDKI. They interact with various CDK complexes, which eventually lead to inhibition of kinase activities of pre-activated G1, cyclin E-CDK2, and cyclin D-CDK4/6. The CIP/KIP family includes p21, p27 and p57. Members of KIP proteins share a great deal of homology, e.g., p27 protein have a 42% amino acid homology with p57 at amino terminal domain. The amino terminal domain mediates inhibition of CDK (10).

P27 was originally identified as an inhibitor of CDK complex that is induced by antimitogenic signals (3, 25). Forced expression of p27 results in G1-phase cell cycle arrest (9). Normally the progression of cell cycle G1/S requires p27 proteolysis, which is initiated by the phosphorylation of p27 on threonine-187 (9, 10).
**The p27 cyclin dependent kinase inhibitor**

P27 is a member of the universal cyclin dependent kinase inhibitor (CDKI) family and its gene was cloned in 1994 (26). P27 expression is regulated by cell contact inhibition and by specific growth factors such as transforming growth factor TGF-β (10). P27 has several functions such as tumor suppression, regulation of drug resistance in solid tumor, the promotion of apoptosis and cell differentiation.

The cycle through which a cell moves as it divides and replicates itself is tightly regulated to control cell growth; cyclins and their partners, the cyclin dependent kinases (CDKs), are instrumental in driving the cell cycle forward. Cyclin D/CDK4 complexes function early in the cell cycle, while cyclin E/CDK2 complexes function later. P27 acts as an inhibitor of CDK2 activity and is required early in the cell cycle for the assembly of cyclin D1/CDK4 complexes. This suggests that p27 permits early progression but suppresses later progression of the cell cycle (11).

P27 was first identified in cells treated with transforming growth factor (TGF-β) or by stimulation of contact inhibition; under these conditions it was found as an inactive form bound to cyclin-E-CDK-2 (10,11). The protein was purified from a cyclin-E-CDK2 affinity column and characterized by its strong inhibitory activity toward cyclin-E – CDK2. P27 can directly inhibit the enzymatic activity of CDK-cyclin complexes and arrest cells in G1 (10). The association of p27 with CDK4- cyclin D or with CDK2-cyclin E complexes prevents phosphorylation of CDK4 on Threonine-172 and CDK2 on Threonine -160 via a CDK activated kinase (9, 10, 11). The level of p27 protein expression is high in G0/G1 resting cells and declines as cell progresses towards the S-
phase. Overexpression of p27 has been shown to inhibit entry into the S-phase in normal and malignant cells (5).

Mutation in genes encoding the KIP/CIP inhibitors p21 and p27 are rare. In mice, p27 behaves like a tumor suppressor. The loss of only one gene copy decreases the levels of p27 and susceptibility to cancer. The increase in levels of p27 in quiescent cells rapidly reduces after stimulation with mitogens (12, 13).

**Endothelin**

Endothelin (ET) is a potent vasoconstrictor/vasopressor peptide originally characterized from the culture supernatant of porcine aortic endothelial cells and consists of 21 amino acid residues with two sets of intrachain disulfide linkages (12, 13). Sequence analysis of the cloned cDNAs for porcine and human endothelin precursors showed that endothelin is produced in endothelial cells from a precursor of approximately 200 residues (preproendothelin) (13, 14). A presumptive 39 residue (porcine) or 38 residues (human) is thought to be generated from preproendothelin. The amino acid sequences of the mature porcine and human endothelin are identical. Since its discovery in 1985 and its first detailed characterization in 1988, 3 ET isoforms have been described: ET-1, ET-2 and ET-3 (13). The vasoconstrictor potency is highest with ET-1. All ET isoforms are composed of 21 amino acids with two intrachain disulfide bridges. This 21-residue (mature) endothelin is produced through unusual proteolytic processing of big endothelin between Trp21 and Val22 residues. A variety of cells including, vascular endothelial cells, heart, brain, spinal chord, pulmonary epithelial cells, glomerular mesangial cells, renal epithelial cells, monocytes and macrophages synthesize ET. After
biosynthesis, ET is released into the circulation or it diffuses towards the underlying vascular smooth muscles cells. ET-1 production in glomerular mesangial cells is stimulated by a variety of cytokines and growth factors, including IL-β, TNF-α, TGF-β, PDGF and vasopressin. Inhibiting factors include nitric oxide, prostacyclin and atrial natriuretic (14).

Endothelins exert their effects by binding to two distinct cell surface receptors, ET-AR and ET-BR. These receptors are found on smooth muscle cells, myocytes and fibroblasts. The binding to the receptor leads to various cellular outcomes i.e. efflux of cellular calcium, release of intracellular stored calcium, and inhibition of Na/K ATPase (12, 13). The ET-BR binds the three peptides isotypes with equal affinity; in contrast, ETAR binds ET-1 with higher affinity than the other isoforms. Both receptors belong to the G-protein coupled receptor (GPCR) system and mediate biological responses from a variety of stimuli, including growth factors, vasoactive polypeptides, neurotransmitters, hormones and phospholipids (12).

The physiological importance of the cleavage of big ET is indicated by the increase in vasoconstrictor potency. However the half-life of the cleavage product is less than a minute (12). It is rapidly removed from the circulation by the kidney, the lung and vascular endothelial cells. In addition to the potent vasoconstrictor and vasopressor actions, endothelin has been reported to produce a wide spectrum of biological effects including contraction of airway and intestinal smooth muscles, release of eicosanoids and/or endothelium derived relaxing factor from vascular beds, and inhibition of renin release from the glomerulus. It can stimulate the proliferation of vascular smooth muscle cells and fibroblasts. (4, 9, 13).
Recently, investigations into the role of the endothelin in mitogenesis have provided evidence of the importance of the ET-1 in cancer. Data suggest that ET-1 participates in a wide range of cancer relevant processes such as cell proliferation, inhibition of apoptosis, matrix remodeling, bone deposition and metastases (12). ET-1 stimulates DNA synthesis and cell proliferation in various cells including vascular smooth muscles, osteoblasts, glomerular mesangial cells, fibroblasts and melanocytes. ET-1 is also a mitogen for different cell types including prostate, cervical and ovarian cancer cells. In primary cultures and established ovarian carcinoma cell lines, spontaneous growth was significantly inhibited in the presence of antagonists, specifically the one which blocks ET-AR (15). The mitogenic activity of ET-1 can be amplified by synergistic interactions with other growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), transforming growth factor and interleukin-6 (IL-6) (16).

A specific ETA receptor, BQ123 is a well-characterized synthetic pentapeptide with high selectivity for the ETA receptor and thus high potency for ETA receptor antagonism (17).
**Hypothesis and Purpose of Research**

A long term potential outcome of this line of investigation is to develop a potential therapy to increase cell proliferation in corneal endothelial cells. The proposed approach is to reduce the expression of p27 by transiently stimulating corneal endothelial cells with endothelin-1 (ET-1). We also investigated whether the specific ET-1 receptor antagonist, BQ123, may inhibit the suppression of p27.

Primary cell culture of bovine corneal endothelial cells (BCECs), a widely used model of the corneal endothelium, will be used to determine whether the expression of p27 can be altered (reduced) in the presence of ET-1. We hypothesize that upon treatment with ET-1, the expression of p27 will decrease. If the expression of p27 is decreased, we expect that the cell cycle arrest will not occur, and the cells will continue to proliferate. This (decrease of p27), will be of potential therapeutic interest (at the moment the only way to correct damaged cornea is by transplantation, which is costly and has potential side effects). An agent that transiently inhibits p27 expression may be useful for treating an injured or diseased cornea by allowing replication of endothelial cells, and hence promoting healing of this tissue.
CHAPTER 2

Materials and Methods

Isolation and culture of bovine corneal endothelial cells (BCECs)

Cow eyes were obtained from Kirby and Poe abattoir (Alvaton, Kentucky). The globe was placed cornea side up in a shallow dish lined with paper towel. A 2X antibiotic solution (from 100x stock penicillin/streptomycin/ nystatin) was dropped on the globes and soaked for 30 minutes at room temperature after being covered with sterile gauze. The cornea was dissected with a 1-2mm scleral rim and transferred endothelium side up to the eyecup and rinsed with dispase dissolved in Earle’s Balanced Salt Solution (EBSS) (Invitrogen, CA). The cornea was incubated with dispase-EBSS at 37°C for 90 minutes. The endothelium was then gently scraped using spatula with a tapered silicon rubber tip. Cells were aspirated with a sterile Pasteur pipette and transferred to a sterile 15ml centrifuge tube containing 5ml of basal medium (Dulbecco’s Eagle’s Medium -DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% calf serum (Hyclone, Logan, UT) and 100μl gentamycin, 100μl fungizone (antibiotic-antimycotic) and pelleted by centrifugation at 600xg for 2 minutes. The supernatant was decanted and the cell pellet resuspended with 5ml of basal media. Cells from two eyes were then transferred to a 25cm² culture flask (T25). The cultured cells were maintained in basal media in a 5% CO₂, 37°C incubator. For subculture, cells were treated with trypsin (0.05% in calcium + magnesium with EBSS containing 0.6mM EDTA) and were pelleted by centrifugation at 600xg for two minutes. The cell pellet was then resuspended in appropriate volume of
basal media and transferred to flasks. After the cells reached approximately 99% confluence, they were deprived of serum for twenty-four hours, some were treated with 100nM ET-1, 1µM BQ123, combined (BQ123 + ET-1) and others left untreated i.e. they were not treated with either ET-1 nor BQ123.

Cell lysis and isolation of proteins

BCECs were washed with cold EBSS, gently scraped from T25 flasks, and washed with ice cold phosphate buffered solution (4.38g NaCl, 1.38g NaPO₄ monobasic in 1Liter) cells were then lysed with lysis buffer (20mM Hepes, 10% glycerol, 0.1mM dithiothreitol (DTT), 1% tritox-100x and protease inhibitor cocktail) on ice for 30 minutes with occasional rocking. The cell lysate was collected and sonicated on ice 2x for 10s, and the contents were aliquoted into 1ml tubes, and stored in a -20°C freezer for further analysis. The protein content was assessed by the Peterson modification of the Lowry protein assay using bovine serum albumin as a standard (18).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Cell lysates were solubilized in 5x solubilizing buffer (0.145M Tris, 0.025% bromophenol blue, 5% SDS, 25% glycerol) at a ratio of 1:4, and subjected to 90 °C for five minutes, followed by addition of beta-mercaptoethanol (BME) to a final concentration of 5%. Samples containing 20-40µl BCECs lysate were then analyzed by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). The 4-12% precast gradient gel was run at a constant voltage of 50V for three hours. The conditions of electrophoresis were
as described by Laemmli (29). Half of the gel was placed in coomassie blue stain to check for protein migration. Before transfer, the unit was assembled as follows: two sheets of Whatman #1 paper and one sheet polyvinylidenedifluoride membrane (PVDF) were cut to 8cm x 5cm. The PVDF membrane was carefully wetted in methanol (to remove hydrophobicity); the rest of the assembly unit then wetted with transfer buffer (1% SDS, 20% methanol in 1x running buffer (0.3g tris base + 1.44g glycine brought up to 1L with DI water) for at least 5 minutes. A blot sandwich was built on the sponge in the following order: Whatman paper, gel, PVDF, Whatman paper, each piece was layered on the sandwich. The sandwich was placed in such a way that proteins will migrate out of the gel and onto PVDF membrane. (PVDF side of the sandwich was towards the positive electrode). The separated proteins were then transferred from the gel to the PVDF membrane at a constant voltage of 30V overnight. The tank transfer blotting method was used to transfer proteins to the membrane.

**Immunodetection of proteins**

After overnight blotting, the PVDF membrane was removed from the transblot apparatus and placed in a flat dish and blocked with 5% non-fat dry milk (i.e. 25g non fat dry milk in 500ml 1x PBS) for one hour at room temperature. The primary antibody rabbit anti-human p27 polyclonal antibody, (Zymed Laboratories, San Francisco, CA) was diluted at 1:100. In this case 6μl of antibody was added to 600μl of medium (PBS + 0.1% Tween-20, i.e. PBS-T). The primary antibody was then added and the membrane was incubated for 1hr, at 37°C. 30ml of PBS-T was then added to the dish while shaking, at 37°C for 5min, PBS-T was decanted and the process repeated until the sheet was
washed 6 times. To test for non specific binding, immunoblots were performed with the primary antibody omitted.

The membrane was then incubated with secondary antibody. The experiment was performed independently using two sets of antibodies, goat anti-rabbit polyclonal antibody diluted at 1:500 conjugated to horseradish peroxidase (HRP) and goat anti-rabbit polyclonal antibody (diluted at 1:200) conjugated to alkaline phosphatase (AP) (Southern Biotech, Birmingham, Alabama) was used. Secondary antibodies were added to the membrane in dish and incubated for 1hr at room temperature with gentle shaking. The antibody was decanted and 30ml of PBS-T then added to the dish with gently shaking at 37°C for 5min. The PBS-T was decanted and the process repeated until the sheet has been washed six times at 5 minute intervals. The membrane was then treated with the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Buckinghamshire UK). The ECL-treated membrane was exposed to a digital imager (Storm). In the second experiment the membrane was treated with enhanced chemifluorescence (ECF) (Amersham Pharmacia Biotech, Buckinghamshire UK) and exposed to digital imager.
**Results**

*Protein concentration*

While the protein concentration in the lysate of ET-1 treated cells was highest, it was not statistically significant, i.e., protein concentrations of the cell lysate were 2.90µg/µl for untreated BCEC, 3.10 µg/µl in 100nM endothelin-1 treated, 2.99µg/µl in BQ123 + endothelin-1 and 2.97 µg/µl in BQ123 treated BCEC.

*Quiescent BCECs express p27*

Expression of p27 was determined by western blot analysis. When BCEC cells reached 90% to 99% confluence, they were placed in serum free medium for 24 hours and proteins harvested. 15µg of total proteins were loaded on the second and lane while 30µg loaded on the 3rd lane the rest of the lanes were left (unloaded). A band below 28.8 molecular weight marker consistent with p27 was obtained. As predicted, the band at approximately 27kDa was thicker in lane 3 (figure 1).

*Endothelin-1 downregulates p27*

To test for the expression of p27 in BCECs, confluent cells were placed in serum free medium for 24 hours. Some were left as control, (untreated) and the rest treated with 100nM Endothelin-1 for a further 24 hours. 30µg of each sample was subjected to SDS-PAGE on a 4-12% gel and immunoblot analysis performed. The endothelin-1 treated cells showed a marked decrease in p27 expression (figure 2).
Non specific binding

To test for possible non specific binding, two membranes from two experiments were compared. When cells reached 90% to 99% confluence, they were placed in serum free medium for 24 hours (left untreated), and sample subjected to SDS-PAGE on a 4-12% gel and immunoblot analysis performed in the absence of primary antibody. Non specific binding to a protein between 75kDa and 48 kDa markers was observed in two experiments (figure 3). The band corresponding to p27 was absent. Although this does not prove the identity of the band as p27, it does show the observed band at approximately 27 kDa (figure 1) was not due to non-specific binding (presence of the secondary antibody) (figure 3). A positive control utilizing purified p27 protein was not performed.

The effect of ET-1 Receptor antagonist (BQ123) is unclear

To elucidate the role of Endothelin-1 receptor antagonist (BQ123), confluent cells were deprived of serum for twenty four hours (untreated) and then treated with 100nM ET-1, or BQ123, or both combined (BQ123 +ET-1). 30μg of each sample was subjected to SDS-PAGE on 4-12% gradient gel and immunoblot analysis performed. The expression of p27 seemed to be downregulated by BQ123, however a poor transfer of proteins to the membrane was observed (Figure 4). The experiment was repeated three times and Statistical analysis was performed with repeated-measures ANOVA, P=0.0026 and post test Student-Newman-Keuls (SNK) (Figure 5).
Quiescent BCECs express p27

**Figure 1**

Expression of P27 (determined by western blot). When BCEC cells reached 90% to 99% confluence, they were placed in serum free medium for 24 hours and proteins harvested. 
A: 15 µg of total proteins loaded on the second lane while 30 µg loaded on the 3rd lane. The third lane has a thicker band. The left arrow shows 28.8 molecular weight markers 
B: Western blot analysis in the absence of primary antibody. There was absent of any band (except the molecular weight standards) when western blot analysis was performed in the absence of primary antibody.
Endothelin-1 treated and untreated BCEC culture

Figure 2
Effect of endothelin-1, on the expression of p27, when cells reached 90% to 99% confluence, they were placed in serum free medium for 24 hours. Some were left as control, (untreated) and the rest treated with 100nM Endothelin-1 for a further 24 hours. 30µg of each sample was subjected to SDS-PAGE on a 4-12% gel and immunoblot analysis performed.
Non specific binding

Figure 3
To test for non specific binding the membranes were compared from two experiments

A: When cells reached 90% to 99% confluence, they were placed in serum free medium for 24 hours (left untreated), and sample subjected to SDS-PAGE on a 4-12% gel and immunoblot analysis performed in the absence of primary antibody.
Lane 1: 30µg, lane 2: 15µg, lane 3: 30µg, lane 4: 15µg

B: When cells reached 90% to 99% confluence, they were placed in serum free medium for 24 hours (left untreated), and sample subjected to SDS-PAGE on a 4-12% gel and immunoblot analysis performed in the absence of primary antibody.
Lane 2: 15µg, lane 3: 30µg, lane 4: 0µg protein, lane 5: 0µg protein

Right arrow: location of non specific binding (band).
Endothelin treated and receptor antagonist treated BCEC proteins

![Image of gel with labeled lanes: 1. ET-1, 2. Untreated, 3. BQ123E, 4. BQ123, 5. Cells treated with ET-1 and BQ123.]

**Figure 4**

Expression of p27 in BCEC treated with ET-1, BQ123+ET-1 and 1μM BQ123. When cells reached approximately 99% confluence, they were deprived of serum for twenty-four hours (untreated) and then treated with 100nM ET-1, 1μM BQ123, both combined (BQ123+ET-1). 30 μg of each sample was subjected to SDS-PAGE on 4-12% gradient gel under reducing conditions, and immunoblot analysis performed. *Lane 2*: cells treated with ET-1; *lane 3*: control-untreated cells; *Lane 4*: cells simultaneously treated with ET-1 and BQ123; *Lane 5*: cells treated with BQ123. The left arrow represents 28.8 MW marker, while the right arrow shows an unknown protein (a non-specific binding between 75MW and 48 MW markers).
Figure 5

Histograms represent relative levels of p27 determined by densitometry analysis of bands from western blots.

All the immunoblot results were expressed as percentage relative to density of p27 bands. The total volume equals the sum of the volumes of all the objects included in one quantitation of all objects estimated using image analyzer. Histograms represent relative levels of p27 determined by densitometric analysis of bands from western blots. Statistical analysis was performed with repeated-measures ANOVA, $P=0.0026$ and post test Student-Newman-Keuls (SNK) Bars: Control; mean ± SD of data from three experiments, $p<0.001$ compared to BQ123, ET-1 and BQ123+ET-1. The expression of p27 in BQ123 treated cells was not clear because proteins transfer efficiency to the membrane was low.

*The integrated intensity of all the pixels in the spot, excluding the background. To calculate volume, ImageQuant subtracts the background value from the intensity of each pixel in the object, and then adds all the values.
CHAPTER 3

Discussion

It has been known that the ability for regeneration of corneal endothelium after injury, age or other stresses is limited in humans because this tissue is thought to be nonreplicating (19). However Joyce et al (6) have shown that corneal endothelial cells cease to move out G1 phase of the cell cycle, which means they have proliferative potential. This investigation explored ET-1’s role as a mitogenic factor in corneal endothelial cells and whether mitogenic activity is mediated through p27, a cyclin dependent kinase inhibitor. Previously it was shown in our lab by indirect immunofluorescence microscopy that p27 is present in bovine corneal endothelial cells (BCEC). Our observation by immunoblot analysis (Figure 1), though not clear, seems to suggest that p27 expression in BCEC was downregulated when the cells were treated with 100nM endothelin-1.

In this study, the role of ET-1 in the expression of p27 was investigated. The preliminary results presented here could suggest a novel mechanism whereby the expression of p27 was downregulated by ET-1. These effects were specific to Endothelin-1 treated cells as they were not observed in untreated cells (Figure 2). Though the action of ET-1 involves activation of signal transduction pathways, the specific action and/ or mechanism of ET-1 on p27 is unknown. However, the results suggest this mechanism might be due to the increased degradation of p27 in the cytoplasmic compartment (15). Earlier studies suggested that p27 degradation in mammals is mediated by both the ubiquitin proteosome pathway (21) and by ubiquitin-independent proteolytic cleavage (19). The ubiquitin pathway is emerging as a major mechanism that
regulates the selective and time control elimination of key short-lived proteins such as CKIs, this mechanism is achieved by phosphorylation of the target protein. In the case of p27, phosphorylation occurs on threonine-187 (19). In breast cancer cells, p27 phosphorylation occurred almost exclusively in the cytoplasmic compartment, it probably reflects impaired nuclear import of p27 (19, 20). Taking this mechanism into account, the findings of this investigation raises the possibility that the effect of ET-1 binding to ETA receptor on the surface of BCEC may result in additional mechanisms which might have an impact on p27 protein. For example, the cytoplasmic phosphorylated p27 has been shown to bind to cytoplasmic proteins such as 14-3-3 protein, which prevents p27 nuclear export, consequently, there is no binding to cyclin D and eventually, the cell proliferates (20).

Elucidating the function of ET-1 is crucial for cancer research; low level of P27 protein due to the administration of ET-1 possibly stimulates cell division (cells enter S-phase), which might coincide with the downregulation of p27 (figure 5). However, in this experiment the level of protein was not significantly different in ET-1 treated and untreated cells. Yang et al (22) could demonstrate that ET-1 alone was not able to increase smooth muscle cell proliferation. The ineffectiveness of ET-1 to stimulate smooth muscle cell proliferation was further confirmed by the result that ET-1 was not able to activate cyclin dependent kinase-2. Furthermore the CDK inhibitor, p27, was downregulated and the retinoblastoma protein, pRb, was not hyperphosphorylated, this being crucial for cell cycle progression (23). The indication is that the binding of ET-1 to the ET<sub>A</sub> receptor is responsible for potentiating its (ET-1) effects (30). However the relationship between ET-1 and the transforming growth factor TGF-β2 is well
documented. TGF-β2 which is present in the aqueous humor of the anterior chamber has been proposed to suppress mitotic activity of cells (19, 22). TGF-β2 exerts its antimitogenic effect through p27, and can inhibit both cyclin-D-CDK4 and cyclin-E-CDK2. Expression and kinase activity of CDK2 enhanced by ET-1 increased significantly the cross-sectional area and number of smooth muscle cell (19).

It is necessary to point out however that in addition to the downregulation of p27 by ET-1, our results suggest that BQ123 downregulates p27 (figure 5). This downregulation is intriguing because BQ123 is a receptor antagonist; there are several possibilities that can explain the effect of BQ123. First, BQ123 may contribute directly to the downregulation of p27 (figure 4) independent of receptor binding. Secondly, BQ123 might prevent p27 localization to the nucleus, which is important for cytoplasmic degradation of p27. This needs further investigation because our results showed poor transfer of proteins (in BQ123 section) from the gel to the membrane.

Considering the importance of the different biological functions of p27: regulation of cell growth, contact inhibition and apoptosis among others, the combined use of both ET-1 and BQ123 (figure 5) might be a useful tool to better characterize their roles in cell proliferation. Recent studies have shown that the expression levels of p27 are decreased in proliferating cells such as aggressive breast cancer cells (7). Taking into consideration the findings of this study, it could be possible that ET-1 stimulates uncontrolled proliferation of cells which might eventually commit the cell to a tumor. In contrast BQ123 affects the overall physiological activity of the cell which indirectly affects the regulation of p27. ET-1 is crucial in stimulating growth in various cancers. Therefore ET-1 activity could account in generating tumor cells. Venuti et al (15) have previously
suggested a role for ET-1 in the regulation and promotion of cervical tumor growth. This growth promotion may result from the ability of ET-1 to act alone or with various growth factors such as EGF.

**Conclusion**

In conclusion, this study indicates that ET-1 may potentially play a role in promoting the regeneration of corneal endothelium after the cells are destroyed due to injury, disease or other stresses. This investigation suggests that the CDK inhibitor, p27, is functionally down regulated by the mitogen ET-1. However, the effect of its receptor antagonist BQ123 was unclear. ET-1 is important in BCEC cells moving out of G1, and may be critical in the continuous proliferation of BCEC. Indeed, the pattern of p27 expression suggests there could be other mechanisms, which regulate proliferating bovine corneal endothelial cells where ET-1’s role needs to be investigated further.

**Further studies**

The effect of Endothelin-1, BQ123 and BQ123 + ET-1 on the expression of p27 in a cell may be a sign of mitogenic effect when they bind independently to and/or compete for ET\(\lambda\) receptor. Though it is not clear how BQ123 could affect cell proliferation, our results indicate that BCEC responded to ET-1 treatment. Hence further investigation is required to determine whether the change in the expression of p27 in the presence of ET-1 and BQ123 is related to cell proliferation.
Literature cited


