Integrins Affecting Mechanical Strain-induced Expression of Egr-1 in Vascular Smooth Muscle Cells

by

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Abstract

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Integrins are transmembrane proteins that anchor cells to the extracellular matrix, ECM, and they are thought to play a very important role in the transmission of signals between the ECM and the cell. The cells of interest in this study are the vascular smooth muscle (VSM) cells, specifically the mouse aortic VSM cells, which are constantly under cyclic mechanical strain. It has been shown in previous studies that this strain induces proliferation of the cells by autocrine action of platelet derived growth factor (PDGF). The PDGF promoter contains a binding site for the transcription factor, early growth response gene-1 (Egr-1). It was previously shown that when the cell undergoes cyclic mechanical strain, Egr-1 mRNA expression is significantly increased. The scope of this study is to determine the integrins that are involved in the mechanical signaling via cyclic mechanical strain. Introducing neutralizing antibodies that block the function of the integrins and subjecting VSM cells to mechanical strain with the Flexercell strain unit may lead to the deduction of the integrins that are involved in transmitting the signal to increase Egr-1 mRNA expression. Egr-1 expression would be expected to decrease when the integrin that is transducing the strain response is blocked. The RT-PCR procedure was developed and used for determination of Egr-1 mRNA expression through the course of this study. There are still many problems that must be assessed before the integrin may deduced. Problems in the procedure including antibody concentrations, in the handling of cells and RNA, and in the basal expression of the Egr-1 mRNA arose through the course of the study. From the latest experiment and from previous studies by Wilson et al., it is suggested that the $\beta 3$ subunit and thus the $\alpha v \beta 3$ integrin may be responsible for transducing the mechanical strain signal and increasing Egr-1 mRNA expression.

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Abbreviations used throughout: VSM, Vascular Smooth Muscle; ECM, extracellular matrix; PDGF, platelet-derived growth factor; Egr-1, early growth response-1; FAC, focal adhesion complex; GAPDH, glyceraldehyde phosphate dehydrogenase.

Introduction

Approximately 20% of the world's adult population or approximately 690 million people suffer from hypertension.[1] Hypertension is one of the major risk factors for atherosclerosis, stroke and heart and renal failure. About 30% of all deaths worldwide are due to cardiovascular diseases,[1] while many more people are disabled by such diseases.[1] Hypertension is associated with the remodeling of arteries and altered function of smooth muscle and endothelial cells.[4] The specific mechanism that leads to these changes in response to increased blood pressure is not totally understood. Much research has focused on the effects of humoral factors such as the Renin-Angiotensin system.[33] At the present time, little is known about the direct role of mechanical factors.

Vascular Smooth Muscle (VSM) Cells are found lining the arterial walls of the cardiovascular system and are constantly exposed to cyclic mechanical strain due to the cardiac cycle.[2] Changes including flow velocity, shear stress as well as axial and circumferential stress lend to the overall strain felt by the vascular smooth muscle cell.[3] During a normal cardiac cycle, the VSM cell is stretched by 10% of its initial length, and with pathogenic conditions such as hypertension, this elongation may be increased to as much as 20%.[3] These mechanical forces are thought to play an important role in the developmental processes of the vascular system as well as in pathological changes such as hypertension by stimulating many different cell signals. For example, thickening of the vascular media is seen after chronic elevation of arterial blood pressure.[4, 5, 6] Due to the advances in technology, it is now possible to study the role of cyclic mechanical strain upon the VSM cells in vitro.

VSM cells are located in the media of the blood vessel and are surrounded by the extracellular matrix (ECM), which the cell secretes and to which it adheres. The ECM holds the cells together in the media and includes proteins such as collagen, elastin, fibronectin, laminin and vitronectin.[2] In normal blood vessels, the primary function of the VSM cell is contractile, regulating vascular tone and blood flow through the vessels and the organs.[2] However, as a result of pathogenic conditions, VSM cells may undergo phenotypic changes which include increased synthesis of ECM, altered responsiveness, hypertrophy or hyperplasia.[7,8,9,10] *In vivo*, this proliferation is associated with a remodeling of the vascular media.[2] *In vitro*, VSM cells subjected to strain show a marked increase in DNA synthesis.[11] This increase is due to the autocrine action of the platelet-derived growth factor (PDGF).[11] Mechanical strain induced production of PDGF-A in VSM cells is dependent upon the ECM to which the cell is bound.[12] These *in vitro* studies are consistent with alterations in mechanical strain to which VSM cells are exposed *in vivo*. These studies also suggest that strain may be a major contributing factor to vascular remodeling seen in hypertension. Thus, understanding the molecular mechanism by which cells sense and respond to mechanical strain may be important in understanding alterations in hypertension.

To begin to understand how mechanical strain alters gene expression, the PDGF-A gene promoter was analyzed.[12] The PDGF-A promoter contains many regulatory sites.[13,14] To identify which of these regulatory sites are affected by mechanical strain, deletion analysis was performed.[12,13,14] VSM cells were transfected with various truncations of the PDGF-A promoter fused to a chloramphenicol acetyl transferase (CAT) reporter gene.[12,13,14] The transfected cells were then exposed to strain. The region of the promoter between -92 b.p. and

-41bp contained the activator of the PDGF transcription as a response to strain.[12] This region of the promoter contains the site for Egr-1 or "early growth response gene 1" binding , and further studies determined that expression of Egr-1 is responsive to mechanical strain.[14, 15]

Egr-1 is an immediate early growth response gene that is involved in differentiation and growth and is expressed as a result of a multitude of diverse signals.[16, 17] The Egr-1protein translocates to the nucleus and is responsible for transcriptional activation of many proteins depending upon the cell type in which it is expressed.[16,17] In VSM cells the expression of Egr-1 is initiated by strain. Upon the cell's exposure to cyclic mechanical strain on elastomer plates *in vitro,* the expression of Egr-1 mRNA increases within as little as a minute.[15] Egr-1 mRNA expression peaks after a period of 30 minutes of stretch, and by 2 to 4 hours of strain the level of Egr-1expression returns to the basal level.[15] In addition to the duration of stretch, the extracellular matrix upon which the cells are stretched affects the level of Egr-1 expression.[15] Cells stretched on collagen and fibronectin showed a marked increase in expression, whereas cells on laminin showed little or no change in the level of expression.[15] Thus, Egr-1 expression is responsive to mechanical forces and is dependent upon the ECM. These studies suggest that the type of ECM protein determines which signals are generated in response to mechanical strain, leading to altered gene expression.

The focus of this study is to determine how the mechanical forces are transferred to the cell from the matrix in order to activate and increase expression of Egr-1. One hypothesis is that integrins, the cell surface receptors for ECM proteins may serve as "mechanotransducers."[18, 19, 20, 21] Integrins have been proposed to fill this role because they are transmembrane proteins that physically

connect ECM with the cytoskeleton of the cell.[22] An integrin spans the cell membrane and is composed of two different subunits, α and β.[22,23] Multiple α and β subunits have now been identified which show specificity for binding to specific ECM proteins.[24] In addition to governing adhesion to ECM proteins, specific integrin heterodimers may interact with different signaling molecules leading to specificity in how cells respond to external stimuli.[24] Based on these facts it has been proposed that alteration in mechanical stimulation can be transferred from ECM to the cytoskeleton through specific integrins.

The intracellular portion of some β subunits binds to the focal adhesion complex (FAC) of the cell. The FAC is a sort of scaffold made up of molecules that bind actin proteins of the cytoskeleton as well as signalling proteins that are involved in stimulus response coupling.[25,26] The organization of the FAC is controlled by the mechanical forces.[26] Some of these proteins in the FAC include paxillin and focal adhesion kinase (FAK), and they are shown to be tyrosine phosphorylated by many different mitogens as well as mechanical strain in aortic endothelial cells.[27,28] Similar pathways may also be activated in smooth muscle cells, in response to mechanical strain.[29] It is suggested that these proteins are phosphorylated and activate signal transduction pathways such as MAP Kinase in VSM cells that may lead to activation of transcriptional events. **Figure 1** shows the interaction between the ECM, the integrin and the focal adhesion complex.[30] It is thus indicated that mechanical signals may be converted to molecular signals through the alterations in the ECM-integrin interaction leading to activation of signaling molecules in the focal adhesion sites resulting in altered gene expression.

As previously mentioned, the mechanical strain induced expression of Egr-1 is also dependent upon the ECM. Thus, cells respond differently to strain based

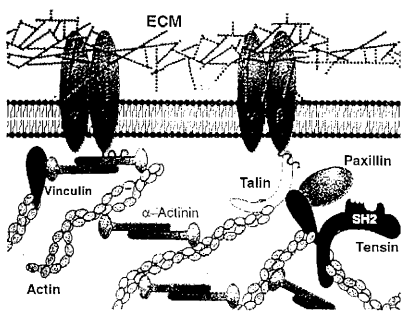

Figure 1. A schematic diagram of integrin interaction with the ECM and the FAC. An integrin is a transmembrane cell surface receptor composed of α and β subunits, which adhere the cell to the ECM and can mediate signaling. The intracellular portion is attached to the FAC made up of actin binding proteins such as vinculin and talin and signaling proteins such as paxillin, tensin and SH2. Cyclic mechanical strain signaling is transduced by the ECM-integrin interaction leading to signal response at the FAC. (Figure courtesy of Clark and Brugge, *Science.* 1995; **268**:233-239.)

upon the matrix protein on which they are plated. Certain integrins bind particular matrix proteins. **Table 1** demonstrates the number of integrins binding a specific matrix protein.

Table 1. ECM-Integrin interactions[24,30]

ECM proteins[a]	Integrin receptors[b]
Collagen	α1β1, α 2 β 1, α 3 β 1
Fibronectin	α3β1, αvβ1, αvβ3, αvβ5, α5β1, α8β1
Laminin	α1β1, α2β1, α3β1, α6β1, α6β4, α7β1

a. These ECM proteins represent the most common matrix proteinsfound surrounding the VSM and endothelial cells.
b. Some cells do not necessarily contain all the integrin receptors for a particular matrix protein

It may be assumed that only particular integrins are involved in transducing a mechanical signal from the ECM to the cell. Only four different integrins on the VSM cell bind to fibronectin, including αvβ1, αvβ3, αvβ5 and α5β1. (Wilson, unpublished data) Because fibronectin increases expression of Egr-1, it may be deduced that at least one of these integrins is responible for mediating strain-induced expression of Egr-1. These integrin-ECM interactions for transducing strain may be further studied by blocking strain. Previous studies have shown that soluble matrix proteins, RGD peptides and anti-integrin antibodies can block the mitogenic effect of strain in the VSM cell. [24] Incorporating a mechanism to block the integrin-ECM interaction should affect the expression of Egr-1. Anti-integrin antibodies should be able to elucidate the integrin involved in transducing the mechanical strain signal activating the expression of Egr-1.

The goal of this study was to utilize similar techniques to identify specific integrins that might mediate strain-induced Egr-1 expression. We are particularly interested in this system because Egr-1 has been shown to be very sensitive to strain, and its expression is a very early transcriptional event. The identification of the integrins mediating strain-induced transcription will be an important step in understanding the signal transduction pathway leading from exposure to cyclic mechanical strain to gene regulation. Therefore, the primary goal of this study was to utilize function blocking antibodies directed against specific fibronectin binding integrins to try to identify which integrin will mediate strain induced increases in Egr-1 expression.

Materials and Methods

Materials

Most materials were purchased from Sigma (St. Louis, MO) except the following materials. Pronectin- coated flex plates were attained from FlexCell Inc. (McKeesport, PA). Neutralizing antibodies against $\alpha 5$, $\beta 1$ and $\beta 3$ integrin subunits were purchased from Pharmingen Inc. The TriZol reagent (Total RNA Isolation Reagent) for harvesting the cellular RNA was purchased from Gibco BRL. The RT-PCR kit, containing enzymes, nucleotides and buffer, was purchased from Boehringer Manheim (Indianapolis, IN). The Egr-1 and GAPDH primers were purchased from Genosys.

Cell Culture

The S1P8 cell line used was derived from mouse aorta. The cells were selected by Fluorescence Activated Cell Sorting (FACS) for the expression of integrin $\alpha 7\beta 1$, which is expressed preferentially in differentiated smooth muscle cells. The cells were a gift from Drs. Randall Kramer and Jane Yao of the University of California, San Francisco. The cells were maintained in minimal essential medium with 10% fetal bovine serum, 2% tryptose phosphate broth, 50U/mL penicillin and 50 U/mL streptomycin. These cells were grown on 6 well silicone elastomer plates coated with Pronectin (a fibronectin-like poly RGD matrix) in the middle of the well confined by a glass ring (~0.5 cm^2). The purpose of growing the cells in a smaller area was to minimize the loss of cells on the exterior of the well due to strain. The cells were grown in a humidified atmosphere with 5% CO_2 at 37°C, and the media was changed every other day

until cells were confluent. Three days before the experiment, the media was replaced with a no serum media, minimal essential medium containing 0.5mg/mL Bovine Serum Albumin, 0.5 mg/L apo-transferrin, 2% tryptose phosphate broth, 50 U/mL penicillin and 50 U/mL streptomycin. The no serum media was replaced every other day and within a couple of hours before cell stretching experiments.

Inhibition of Integrins with neutralizing antibodies and application of cyclic mechanical strain to S1P8 cells

After changing the no serum media for the last time, neutralizing antibodies against the integrin subunit of interest were introduced to the media. Since each plate contained six wells, the antibodies were added at a high concentration (2.5 μg for every 2 mL of media) to three wells and at a low concentration (0.5μg for every 2 mL of media) to the other three. Antibodies against the α5, β1 and β3 subunits were introduced to separate plates. The negative control plate contained no antibodies and did not undergo stretch. The positive control plate contained no antibodies but was subjected to cyclic mechanical strain.

The cells, with the exception of the cells in the negative control, were subjected to mechanical derformation with the Flexercell Stess Unit (Flexcell, Inc., Mc Keesport) The stress unit is a modification of the unit described initially by Banes and coworkers[31,32] and consists of a computer controlled vacuum unit and a base plate to hold the culture plates. Each well on the plate sits on a loading post, which allows equibiaxial strain to be applied to the cells. (See **figure 2**) The computer controls the frequency and the intensity of the mechanical deformation. The negative pressure produced allows for a maximum of 18% elongation of the cells. The frequency of the cyclic mechanical strain was 1 Hz. In most

experiments cells were subjected to 30 minutes of cyclic mechanical strain. An exception occurred when we determine the time dependence of stretching. In this case the cells were stretched for different time intervals prior to RNA harvesting.

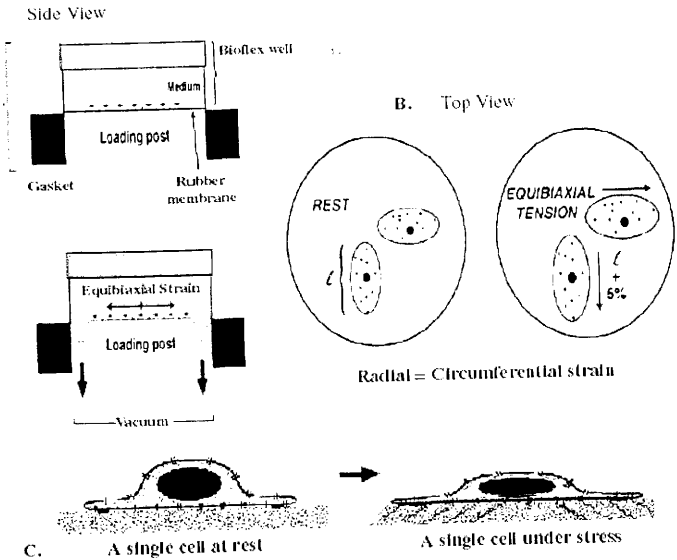

Figure 2. Ilustration of cyclic mechanical strain using the Flexercell Stretch Unit. A. The plate containing the cells and media is positioned upon the loading post in the gasket. Strain is induced when vacuum is applied creating equibiaxial strain upon the cells. **B.** When viewing the stetched cells from above, the stretch is radial or circumferential. **C.** The illustration shows the elongation of the cell when stress is induced. (Figure courtesy of Flexcell, Inc. (McKeesport, PA))

RNA Harvesting

Upon stretching of the cells, the media was removed and replaced by TriZol (Gibco BRL) (0.67 mL for each well). TriZol is a solution containing monophasic phenol and guanidine isothiocyanate allowing for lysis of the cell and degradation of nucleoproteins. The cells were homogenized and were subsequently scraped off the well. Addition of chloroform followed by centrifugation allowed for separation into two phases. The aqueous phase contained the cellular RNA and was precipitated with isopropyl alcohol and washed with 75% ethanol. The RNA was diluted with water, and concentrations were measured by spectrophotometer using absorbance at 260 nm and an assumed concentration of 40 μg/ O.D. unit for single stranded RNA.

Reverse Transcriptase- Polymerase Chain Reactions

Once total cellular RNA concentrations were found, the relative expression of Egr-1 mRNA could be determined with RT-PCR. The Titan One Tube RT-PCR system from Boehringer Manheim was used, and a reaction was set up for each set of RNA harvested from different antibody concentration/stretch parameters. The reaction called for 1 μL of the enzyme mix which included reverse transcriptase AMV, Taq DNA polymerase and Pwo DNA polymerase to convert RNA into DNA and then amplify the DNA. The primers for Egr-1 and GAPDH, a control gene to determine if the RT-PCR worked, were designed using the Oligo Primer Design program and Blast homology search program. Oligos were selected based on the size of the expected amplified fragment and melting temperature of the oligos. The primers were synthesized by Genosys (Houston, TX). The upstream and downstream primers for each product were added at a

final concentration of 0.4μM each. A nucleotide mix containing each nucleotide (dATP, dGTP, dCTP and dTTP) was added so that the final concentration of each nucleotide was 0.2 μM. Buffer containing $MgCl_2$ at a final concentration of 1.5 mM as well as DTT at 5mM was added. Approximately 1 μg of harvested RNA was added to the reaction; and sterile, RNase free water was added to bring the reaction mixture up to the appropriate volume.

The reactions were placed in a Techne Genius thermocycler where the reverse transriptase reaction occurs at 50°C for 30 min. Upon completion of the RT cycle, the PCR reaction amplifies the DNA. Upon completion of the PCR portion, the DNA was visualized on an agarose gel.

Visualization and Quantification of DNA fragments

The primers designed for Egr-1 and GAPDH produced 430b.p. and 225 b.p fragments respectively. The DNA was run on a 1.5% agarose gel containing Ethidium Bromide and visualized using the Gel Doc instrument and program designed by Bio Rad. The bands from the gel could be quantified based on the optical density of the band. The amounts of Egr-1 for each reaction are expressed in relative units by dividing the Egr-1 densities by the control (GAPDH) densities for each reaction.

Results

Egr-1 mRNA expression after timed stretch intervals

S1P8 mouse aortic VSM cells plated on pronectin are exposed to varying time intervals of cyclic mechanical strain at 1Hz and 18% strain. The RNA is harvested, and RT-PCR is performed. Visualization on a 1.5% agarose gel is shown in **figure 3**, and quantification of Egr-1 mRNA expression as compared to GAPDH is shown in **figure 4**. **Figures 3 and 4** show a dramatic increase in expression of Egr-1 mRNA between 15 minutes and 30 minutes. In **figure 3** the Egr-1 band appears at 430 b.p., and the GAPDH band appears at 225 b.p. First and foremost, the RT-PCR protocol worked as can be demonstrated by the bands. Secondly, the Egr-1 fragment band at 15 minutes is much less intense than the bands at 30 minutes and 1 hour of stretch. The GAPDH is consistently expressed in all three time periods.

Quantitative analysis is performed on the bands using densitometry as a part of the Gel Doc program. The bands are expressed in volume units, i.e. optical density by square meters. The Egr-1 volume is divided by the control GAPDH volume to give the values in relative units. The Egr-1 mRNA expression in relative units is 0.453 for 15 minutes, 1.086 for 30 minutes and 0.955 for 1 hour of stretch. These data confirm that the mouse VSM cells analyzed in this experiment show a similar induction profile in response to strain as the rat aortic cells previously reported.[12, 15]

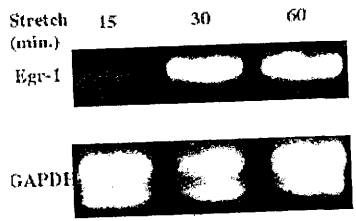

Figure 3. Egr-1 mRNA expression after timed intervals of strain. The Egr-1 mRNA expression increases between 15 and 30 minutes of cyclic mechanical strain. The presence of the bands demonstrate that the RT-PCR protocol for Egr-1 expression works. The Egr-1 fragment is approximately 430 b.p. whereas the GAPDH fragment is 225 b.p.

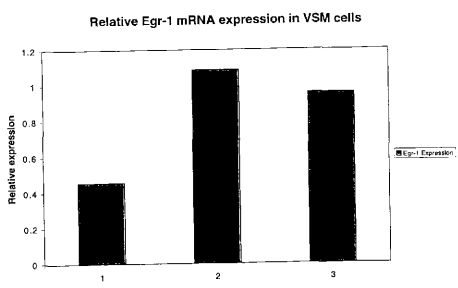

Figure 4. Densitometry analysis for Egr-1 expression after timed intervals of strain. The relative expression of Egr-1 mRNA is measured by comparing the density of the Egr-1bands to the GAPDH bands. The Egr-1 mRNA expression more than doubles between 15 and 30 minutes of strain. 1.) 15 minutes of strain - 0.453 Egr-1 expression. 2.) 30 minutes of strain - 1.086 Egr-1 expression. 3.) 60 minutes of strain – 0.955 Egr-1 expression.

Use of integrin function blocking antibodies to indentify which integrin(s) is/are responsible for transducing strain signals

Once it was established that the RT- PCR protocol worked and is an efficient method for analyzing multiple treatment groups, our new approach is to utilize neutralizing antibodies against fibronectin-binding integrins to inhibit strain-induced Egr-1 expression. The rationale behind these studies is that if a specific integrin is responsible for mediating strain-induced increases in Egr-1 expression then addition of function blocking antibodies to that integrin should block or decrease strain-induced Egr-1 expression. The first step in this process is to identify appropriate antibody concentrations. Initial studies utilized 12.5 μg and 2.5 μg of antibody. Both concentrations completely block Egr-1 expression suggesting that the concentrations of antibodies are too high. (**Fig. 5**) The negative and positive controls show some amount of expression. In all cases the GAPDH fragment was expressed. The only sample not showing significant expression of GAPDH is the sample that is blocked with antibody against β1, suggesting that there may have been some RNA degradation. Further studies are performed utilizing lower antibody concentrations.

The antibody amount added to the media is lowered to 2.5μg and 0.5 μg for the high and low concentrations respectively. **Figure 6** shows that there is expression of Egr-1 for the cells that were treated with antibody. The control samples to which antibodies are not added show dark bands for Egr-1 expression as well as the sample with antibody against α5 at a low concentration and the sample with antibody against β1 at a high concentration. The other samples show little or no Egr-1 expression. The GAPDH is expressed in all the samples. Again, some samples may have experienced some RNA degradation during handling.

When the data is analyzed quantitatively (**fig. 7**) using the same method that was described in the previous section, the unstretched and stretched controls show similar expression of Egr-1 mRNA with .7821 and .7683. When the antibody against $\alpha 5$ is added at the aforementioned concentration, the Egr-1 mRNA expression is 0.3717 for the high concentration and 0.7817 for the low. For the antibody against $\beta 1$, the high concentration of antibody gives an Egr-1 mRNA expression of 1.128 while the low concentration gives an expression of 0.3834. Finally, when the antibody against subunit $\beta 3$ is used, the relative Egr-1 mRNA expression is 0.3147 for the high concentration and 0. 6543 for the low concentration.

Further studies are necessary to obtain more consistent results and to attain complete analysis without any RNA degradation. The correct antibody dosage should also be addressed before the relative Egr-1 mRNA expression is further studied and before the integrin that is mediating strain-induced Egr-1 expression can be deduced. The data obtained and methods that were established during these studies has set the foundation for completion of this project.

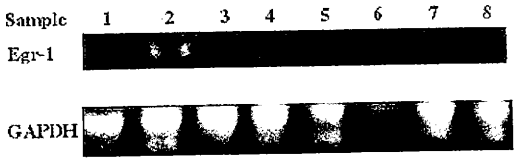

Figure 5. Egr-1 expression with the initial concentration of neutralizing antibodies. Neutralizing antibodies against α5, β 1 and β 3 are added to each sample media at either a high concentration (12.5 μg antibody) or a low concentration (2.5 μg antibody). There is no Egr-1 mRNA expression in any of the samples treated with antibody, indicating that the antibody concentration may be too high and may detach the cells from the plate, thus not allowing strain to activate Egr-1 expression . The controls exhibit little Egr-1 expression. (1.) no stretch, 2.) stretch, 3.) stretch + high anti- α 5, 4.) stretch + low anti- α 5, 5.) stretch + high anti- β 1, 6.) stretch + low anti- β 1, 7.) stretch + high anti- β 3, 8.) stretch + low.

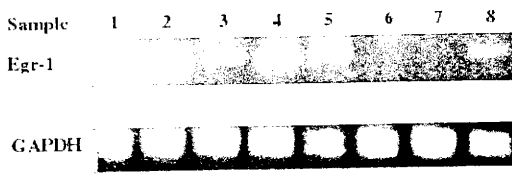

Figure 6. Egr-1 expression with lower concentrations of neutralizing antibodies. The amounts of the antibodies against α5, β1 and β 3 are lowered to 2.5 μg for the high concentration and 0.5 μg for the low concentration. There is Egr-1 expression for most of the samples. The results suggest these antibody concentrations are more beneficial for this experiment. (1.) no stretch, 2.) stretch, 3.) stretch + high anti- α 5, 4.) stretch + low anti- α 5, 5.) stretch + high anti- β 1, 6.) stretch + low anti- β 1, 7.) stretch + high anti- β 3, 8.) stretch + low.

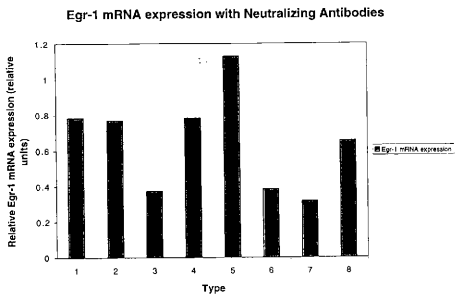

Figure 7. Densitometry analysis for Egr-1 expression with neutralizing antibodies. Upon quantitative analysis of the results from **fig. 6**, the negative and positive controls demonstrate a similar relative Egr-1 expression, suggesting that the mouse cell line may differ with the rat cell line used in previous studies. The Egr-1 expression varies for the samples treated with antibody except that the two samples treated with anti- β 3 appear lower than both the positive and negative controls. (1.) no stretch, 2.) stretch, 3.) stretch + high anti- α 5, 4.) stretch + low anti- α 5, 5.) stretch + high anti- β 1, 6.) stretch + low anti- β 1, 7.) stretch + high anti- β 3, 8.) stretch + low anti- β 3.)

Discussion

VSM cells undergo significant amount of mechanical strain during the cardiac cycle. This strain induces a plethora of intracellular signals which affect the proliferation, the differentiation and the function of the cell. Recently, many studies have been performed demonstrating the importance of mechanical forces upon VSM cells and how strain may play a role in pathological changes as seen in hypertension. Prior studies have focused upon the humoral factors such as angiotensin II,[33] and the mechanical factors were largely ignored because of the inability to study strain *in vitro*. With advanced technology now, it is possible to study the role of strain on VSM cells and determine changes in the cell as a result of mechanical forces.[31, 32] Hypertension is a worldwide epidemic.[1] Having a broader perspective about the cause of hypertension may prove most beneficial in reducing the number of deaths that occur due to cardiovascular and other diseases triggered by hypertension.

The remodeling of the artery as well as change in function in the VSM cell are some of the effects of hypertension.[4] The VSM cells undergo phenotypic changes including increased synthesis of the ECM, altered responsiveness, hyperplasia or hypertrophy.[7,8,9,10] Wilson et al[11] showed that rat VSM cells undergoing strain demonstrate an increase in DNA synthesis due to the autocrine action of PDGF. They showed that the production of PDGF-A increased with intervals of cyclic mechanical strain.[11] Upon further study of the promoter of PDGF-A, they found that a region between -92 b.p. and -41 b.p contained the transcription activator for PDGF as a response strain.[12] They deduced that the activator was Egr-1 when it also showed an increase in expression as a response to

strain. Egr-1 mRNA expression peaked after 30 minutes of cyclic mechanical strain and returned to basal level of expression after 2-4 hours of stretch.[12] Wilson et. al.[24] also showed that the ECM upon which the cell is plated plays an important role in the increase of PDGF-A and cell proliferation. Morawietz et al.[15] continued with these experiments and determined that the ECM to which the VSM cell adheres may play a role in the Egr-1 mRNA expression as well. They found that when cells are plated on collagen and fibronectin (pronectin) and subjected to cyclic mechanical strain, there is a marked increase in Egr-1 expression after 30 minutes. When the cells are plated on laminin and stretched, there appears to be no change in Egr-1 mRNA expression. The dependence of Egr-1 expression upon the ECM suggests that certain integrins may be involved in the transduction of the stretch signal for activation of Egr-1 transcription.

The focus of this study was to determine which integrins may be implicated in activation of Egr-1 mRNA expression as a result of cyclic mechanical strain. Since strain induces Egr-1 upregulation in cells plated on collagen and fibronectin, the integrins that bind these particular ECM proteins may be studied further. Since only four different integrins of the VSM cell bind to the fibronectin matrix protein, the subunits of these integrins were the prime target of this study. To determine which subunits may possibly transduce the stretch signal, we added neutralizing antibodies to the no serum media prior to stretching. Upon blocking the mechanical strain signal and then stretching the cell, the level of Egr-1 mRNA expression should decrease. By measuring the extent to which expression decreases, a single integrin may be deduced as the mechanotransducer, transferring strain signaling from the ECM to the cytoskeleton of the cell and

initiating intracellular signaling. To determine this integrin in a timely manner, an efficient protocol was eventually developed.

Determination of Egr-1 mRNA expression via Northern Blot

The first protocol used in the determination of Egr-1 mRNA expression was one that required handling of RNA throughout the entire experiment. The cells were stretched, and the RNA harvested as was explained in the Materials and Methods section. After harvesting the RNA with TriZol, the total cellular RNA was run on an RNase free gel. The bands were transferred to nitrocellulose via Northern blotting apparatus provided by Ambion, Inc. A radio- labeled probe for Egr-1 mRNA would be prepared to determine the relative expression. The radio-labeled probe required for the Northern blot was very burdensome to prepare. In fact, it was never successfully prepared and was the major reason that the Northern blot protocol was abandoned after a considerable amount of time. With the number of experiments that were required to deduce the integrin involved in mediating strain, the protocol was by no means an efficient way to determine the expression of Egr-1 mRNA. There were many factors involved in determination of an integrin. With the tedium of the Northern Blot protocol, the experiment would have yielded nothing useful, as errors were bound to occur. An easier, less tedious protocol was substituted for the Northern blot procedure to allow for error without losing a considerable amount of time.

Determination of Egr-1 mRNA expression via RT-PCR at different time intervals

As described before, the RT-PCR protocol was substituted for the Northern blot protocol because of its efficiency.. The first experiment that was performed with RT-PCR was one to determine whether this procedure worked. By stretching VSM cells at different time intervals, the experiments of Wilson et al.[11] and Morawietz et. al.[15] were simply repeated. Like these previous experiments, Egr-1 mRNA expression peaked after 30 minutes of cyclic mechanical strain. According to **figure 5**, the relative expression of Egr-1 mRNA more than doubles between 15 and 30 minutes, which is what Moraweitz et al.[15] also found. The expression stays about the same with a slight decrease between 30 minutes and an hour.

The primary reason for this experiment was to show that the RT-PCR protocol actually worked and can be used for subsequent experiments. This experiment also showed that strain increased Egr-1 mRNA expression between 15 and 30 minutes in accordance with previous studies. The main problem with this experiment is that the control which was not subjected to strain was not included. The control did not produce an appreciable amount of RNA after the RNA was harvested. Thus, cells that have undergone stretch cannot be properly compared to the cells that have not experienced stretch.

The control was necessary to measure the basal Egr-1 mRNA in VSM cells that were not stretched. The difference between this study and previous studies is that this study used the mouse aortic VSM cells. All the previous studies using cyclic mechanical strain on VSM cells used the R22D, the rat aortic cell line. The

rat line is usually used because it has been the model upon which most physiological studies have been made. This study utilized the mouse cell line because of its closer relation to the human system and because mouse lines are usually more useful for genetic studies. To further study the signal transduction pathways involved in activating transcription as a response to strain, the mouse strain would prove to be more useful than the rat because future studies may involve isolating and utilizing smooth muscle cells from integrin "knockout" mice to verify the role of a specific integrin. When this experiment was performed, the mouse and rat lines were assumed to yield the same results; and when comparing the Egr-1 expression after intervals of cyclic mechanical strain from the studies of Wilson et al. with the expression in this study, it looked as if the two lines did yield very similar results. There is, however, some indication that the two cell lines do not behave exactly the same. For example, the experiments described in the next section show that the S1P8 mouse line has a high basal expression of Egr-1. This high expression may be the case because we have not identified the optimal protocol for quiescing these cells, or they may be more sensitive to other mechanical stimulation such as changing the medium or handling than the rat cells are.

Determination of Egr-1 mRNA expression with RT-PCR and with neutralizing antibodies.

The neutralizing antibody functionally blocks the integrin to which it binds. From this experiment, it was shown that the concentration of neutralizing antibodies is a signficant factor influencing Egr-1 expression. From **figure 6**, the effect of very high concentrations of antibodies demonstrates no Egr-1 mRNA

product. The lack of expression may have been a result of cell dissociation from the plate itself before strain was induced. That is, the high concentration of antibodies may have caused functional block of integrins to the extent that the cells detached from the matrix. Deciding upon antibody concentration is rather difficult. The inital antibody concentrations were simply estimates. Because the result after RT-PCR was inadequate, the concentrations of the added antibodies were lowered.

The lower antibody concentrations showed slightly better results in that there was some Egr-1 expression after strain when the cells were treated with antibodies. Not much, however, can be deduced from such results. The sample with 0.5 μg (low concentration) of anti-α5 and with 2.5 μg (high) of anti-β1 demonstrated as much, if not more, Egr-1 expression than the stretch control which was subjected to strain but not treated with any antibodies. This result should suggest that α5 and β1 can be eliminated from consideration as being the strain-transducing subunits because Egr-1 mRNA was expressed. When the samples with opposite antibody concentrations for α5 and β1 are analyzed, i.e. high anti-α5 and low anti-β1, there is not significant Egr-1 mRNA expression, which suggests either the two subunits were functionally blocked or that the RT-PCR did not work. Thus, these two integrin subunits may still play a role in strain mediation and cannot be eliminated. When analyzing the sample treated with antibody against β3 at high and low concentrations, there is no expression of Egr-1 for the sample treated with 2.5 μg of antibody. There is some amount of expression of Egr-1 when 0.5 μg of anti-β3 was added. The expression, however, was not near the value of expression of the positive control. Of these three integrins, the best possibility would be β3 to mediate strain between the ECM and

the cell. This deduction is still rather preliminary as there are many problems that still need to be corrected. However, this observation is consistent with previous studies in which Wilson et al (15a) showed that the PDGF-mediated strain-induced cell proliferation was blocked by neutralizing antibodies to the β3 integrin.

More studies are still necessary before the strain-transducing integrin can be elucidated. There are many areas which must be addressed before further experiments may occur. First of all, as mentioned before, the negative controls in the last two studies showed high basal expression of Egr-1 mRNA. The negative control plate (no strain) actually showed slightly higher relative expression of Egr-1 than the positive control plate (30 minutes of strain). It is suggested that the basal levels between the rat line and the mouse line are different or that the mouse VSM cells are more sensitive to strain and may increase Egr-1 expression in response to handling and/or the media change. More studies should be performed on the S1P8 cell line to establish a correct protocol for handling. Secondly, the best antibody concentrations still need to be determined. The low concentration from the first study with antibodies was the same as the high concentration of the second study (2.5μg), yet the results were slightly different. A concentration which would give the most consistent results and would not interfere too much with the adhesion of the cell to the plate must be found. Another reason why these results would differ includes RNA degradation. If cellular RNA or primer RNA is degraded, the expression of Egr-1 mRNA may be misrepresented on the gel when visualized. Finally, the RT-PCR was shown to work as seen in **Fig. 4**, but there were many experiments where the GAPDH was expressed but the Egr-1 fragment was not expressed. A possible reason could include problems with the primers.

The fact that the annealing temperature for the lower Egr-1 primer is almost 5°C different from the rest of the primers may be an explanation for the many RT-PCR reactions which showed no Egr-1 expression, even for the controls. The number of problems and possible mistakes that may rise as a result of RT-PCR, the cell line, the antibody concentration and RNA degradation is immense. With the RT-PCR protocol, however, the problems may be overcome more quickly than with any other protocol.

Conclusion

The integrins involved in cyclic mechanical strain induced expression of Egr-1 have yet to be found. There is a slight possibility that the $\alpha v\beta 3$ integrin may be involved, but many more studies are required to justify this possibility. This study established that the RT-PCR protocol is the more beneficial and efficient procedure to use to determine Egr-1 mRNA expression. The study has laid the foundation for further study in determination of the integrin and the signal transduction pathways involved in Egr-1 up regulation as a result of cyclic mechanical strain in vascular smooth muscle cells. These future studies will be important in furthering the understanding of mechanical forces on conditions such as hypertension.

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