2009

Enumeration of Endothelial Progenitor Cells in Hind Limb Suspended Rats and the Mechanics of Endothelial Wound Healing

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Thesis submitted to

the Graduate College of

Marshall University

In partial fulfillment of

the requirements for the degree of

Master of Science

by

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Marshall University

August 2009

ABSTRACT

Enumeration of Endothelial Progenitor Cells in Hind Limb Suspended Rats and the Mechanics of Endothelial Wound Healing

By Jarrod Pennington

The endothelium repairs itself through two methods. One is by the activity of circulating endothelial progenitor cells (EPCs). EPCs are immature endothelial cells that circulate the bloodstream that are capable of proliferation and differentiation into mature endothelium. It is thought that EPCs contribute to the repair and replacement of damaged endothelial cells in the process of reendothelialization. Physical inactivity and bed rest are known to be deleterious to the endothelium. It is possible that this inactivity is also deleterious to the number of viable EPCs, which would reduce the ability of the endothelium to repair itself. We used hind limb suspended rats as a model for physically inactive patients to examine its effects on circulating EPC populations. We hypothesized that HLS rats will exhibit fewer circulating EPCs than control rats. After obtaining a peripheral blood sample, the mononuclear cell fraction was stained with fluorescent antibodies and observed via flow cytometry. In addition, a fraction of mononuclear cells were cultured to determine colony forming potential. We did not find any significant differences in EPC numbers between control rats and HLS rats. The second means by which the endothelium repairs itself is through the migration of existing mature endothelial cells. To further understand the mechanics of the in vivo healing process, we subjected cultured human umbilical vein endothelial cells (HUVECs) to various drugs treatments and observed their effects on *in vitro* wound healing. We hypothesized that endothelial migration as a part of wound healing is controlled by cytoskeletal elements as well as secondary signals. Wounded cells were photographed on a time course and rate of healing was measured at each hour. We found that

the rate of healing slows over time, and that disruption of cytoskeletal filaments slows wound healing. We concluded that HLS is not sufficient to induce a change in circulating EPC populations. In addition, we conclude that the microtubule components of the cytoskeleton are critical to endothelial resurfacing.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Elmer Price for the opportunity to work in his lab and for his guidance for the last two years. In addition I would like to thank committee members Dr. Eric Blough and Dr. Vincent Sollars for their help on various aspects of this project. I would also like to thank my supervisor Dr. Jason Black for his patience and help every step of the way. I wish to thank my colleagues Dr. Nadja Spitzer and Hari Addagarla for their advice. Finally I want to thank the members of my family who have always supported me 110%.

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CHAPTER ONE

The endothelium, the layer of simple squamous epithelial cells lining the lumen of blood vessels, is critical to overall vascular health. It forms a barrier between the flowing blood and the surrounding vessel wall and covers the entire lumen of the circulatory system from the inner chambers of the heart to every capillary in the body. Not just acting as a simple barrier, the endothelium plays an active role in regulating the many functions of the vascular system.

The capillaries that perform gas, water, nutrient and waste exchange for all tissues in the body are composed solely of endothelial cells. The endothelium is critical to the migration of leukocytes from the bloodstream to surrounding tissues. E-selectin, among other molecules expressed on the surface of endothelial cells, increase in response to cytokines secreted by damaged cells and allow leukocytes to adhere to the vessel wall where they migrate through the endothelium to the damaged site. During inflammation, the endothelium dilates surrounding vessels and increases in permeability allowing plasma to flow from circulation into the tissues. These changes lead to blood flow stasis, which facilitates the adhesion and transmigration of leukocytes. The endothelium is capable of nitric oxide production via endothelial nitric oxide synthase (eNOS). Upon activation, nitric oxide causes relaxation of the smooth muscle tissue surrounding certain blood vessels (Lloyd et al., 2001). This endothelium-dependent vasodilation in turn gives them a role in blood pressure regulation. Endothelial cells also play a role in thrombosis, or blood clotting. Damage to the endothelium triggers platelet activation and clot formation in response to the presence of proteins normally covered by the endothelium, most typically collagen. Endothelial cells produce tissue plasminogen activator,

which catalyzes the cleavage of plasminogen into plasmin, a protein that degrades fibrin clots. Thus the endothelium plays a role in the regulation of thrombosis and preventing the formation of potentially dangerous blood clots.

Endothelial cells are capable of angiogenesis, in which existing cells rearrange themselves into new vessels (Lloyd et al., 2001). When a change in vascular function occurs, whether due to injury to an existing vessel or ischemia (insufficient blood supply) in tissues, the vascular system is capable of remodeling itself. Specifically, it involves the growth of new capillaries within a given tissue. This can be in response to tissue ischemia or can be triggered by an obstruction of blood flow, in which case the capillaries proliferate in order to bypass the obstruction. The main stimulus for onset of angiogenesis is thought to be vascular endothelial growth factor (VEGF), which has also been demonstrated to increase endothelial proliferation *in vitro* (Karamysheva, 2008).

Because the endothelium is so intimately connected to vascular health, it requires a means of repairing itself. Cells damaged by injury or age have to be replaced. In areas where blood vessels bifurcate, or branch off, the normal shear stress experienced by most of the vessel becomes turbulent. This turbulent stress can damage or strip off endothelial cells. While existing endothelial cells are capable of proliferation and can migrate to the injured area, mature endothelial cells have a limited proliferative capacity. The endothelium is repaired in part by means of endothelial progenitor cells (EPCs). EPCs are an immature form of endothelial cells that arises from the bone marrow and are thought to be derived from the hemiangioblast (Nishikawa, 2001). These cells have the potential to differentiate into mature endothelial cells

to replace lost endothelium. While there is still not a consensus within the scientific community as to the phenotype of an EPC, it is commonly accepted that EPCs express CD (cluster of differentiation) 133, CD34 and vascular endothelial growth factor receptor-2 (VEGFR2). Cells positive for these markers appear to be a phenotypically distinct population of circulating cells (Peichev et al., 2000). We also know that CD133+ cell give rise to endothelial colonies *in vitro* (Gehling et al., 2000). It is known that limb ischemia or vessel injury due to coronary thrombosis, burn injury or coronary artery bypass increases the number of circulating EPCs (Gill et al., 2001). This increase in EPC numbers correlates with an increase of endogenous VEGF, the major factor in angiogenesis (Hristov et al., 2003).

EPCs appear to have some homing capabilities. If they encounter an area of denudation where the endothelium has been damaged or stripped off, the EPCs will adhere to this damaged area, proliferate, and differentiate to a mature, functional form. It is known that after thrombotic microangiopathy and balloon angioplasty, procedures which damage or strip away the endothelium that EPCs contribute to the repair of the damaged endothelium (Hristov et al., 2003). It is thought that E-selectin, which plays an important role in leukocyte adhesion and transmigration, also plays a role in EPC homing (Oh et al., 2007). Some studies have shown that E-selectin expression promotes angiogenesis in ischemic limbs partially due to the mediation of EPC adhesion. The inter-cellular adhesion molecule 1 (ICAM1) also appears to be involved in EPC homing. Increased ICAM1 expression in ischemic limbs was associated with increased EPC recruitment. Thus, the ability of EPCs to target areas of damaged endothelium appears to depend on the expression of EPC surface molecules and their counter-ligands on the damaged endothelial cells and other underlying tissues (Zampetaki et al., 2008).

In order to contribute to the repair of the endothelium, EPCs must be able to differentiate to a mature endothelial phenotype. While the aforementioned markers CD133, CD34 and VEGFR-2 are the most commonly accepted markers of an EPC phenotype, it is thought that there is a heterogeneous population of peripheral blood mononuclear cells (PBMCs) exhibiting this phenotype, of which EPCs make up only a fraction. These cells may, under the correct conditions, differentiate into smooth muscle cells and non-endothelial blood cells as well as endothelial cells. It is thought that the number of circulating cells capable of an endothelial phenotype is very small, with some estimates as low as 0.002% of all PBMCs (Hristov et al., 2003). It is known that VEGF, in addition to increasing EPC recruitment from the bone marrow, can also induce endothelial phenotype in EPCs. The shear stress generated by the flow of blood over the endothelium is also believed to be a major factor in determining endothelial phenotype. Laminar flow experiments have been shown to enrich $CD133^{+}/CD34^{+}/VEGFR\text{-}2^{+}$ cells for an endothelial phenotype (Zampetaki et al., 2008). Cells seeded in a laminar flow chamber designed to mimic the shear stress experienced by the endothelium *in vivo* demonstrate more robust growth as compared to cells in static medium. It is possible that the endothelial response to shear stress is mediated by PECAM-1, also known as CD31. This is a common marker found on mature endothelial cells, and may also be found on some EPCs (Woodfin et al., 2007). This positive response to shear stress may be the primary reason that EPCs are seen to increase in response to physical activity. Multiple studies (Hoetzer et al., 2007; Laufs et al., 2005) have shown that aerobic exercise increases EPC clonogenic and migratory capacity. The mechanism for increased EPC recruitment in response to activity is unknown. It

is possible that the increased shear stress induces the production of soluble factors that increase EPC migration from the bone marrow into circulation.

Cardiovascular disease is the leading cause of death in the US today. One form of CV disease, peripheral arterial disease (PAD) affects more than 8 million Americans (American Heart Association). PAD is characterized by occluded blood flow to the limbs, particularly the legs. The ischemia in PAD is usually attributed to atherosclerotic plaque buildup in the peripheral arteries. However, research indicates that there may be an additional contributing factor. The various deleterious effects known to be caused by physical inactivity could have an adverse effect on the number and function of circulating EPCs as well as on the vascular system itself. As EPCs are a major factor in the repair and maintenance of the circulatory system, a deficiency of EPCs means the vascular system is more susceptible to long-term damage and dysfunction (Hill et al., 2003). When endothelial damage occurs, it triggers a number of signals which lead to plaque formation and atherosclerosis. If EPCs are too low in number or do not function correctly, this endothelial damage may not be repaired and could exacerbate the effects of PAD. In patients with PAD, walking becomes painful and difficult, discouraging them from moving around. However, this lack of physical activity may further contribute to the disease. The long-term ischemia may further contribute to EPC dysfunction, which in turns leads to further vascular dysfunction (Alobaid et al., 2005). Indeed, PAD is more likely to occur in individuals who are on bed rest, paralyzed or live a sedentary lifestyle. Conversely, we know that EPC populations benefit from physical activity. Mice subjected to physical activity exhibited greater numbers of circulating EPCs and increased angiogenesis compared to sedentary mice (Laufs et al., 2004).

Because animal subjects cannot be confined to bed rest like a human, we must use an alternate method of recapitulating a lack of physical activity. This method of immobilization is called hind limb suspension (HLS). HLS is a model used to simulate bed rest or immobility. It is also used to examine the effects of microgravity and weightlessness while on Earth. In this model, the rats' hind limbs are suspended off the ground, preventing them from bearing any weight. HLS has been demonstrated to cause vascular problems in animals such as decreased vascular contractility (Hwang et al., 2007), possibly due to changes in eNOS signaling (Wilkerson et al., 2005). Due to the presence of these vascular problems, it is possible that dysfunctional endothelium is involved, which could in turn be caused by a reduction in number or functionality of circulating EPCs.

In an effort to better understand endothelial dysfunction, especially in PAD, and *in vivo* endothelial repair we took two approaches. Our objective in the first study is to determine whether hind limb suspension lowers the number of circulating EPCs in peripheral blood. The model for this study will be hind limb suspended rats. Since these animals experience cardiovascular dysfunction and such problems can result from a dysfunctional endothelium, we hypothesize that hind limb suspended rats will exhibit lower numbers of circulating EPCs than control rats.

To examine EPC numbers we used flow cytometry to observe expression of certain cell markers and compared relative expression in control and HLS rats. We examined four different markers: CD31, CD45, CD62 and CD34. CD31 is also called platelet endothelial cell adhesion molecule-1 (PECAM-1). PECAM-1 is an adhesion molecule found on many blood cells, including platelets,

monocytes, megakaryocytes and leukocytes as well as endothelial cells (Woodfin et al., 2007). In endothelial cells, it is concentrated at cell-to-cell junctions and is thought to be responsible for anchoring the endothelium into a single layer (Fujiwara et al., 2001). It also facilitates leukocyte transmigration via hemophilic interactions between endothelial CD31 and leukocyte CD31. It may even select for certain sub-populations of leukocytes in response to injury or infection. It has also been implicated in the pathogenesis of various inflammatory and vascular disorders, including atherosclerosis (Woodfin et al., 2007). Experiments have shown that CD31 may function as a transducer of mechanical stress, activating signals such as ERK1/2 (Osawa et al., 2002) as well as enriching EPC populations. ERK1/2 is a mitogen-activated protein kinase (MAPK) that transduces extracellular signals, specifically those that activate mitosis (Meloche and Pouyssegur, 2007). However, there is also evidence that these signals may be activated by shear stress independently of CD31 stimulation (Sumpio et al., 2005). In some literature, CD31 is considered a marker for late-stage EPCs (Hristov et al., 2003). While not found exclusively on endothelial cells, it could still be an indicator of more mature progenitor cells and thus be useful in detecting changes in circulating EPC levels.

CD45 is a leukocytic marker, also called the leukocyte common antigen. It is a protein tyrosine phosphatase and is one of the most abundant molecules found on lymphocytes (Zamoyska, 2007). CD45, to date, has not been found on EPCs or mature endothelial cells. However, it is thought that EPCs are found in the mononuclear cell fraction (also known as the buffy coat) of peripheral blood (Ingram et al., 2004). CD45+ leukocytes are also found in this population. Since we will be studying this population of blood cells, we will observe CD45 expression levels to detect any other changes that might occur during HLS.

CD62L is also called L-selectin, and plays a role in recruiting leukocytes to areas of inflammation. It acts as an anchor during leukocyte adhesion by binding to ligands on the surface of endothelial cells, thus allowing leukocytes to transmigrate through the endothelium and move to areas of infection or damage. L-selectin is typically found on leukocytes, however, it has been found expressed on the surface of *ex vivo* expanded EPCs (Biancone et al., 2004). As it may be found on *in vivo* EPCs, we will also observe any changes in CD62L expression.

CD34 is relatively well-accepted as an EPC marker. While its function is unknown, there is some information on structure of this protein that suggests an adhesion function. It is one of the surface markers EPCs are believed to express after leaving the bone marrow and entering circulation (Bauer et al., 2006). CD34 is commonly used as an immunophenotypic marker to purify cells used for bone marrow transplants, being found on hematopoietic cells as well as EPCs. As such, it is an important marker for us to observe during this study.

In our second study, we used the endothelial wound healing assay to study the repair of an endothelial cell layer *in vitro*. Our objective was to determine the effects of different drug treatments on the healing of an endothelial monolayer. The endothelial wound healing assay is one of the earliest developed assays for studying cell migration. In this assay, a confluent endothelial cell monolayer is injured using a tool such as a pipet tip, and the remaining cells are observed as they migrate towards the now-uncovered space and replace the injured cells. Despite its wide use however, many of the underlying mechanics of the wound healing are unknown. It is known that migration and proliferation are involved. However, it is unknown what signals alert cells further away from the wound to begin migrating or dividing.

Our objective for this project was to determine what factors play a role in endothelial wound healing. We hypothesized that the migration of endothelial cells due to wound healing is controlled by remodeling of the cytoskeleton. We also hypothesized that the signaling pathway used for migration is linked to the pathway used to signal angiogenesis. To this end, we exposed cultured human umbilical vein endothelial cells (HUVECs) to a variety of drugs to determine specifically what factors influence wound healing. Because endothelial wound healing requires cell migration, it is possible that wound healing uses some of the same signaling pathways as the process of angiogenesis. Angiogenesis involves existing endothelial cells rearranging themselves to form new blood vessel-like structures. This process is usually initiated in response to tissue ischemia or to bypass an occlusion in the vasculature. We tested the angiogenic inhibitors angiostatin and endostatin, as well as the matrix metalloproteinase inhibitor GM6001, which also is an angiogenic inhibitor. If wound healing uses a process similar to angiogenesis, then wound healing should be noticeably slowed. We also tested the drugs colchicine, cytochalasin D and cycloheximide. The cytoskeleton which gives a cell its shape is a dynamic structure, being constantly remodeled as the cell moves and adjusts to its current conditions. This process is done by breaking down cytoskeletal elements into their constituent monomers (α-tubulin and β-tubulin for microtubules, actin for microfilaments), removing them from one end and adding them to the other. Colchicine acts by "capping" the end of a microtubule. While α-tubulin monomers can be removed from one end, they cannot be added to the capped end, resulting in an overall breakdown of the microtubule and an accumulation of α-tubulin monomers. Cytochalasin D acts in a similar fashion upon microfilaments, preventing actin monomers from polymerizing. If the cytoskeleton plays a vital role in the

process of wound healing, either of these drugs should result in an inhibition of wound healing. Cycloheximide is a protein synthesis inhibitor, preventing the formation of peptide bonds during the elongation step of synthesis. While cycloheximide only acts as a general inhibitor of protein synthesis, if wound healing requires the synthesis of additional proteins not normally expressed during typical cell activity, then cycloheximide should exhibit an effect. By understanding the basic mechanics of how the endothelium recovers and resurfaces a denuded area, we can better understand how the endothelium is repaired *in vivo*. This knowledge will be beneficial in determining treatment option for endothelial damage and dysfunction.

CHAPTER TWO

Introduction

Increased physical activity has a beneficial effect on the vascular system. It leads to improved angiogenesis and, in patients who have undergone balloon angioplasty, decreases neointima (a thickened layer of arterial intima caused by proliferation of smooth muscle cells) formation, one of the contributing factors to cardiovascular disease (Hutter et al., 2003). Beyond the immediate effects on the vascular system, it also affects endothelial progenitor cells (EPCs). It increases the bioavailability of eNOS, which acts as a regulator of EPC populations (Laufs et al., 2004) and improves clonogenic and migratory capacity (Hoetzer et al., 2007). It is also known that long-term physical inactivity can have deleterious effects on the vascular system. It can accelerate atherosclerosis and lead to increased endothelial dysfunction (Laufs et al., 2005). It is possible that lack of physical activity has a corresponding negative effect on EPC number and function. To test this possibility, we used hind limb suspension (HLS). HLS is a method used to mimic the effects of long-term physical inactivity, bed rest or a sedentary lifestyle. In HLS, the animal's hind limbs are suspended off the floor of the cage with a brace of some sort. The animal's hind limbs bear no weight during this period, thus the muscles in the hindquarters experience no load-bearing. By taking a blood sample from an HLS animal and using flow cytometry to look for changes in the expression of certain markers, we can observe possible changes in EPC numbers. We hypothesized that HLS rats would exhibit a lesser number of circulating EPCs than control rats. We chose the following markers to observe via flow cytometry: CD31, CD45, CD62L and CD34.

CD31 is an adhesion molecule most commonly found on endothelial cells. It is usually found concentrated in the cell-to-cell junction of the endothelium. While typically associated with mature endothelial cells, it is sometimes used as a marker for late-stage EPCs (Hristov et al., 2003). Thus, it could be useful as an indicator for circulating EPC levels. CD45 is a leukocytic marker. While it is not known to be found on endothelial cells, mature or otherwise, it is found in the mononuclear cell fraction of blood in which EPCs are also thought to be found. Therefore, we examined its expression to see if any changes occurred in response to HLS. CD62 is found on endothelial cells and leukocytes. It is thought to be involved in leukocytic adhesion and transmigration through the endothelium. It is not a typical EPC marker, but it is possibly found on *in vitro*-cultured EPCs (Biancone et al., 2004). Therefore, we will also examine its expression. CD34 is a common marker used to identify EPCs. Its function is currently unknown. It is thought to be one of the markers circulating EPCs retain after leaving the bone marrow, and thus it is an important marker for us to observe (Hristov et al., 2003).

Studies on the effects of HLS on the vascular system (Jasperse et al., 1999; Woodman et al., 2001) indicate that a decrease in eNOS takes place, with a corresponding decrease in endothelial-dependent vasodilation. EPC recruitment is mediated in part by the bioavailability of nitric oxide. There has also been evidence that HLS can lead to increased apoptosis of endothelial cells in muscle capillaries (Fujino et al., 2005). If endothelial cells integrated into the endothelium are affected, circulating EPCs might also be affected. This leads us to hypothesize that there will be a difference in the number of cells expressing the endothelial markers CD31, CD34 and CD62 in control rats as compared to HLS rats.

Materials and Methods

Animal Care

Six-month old male Fisher 344-Brown Norway crossed (F344XBN) rats averaging 300 gm were used for this study. All rats were housed in Marshall University's approved animal care and housing facility. Animal housing used a 12 hour light–dark cycle and temperature was kept at 22 °C. Animals were fed standard rat chow pellets. Food and tap water were kept freely available. . All rats were housed in grating-bottomed cages. All animal protocols and facilities were reviewed and approved by Marshall University's Institutional Animal Care and Use Committee.

Hind limb Suspension

HLS rats were suspended by placing their tails in a plastic brace attached to a pulley. The pulley was mounted on a bar that ran across the top of the rat's cage. The rats were able to access food and water while suspended, but did not bear any weight on their hind limbs. Rats were suspended for 3, 7 or 14 days. Control rats were kept two to a cage without suspension braces.

Blood Collection

Rats were anesthetized with a ketamine (40 mg/kg)/xylazine (10mg/kg) injection. The chest cavity was opened with surgical scissors. Blood was obtained from the left ventricle using a heparinized syringe. The blood was diluted 2:1 with a 2% fetal bovine serum/PBS solution, and then layered over 15 mL of Ficoll-Paque density gradient solution in a 50 mL tube. The solution was centrifuged at 400*g* for 30 minutes without braking. After centrifugation, the mononuclear

cell fraction was removed and washed with 30 mL of 2%FBS/PBS solution. The solution was centrifuged for 10 minutes at 300*g*. The supernatant was aspirated and the cell pellet was resuspended in 10 mL of 2% FBS/PBS. A 20 µL aliquot was used on a hemacytometer to obtain a rough cell count.

Cell Culture

$1x10^7$ cells were used for flow cytometric analysis, while the remaining cells were seeded on 1 µg/cm^2 fibronectin-coated 100 mm tissue culture plates. Cells were cultured in EGM-2 complete media (Lonza, Basel, Switzerland) which was changed every two days. Culture plates were observed at 7 days after seeding to look for endothelial colonies. Colonies exhibiting a potential endothelial morphology were harvested with a sterilized cloning cylinder and seeded in a 24-well tissue culture plate.

Flow Cytometry

Cells were suspended at a density of $5x10^6$ cells per mL in a PBS/ 2% FBS/ 0.1% sodium azide solution. A 200 µL aliquot of suspension was added to each prepared 15 mL tube. Each tube contained the predetermined antibody suspended in 20 µL of 2% FBS/PBS, except the control which only received 10 µL of buffer. The following antibodies were used: 0.002 µg/µL AlexaFluor® 488-conjugated mouse IgG1 negative control, 0.004 µg/µL FITC-conjugated mouse IgG1 negative control, 0.002 µg/µL AlexaFluor® 488-conjugated mouse anti-rat CD31, 0.002 µg/µL AlexaFluor® 488-conjugated mouse anti-rat CD45, 0.004 µg/µL FITC-conjugated mouse anti-rat CD62L (AbD Serotec, Oxford, UK), 4 µL per $1x10^6$ cells of PE-conjugated mouse IgG1 and 0.004 µg/µL PE-conjugated mouse anti-CD34 (Santa Cruz Biotechnology, Santa Cruz, California).

Cells were incubated on ice for 60 minutes. After incubation, cells were washed 3 times with 5 mL of 2% FBS/PBS solution per tube. Each wash consisted of being mixed by vortex and spun in a centrifuge at 380*g* for 5 minutes. Cells were suspended in 300 µL of PBS/ 2%FBS/ sodium azide solution and transferred to a polystyrene tube. Analysis was conducted using a BD Biosciences (San Jose, CA) FACSAria cell sorter. Fluorescence was recorded for $1x10^5$ cells per sample. Isotype control fluorescence was subtracted from positive marker fluorescent to account for non-specific antibody binding. One-way ANOVA was performed to determine statistical significance with $P<0.05$.

Results

Flow Cytometry

During flow cytometry the percent positive cell count for each marker was recorded and evaluated for any statistically significant differences. This allows us to quantitatively evaluate any possible changes in cell marker expression that occurred due to HLS. We did not find any significant differences in our CD45 flow analysis data (**Fig. 1)**. While CD45 would not be found on endothelial cells, it is found on lymphocytes, which compose the majority of the peripheral blood mononuclear cell fraction we obtained during blood collections. We examined this marker to see if any changes in its expression would occur in response to HLS. We did not find any statistically significant differences.

CD31 is a very common mature endothelial marker, and is considered in some literature to be a late-stage EPC marker (Hristov et al., 2003). If its expression changes due to HLS, this may

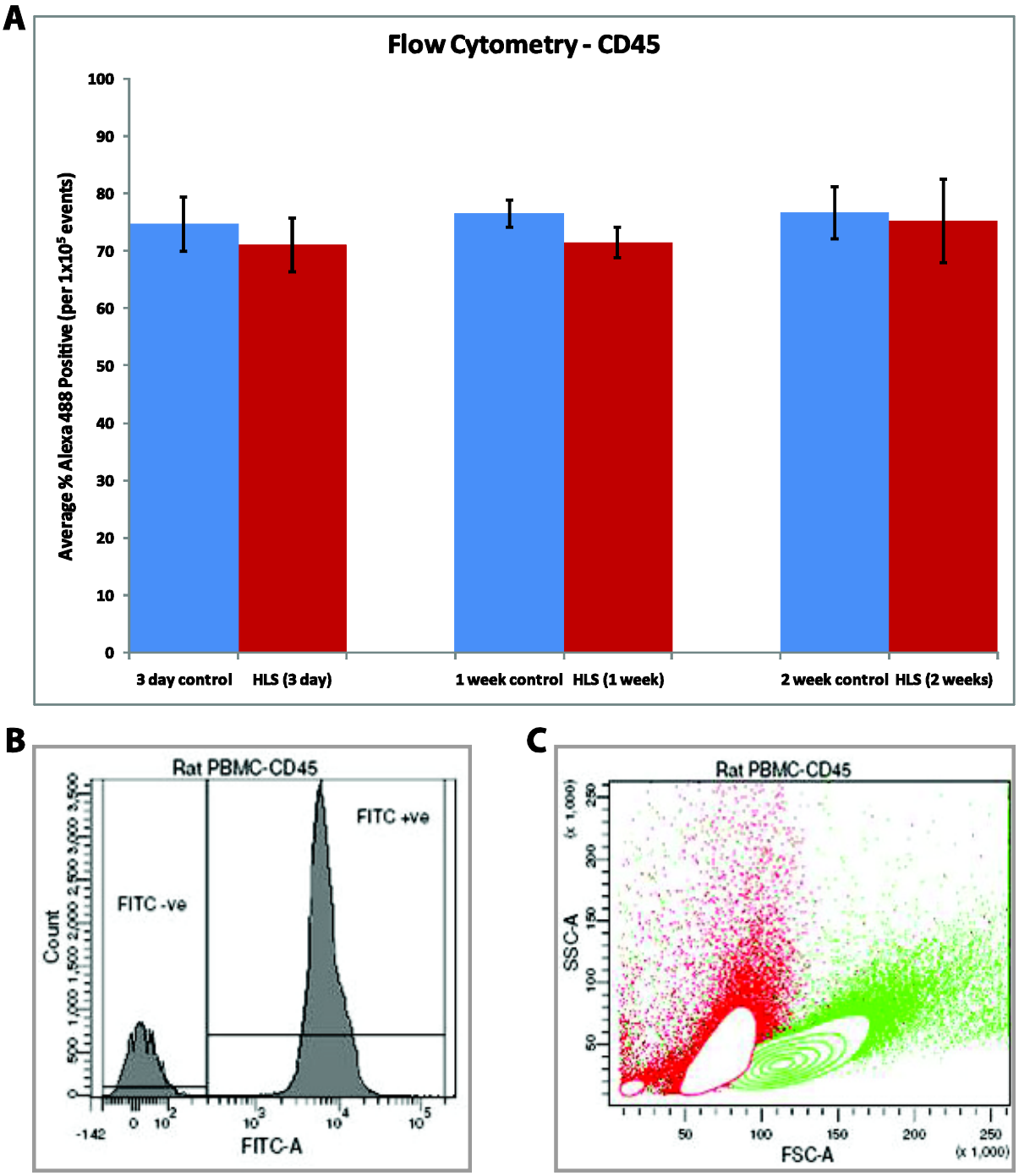

Figure 1. (**A**) Average CD45 expression according to flow cytometric analysis. CD45 expression was found to be lower in HLS rats than in control rats, however these results were not found to be statistically significant. Error bars represent standard error of the mean. (**B**) Example histogram of CD45 analysis. (**C**) Example of CD45+ (green) and CD45- (red) populations.

indicate a change in the endothelium or in circulating EPC numbers. However, our data did not show any significant changes in CD31 in response to HLS (**Fig. 2**).

We did not find any statistically significant differences in CD62L expression (**Fig. 3**). CD62L is found on several cell types, including leukocytes and endothelial cells. As mentioned before, it might be found on EPCs. If any changes were found as a result of HLS, it might indicate a change in circulating EPC numbers. We examined this marker to look for potential changes in the PBMC fraction, similar to CD45. However, we found no significant differences.

Our CD34 flow analysis (**Fig. 4)** did not yield any significant differences. CD34 is a marker more commonly associated with immature EPCs as opposed to mature endothelial cells, and is thought to be expressed as EPCs leave the bone marrow. At seven days, we found a slightly higher expression of CD34 in HLS animals than in control animals. However, this difference was not found to be statistically significant. We took special note of this marker, as it is most likely to be found on an EPC (Peichev et al., 2000). Our data indicated 5000 CD34+ positive cells per $1x10^5$ PBMCs.

Cell Culture

Our cell culture experiments (**Fig. 5**) did not indicate a consistent change in colony-forming EPCs (CF-EPCs) in response to HLS. The total number of colonies obtained is compared to the number of colonies that actually exhibited endothelial morphology. Because we examined the PBMC fraction, some of the colonies that grew exhibited a non-endothelial morphology. Thus, the total number of colonies did not reflect the actual number of CF-EPCs. Our data indicates that in most cases only one endothelial colony managed to adhere and proliferate. The colony

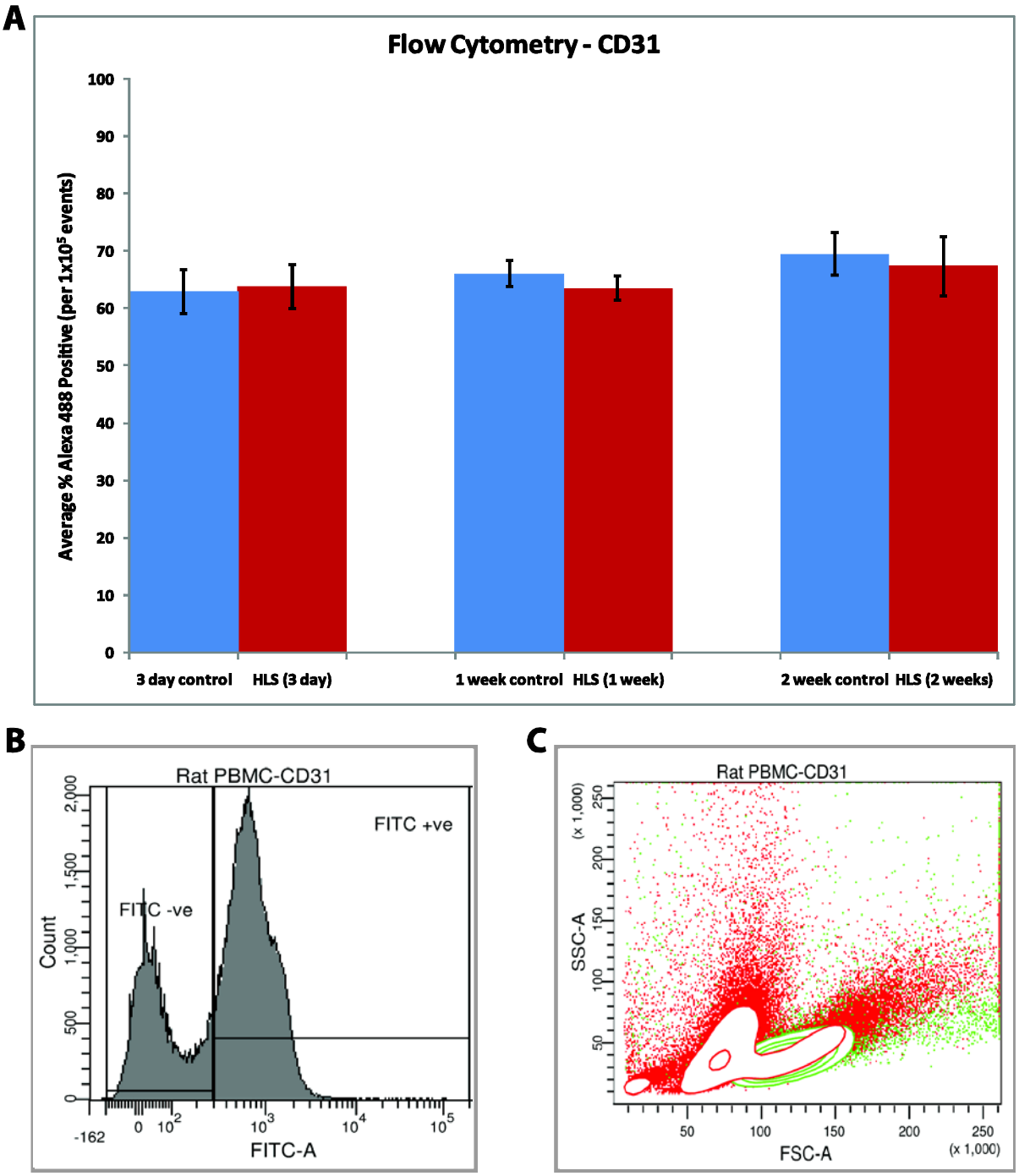

Figure 2. (**A**) Average CD31 expression according to flow cytometric analysis. CD31 analysis results were not found to be statistically significant. Error bars represent standard error of the mean. (**B**) Example histogram of CD31 analysis. (**C**) Example of CD31+ (green) and CD31- (red) populations.

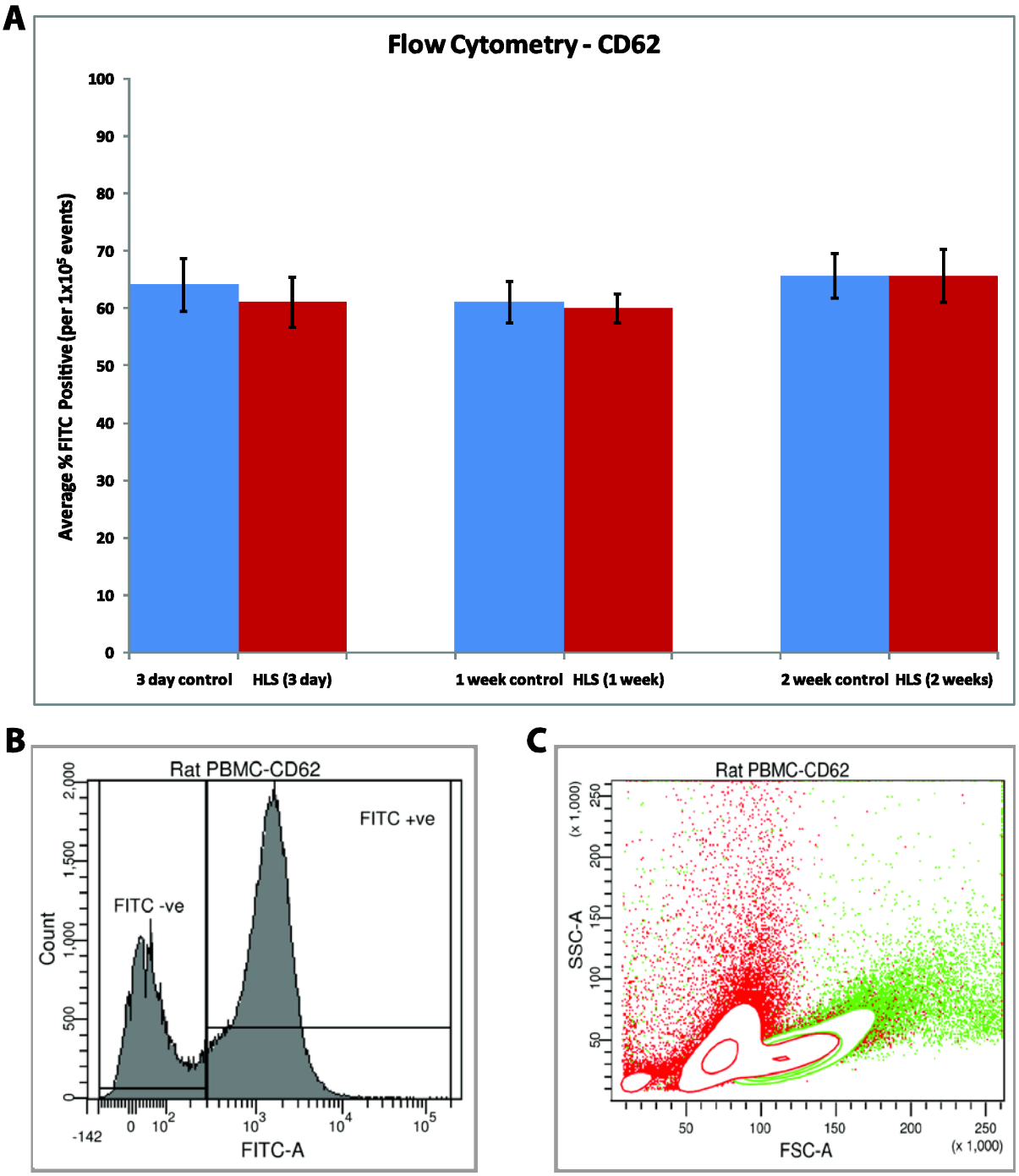

Figure 3. (**A**) Average CD62 expression according to flow cytometric analysis. CD62 analysis results were not found to be statistically significant. Error bars represent standard error of the mean. (**B**) Example histogram of CD62 analysis. (**C**) Example of CD62+ (green) and CD62- (red) populations.

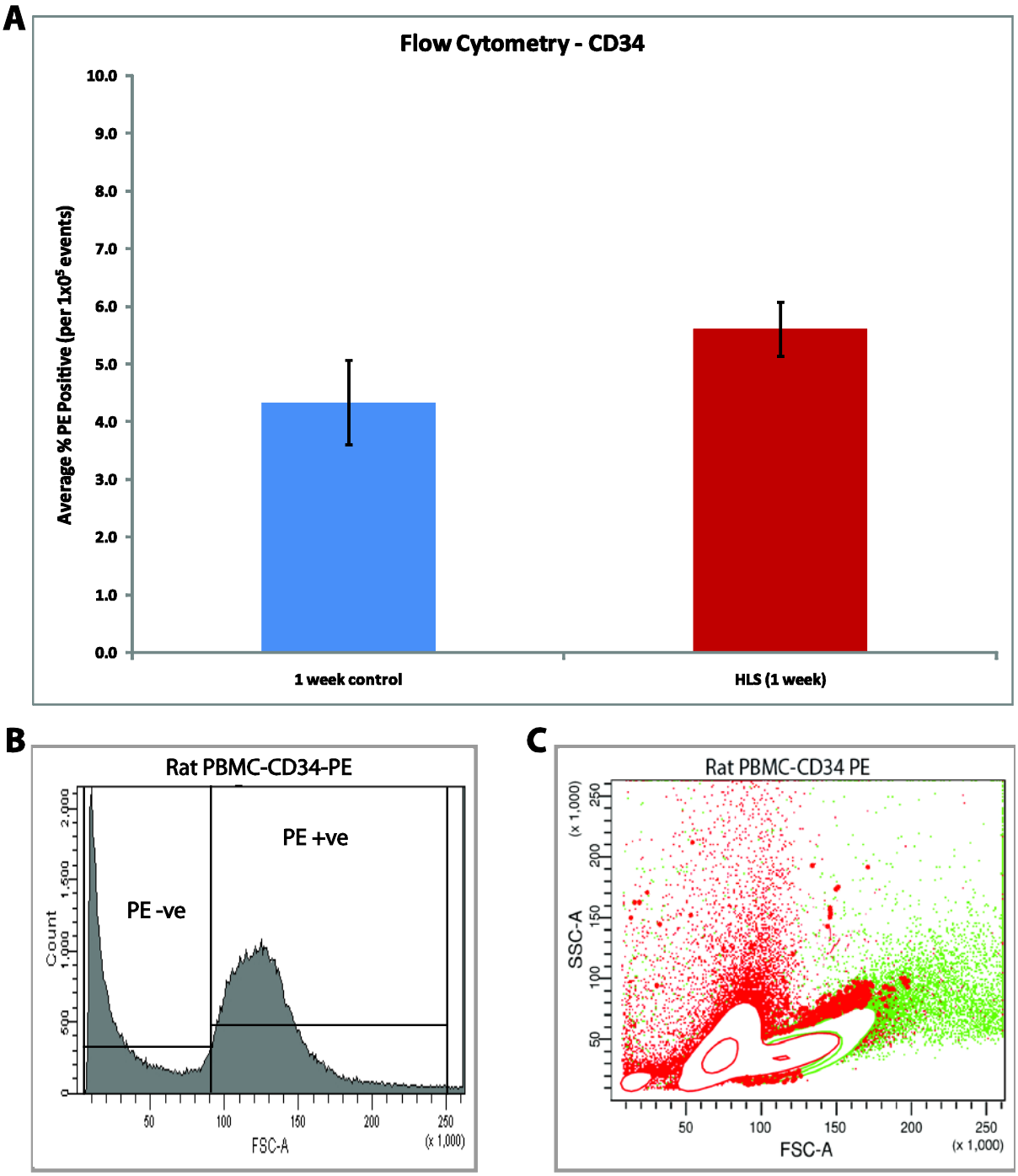

Figure 3. (**A**) Average CD34 expression according to flow cytometric analysis. CD34 analysis results were not found to be statistically significant. Error bars represent standard error of the mean. (**B**) Example histogram of CD34 analysis. (**C**) Example of CD34+ (green) and CD34- (red) populations.

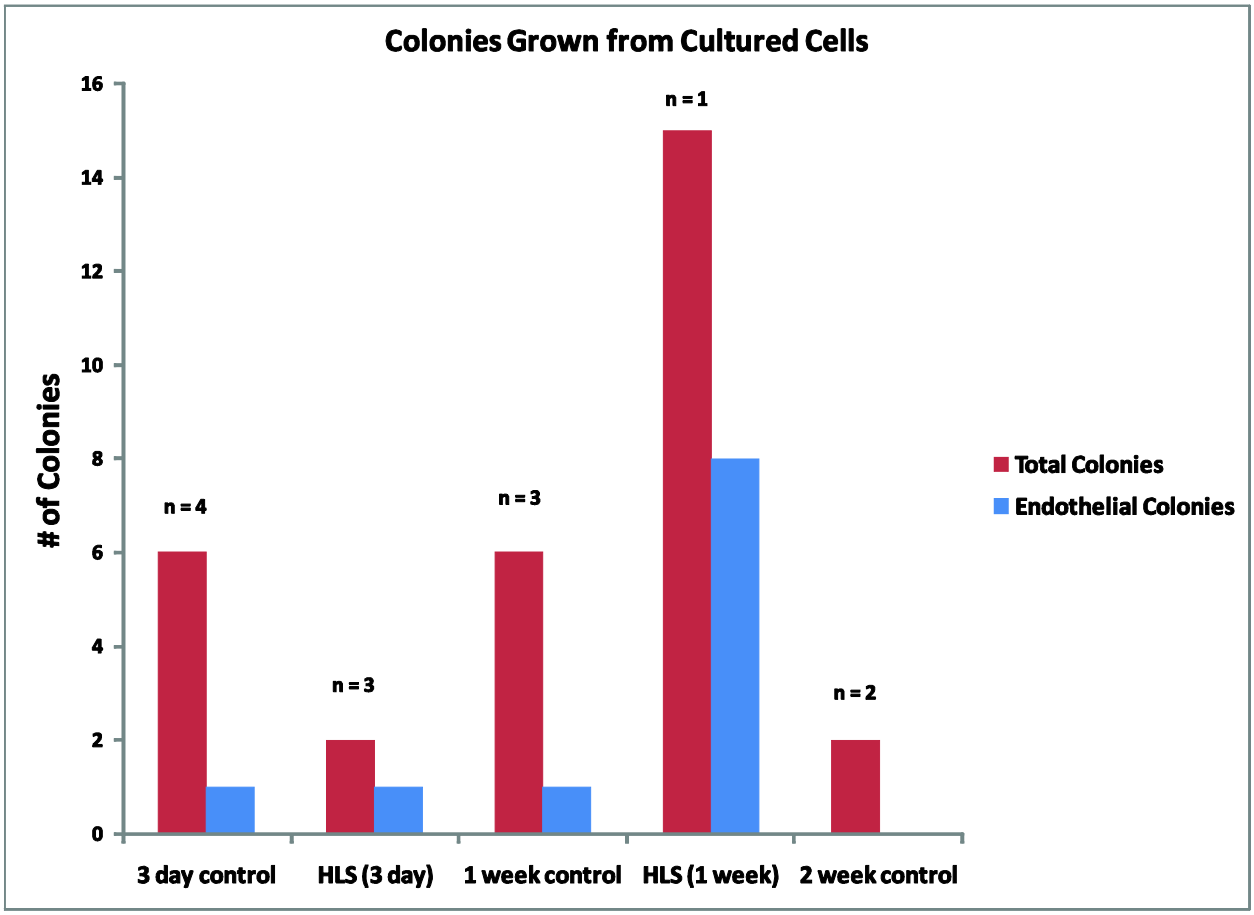

Figure 5. Results of cell colony experiments. Total number of colonies from each experimental group is shown next to number of colonies displaying endothelial morphology.

data for 1 week HLS is unusual, both in the high number of total colonies and high number of actual endothelial colonies. It should be noted that the majority of those colonies came from a single rat, and are not indicative of the 1 wk HLS population as a whole. Therefore, we believe the cell culture data from 1 week HLS rats to be erroneous.

Discussion

No significant differences were found in control animals among any of the three time courses (3 days, 7 days, and 14 days). Numbers did not drop significantly in HLS animals, nor did they vary much between the time courses. This appears to indicate that HLS does not affect numbers of circulating cells positive for CD31, CD45 and CD62. However, it must be noted that while the numbers may not change, we do not know how the functionality of these cells has been affected. It is possible that HLS animals have the same number of circulating cells positive for the aforementioned markers as control animals, but they do not function as well.

We had previously used a FITC-conjugated antibody, but were unable to use the data as the isotype controls displayed greater fluorescence than the anti-CD34 samples. This indicated a problem with the staining, and so we were forced to disregard that data. Although we did not find any significant differences in the number of CD34 positive cells between control and HLS, our findings did seem to indicate a higher number of CD34-positive cells than what would be expected from the literature. Yao et al. found 2700 CD34-positive cells per $1x10^5$ PBMCs in the WKY rat strain, and 1000 CD34-positive cells per $1x10^5$ PBMCs in the SHR-SP strain (Yao et al., 2007). Our data indicated 5000 CD34- positive cells per $1x10^5$ PBMCs. It must be noted however that a CD34-positive cell is not necessarily an EPC, as CD34 is also found on non-

endothelial hematopoietic progenitor cells. Indeed, our cell culture results indicated only a few cells per sample were capable of forming colonies. While those colonies might indicate an EPC origin, it is still uncertain.

It should also be noted that there are more differences between control and HLS animals besides simply being suspended. The control rats were kept two to a cage, while suspended animals caged alone. Rats are social creatures, and interact with each other on a regular basis. The control rats were more active and probably expressed a different biochemical profile than the suspended rats that spent most of their time relatively immobile. It should also be noted that the control rats did not have the suspension harness attached to their tails. The harness may have played a role in the partial constriction of circulation in the hindquarters and distressed the rat simply by being attached to its tail, thus creating another stress to which the control rats were not exposed. The end result of these compound stressors may have confounded our results by activating some form of compensatory mechanism.

Our cell culture experiments did not shed any information to contradict these findings. In most cases, only one sample from each treatment group yielded cells that grew into colonies. Of the colonies that did grow, we typically found only one colony that exhibited endothelial morphology. This may be an indicator that in spite of our CD34-positive cell numbers, few of those cells were actually EPCs. Some of those cells may have been mature endothelial cells, which are capable of only limited proliferation. While we did seem to get an unusually high number of endothelial colonies from the 1 wk HLS sample group, it should be noted that all those endothelial colonies came from a single sample. We found no samples in any of the

other groups exhibiting this kind of colony growth, nor did we find any other case where a sample yielded more than one endothelial colony in any of the treatment groups. Indeed, most of the samples yielded no colonies whatsoever. This single case could most likely be attributed to the variation inherent in using animal models, even strains bred to be identical to each other. It is most likely not an indicator that HLS induces higher numbers of colony-forming EPCs than control animals.

While we did not see significant changes in our observed markers, we cannot say for sure whether or not HLS had an effect on EPC numbers in rats. Most of our markers are found on multiple cell types, thus no single marker can serve as an indicator for a change in a given cell type. A more definite method would be multiple marker analysis. By using more than one surface marker antibody, we could pinpoint a precise phenotype, and thus be better able to positively identify an EPC. In addition, EPCs are thought to be a rare cell population. It is possible that we did not analyze enough cells to give us an idea as to whether cell population experience any change. In addition, we saw no significant differences in the number of colony-forming units between control rats and HLS rats. However, we do not know how HLS has affected the functionality of the EPC populations in these rats. Because we could not consistently grow colonies from the harvested PBMCs, we cannot say anything in regards to the proliferative capacity of the circulating EPCs. Our original intent was to perform functional assays, such as the wound healing assay, with EPCs from both control rats and HLS rats to compare their relative wound healing capacity. However, we were unable to culture any EPC colonies from the animals. Therefore, it may still be possible that the dysfunction seen in PAD patients results from decrease in EPC function instead of a decrease in numbers. If the number

of EPCs remains the same, yet they cannot perform their intended function, it would result in an overall increase in endothelial dysfunction.

CHAPTER THREE

Introduction

The endothelium is critical to vascular health. Because blood is constantly flowing, the endothelium experiences a constant shear stress. At areas where vessels branch and bifurcate, that shear flow becomes turbulent. This turbulent stress can damage endothelial cells or strip them off the underlying basement membrane completely. Because endothelial damage can lead to cardiovascular problems such as atherosclerosis, it is vital that the endothelium has a means to repair itself. The endothelial wound healing assay is a very common and well-known model for studying endothelial cell migration and resurfacing *in vitro*. In this assay, a cultured layer of endothelial cells is scratched with an instrument such as a pipet tip, and the cells are observed as they recover the injured area over time. Despite its widespread use, many of the underlying mechanics of the endothelial healing process have not been thoroughly examined. Our objective in this study was to discover some of the factors that play a role in wound healing. We hypothesized that migration due to wound healing is controlled by cytoskeletal remodeling, in additions to secondary signaling factors.

We treated human umbilical vein endothelial cells (HUVECs) to drugs known to inhibit various cellular processes. We used the anti-angiogenic drugs angiostatin, endostatin and the matrix metalloproteinase inhibitor GM6001 to investigate whether endothelial wound healing used the same pathways as angiogenesis. The cytoskeleton disruptors cytochalasin D and colchicine were used to investigate what parts of the cytoskeleton are involved in endothelial wound

healing. The protein synthesis inhibitor cycloheximide was used to examine to what degree new proteins must be built in order to facilitate wound healing.

Some studies have shown that ERK1/2 activation is changed during cell monolayer wound healing. In previous studies two waves of MAPK phosphorylation have been observed during the healing of a monolayer of canine kidney cell monolayers(Nikolic et al., 2006). This prompted us to study the phosphorylation status of the MAPK ERK1/2 (an indicator of activity) in our HUVECs. We used the phosphor-ERK1/2 inhibitor UO126 to examine the role of ERK1/2 phosphorylation in endothelial wound healing.

Materials and Methods

Cell Culture

HUVECs (Lonza, Basel, Switzerland) were grown on a T-150 tissue-culture-coated flask (BD Falcon, San Jose, CA). Cells were cultured in 30 mL of EGM-2 Complete Media (Lonza) with 2% FBS every two days. Cell passaging was performed every four days using 0.05% trypsin-EDTA solution to remove cells from culture coating and 0.5% soybean trypsin inhibitor to stop trypsin activity.

Wound Healing Procedure and Treatments

HUVECs were grown to confluence on a 24-well tissue culture-coated plate (BD Biosciences, San Jose, CA). Cell monolayers were treated with one of the following drugs diluted in 500 µL EGM-2 complete media 15 minutes prior to wounding: 0.2 µg/mL cytochalasin D, 1 µg/mL colchicine, 1 µg/mL cycloheximide, 30 µg/mL endostatin (Sigma-Aldrich, St. Louis, MO), 26 µM GM6001

(Millipore, Billerica, MA), 10 µM U0126 and $2.0x10^{-3}$ µg/mL angiostatin (Calbiochem, San Diego, CA). Control cells received 500 µL EGM-2 complete media for 15 min. Monolayers were scratched with a 200 µL pipet tip and incubated for particular amounts of time. Cells were washed twice with PBS. Cells were either fixed with 4% paraformaldehyde for 15 min and stored at 4°C in PBS for ICC, or protein was collected with addition of Laemmli buffer and stored at -20°C for immunoblot analysis.

Measurement of Rate of Wound Healing

After wounding, cell culture plates were placed in the incubation hood (37°C, 5% CO_2) of a Zeiss AxioObserver bright field/fluorescent microscope. A 3x3 mosaic image was captured every hour for 24 hrs using the 10x objective. Images were observed and wound width was measured with AxioVision 4.6 software. The initial wound width was measured as the distance between the leading edges of the wound. The wound was measured at each subsequent hour and the width subtracted from the width of the previous hour to find the amount of wound closure. The average rate of healing was found by dividing the initial wound width by the number of hours the monolayer required to fully heal. If the monolayer had not healed by 24 hrs, the width at 24 hours was subtracted from the initial width and the difference divided by 24. Change in rate and average rate of wound healing were calculated and compared for statistical significance on SigmaStat 3.5 software using one-way ANOVA.

Immunocytochemistry

PFA-fixed cell monolayers were incubated with 0.1% Triton X 100 in PBS solution at room temperature for 20 min, and then washed with 1 mL PBS for 5 min. Cells were then incubated

with 0.1% BSAc in PBS for 30 min followed by a 2 hour incubation in 5% BSA, 5% goat serum, 0.1% Micro-O-Protect in PBS at room temperature. After a brief wash with 1 mL PBS, primary antibodies were diluted in 1:10 blocking buffer, added to cells and incubated overnight in a humidified chamber. The following primary antibodies were used: 100 μg/mL rabbit anti-Ki-67 polyclonal antibody (Abcam Inc, Cambridge, MA), 1 μg/mL mouse anti-α-tubulin mAb (Sigma), and 1:200 rabbit anti-phospho-ERK1/2 mAb (Cell Signaling Technology, Beverly, MA). Non-primary antibody-treated control wells received 1:10 blocking buffer only. These wells were used to distinguish between non-specific antibody staining and actual staining. Cells were washed three times with 1 mL PBS, 5 min each, then exposed to secondary antibodies also diluted in 1:10 blocking buffer for 4 hrs. The following secondary antibodies were used: 5 μg/mL AlexaFluor 647-conjugated goat anti-mouse IgG_1 and 5 μg/mL AlexaFluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, Oregon). During the last 30 min of secondary incubation, a 1:40 dilution of AlexaFluor® 568-conjugated phalloidin (Invitrogen, Carlsbad, CA) was added to the cells. Cells were washed three times with 1 mL PBS, and then incubated with 1 mL of 0.002% DAPI (Santa Cruz) for 10 min. After a final wash with PBS, cells were stored in 300 μL of 0.1% Micro-O-Protect (Roche Diagnostics, Mannheim, Germany) at 4°C until observed.

ERK1/2 Immunoblot Quantification

Cells were treated with colchicine, cytochalasin D, cycloheximide or U0126 and wounded as previously noted. Cell protein was harvested with 200 μL Laemmli buffer at the following time points post-wounding: 0 min, 1 min, 3 min, 5 min, 10 min, 30 min, 60 min, and 120 min.

Triplicates of all samples were collected. Equal amounts of protein of all time points was subjected to SDS-PAGE along with a non-treated control and Novex® Sharp Pre-stained Protein Standard (Invitrogen) on a NuPAGE® 4-12% Bis-Tris Gel (Invitrogen). Protein was transferred to a PVDF membrane (Millipore) and blocked with 5% dry milk/ 0.1% Tween-20 in TBS solution. Primary staining was done overnight at 4°C with rabbit anti-ERK1/2 mAb (1:2000). Membrane was washed with 0.1% Tween-20 TBS solution and incubated with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma) diluted 1:100,000 for 60 minutes followed by a second round of washes with 0.1% Tween-20 TBS solution. Protein bands were visualized by treating the membrane with ECL detecting reagents (GE Healthcare, Waukesha, WI) and exposure to photographic film for 30 minutes. Densitometric comparisons of immunoblots were performed with ImageJ software (NIH). Bands of treatment samples were normalized to expression in control sample. Membranes were stained with Coomassie Blue to confirm equal protein loading.

Results

Measurement of Rate of Wound Healing

We examined the impact of the anti-angiogenic drugs angiostatin, endostatin and GM6001 on endothelial wound healing. The average rates of wound healing were compared to the average rate of healing for control cells and evaluated for statistically significant differences. We found that these drugs do not slow healing of wounded HUVEC monolayers (**Fig. 6**). Most control monolayers had healed completely by 12 hours, as had the treated cells. We also compared control cells versus cells treated with the cytoskeleton-disrupting drugs cytochalasin D and

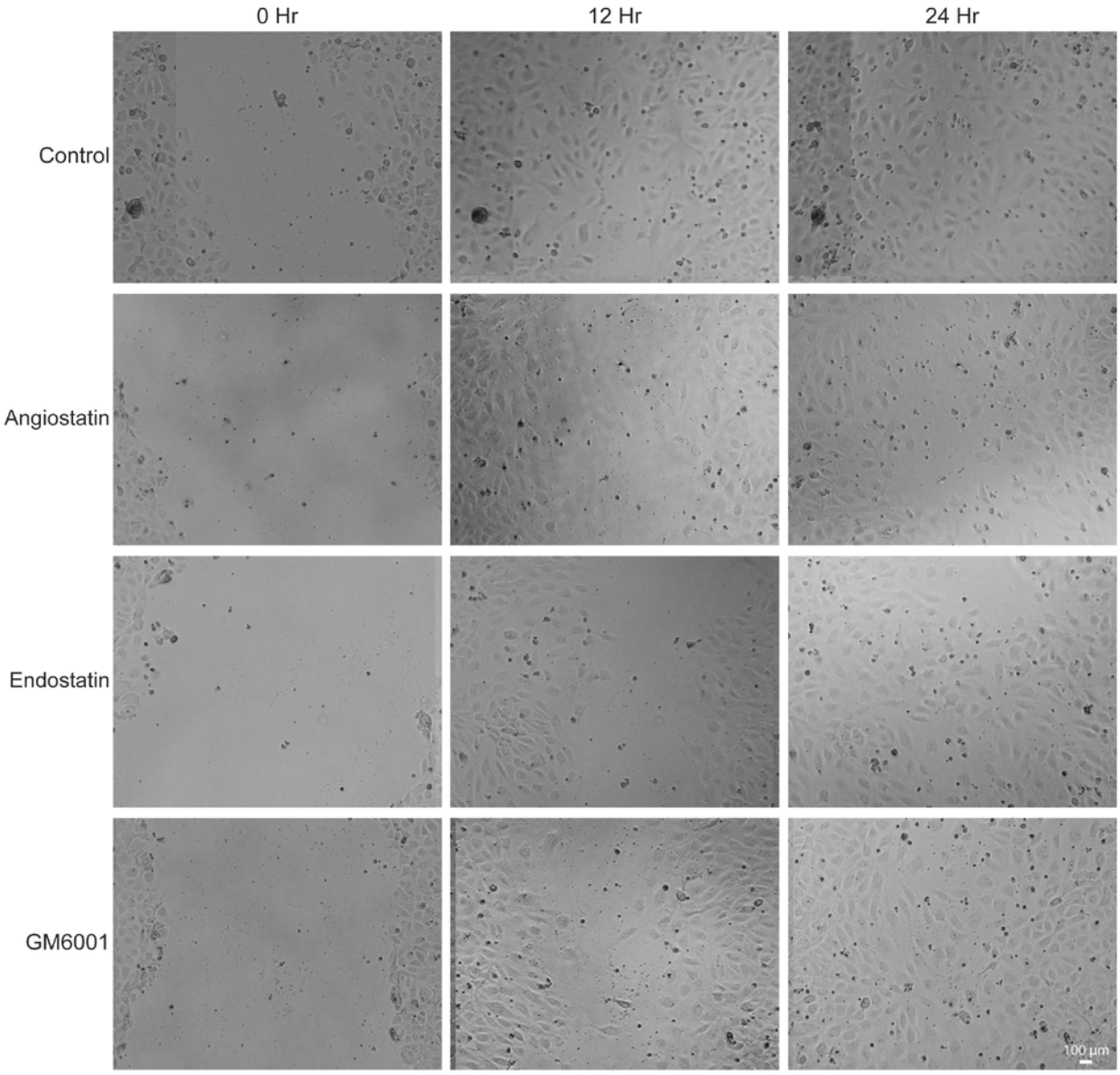

Figure 6. Phase contrast images of wounded HUVEC monolayers taken at specified time points after wounding. Control HUVECs had mostly healed by 12 hours, while HUVECs treated with anti-angiogenic drugs angiostatin, endostatin and GM6001exhibited healing to varying degrees. By 24 hrs after the initial injury, all monolayers had completely healed.

colchicine, as well as the protein synthesis inhibitor cycloheximide (**Fig. 7**). The disparity between rates of wound healing in response to treatments is readily seen by the still-open injury in the treated cells at 12 hours. Cytochalasin D and cycloheximide both significantly slowed wound healing, while colchicine completely inhibited it. During wound measurement the colchicine-treated cells were observed to move into and away from the wounded area, hence the negative rate of change at certain time points.

At the end of the 24 hour time course there was very little overall migration into the injured area. We compared the rate of change in control cells to cells treated with anti-angiogenic drugs (**Fig. 8A**). This demonstrates the rate of wound healing is quantifiably the same for both control and treated cells. It also reveals that rate of wound healing slows over time regardless of treatment. We also compared rate of change of control HUVECs versus colchicine, cytochalasin D and cycloheximide-treated HUVECs (**Fig. 8B**). During the early time points of wound healing the cycloheximide, cytochalasin D and U0126-treated cells all start at a slower rate of healing than the control cells. However, at about 9 hours into healing the control and U0126 cells fall into approximately the same rate. The cytochalasin D and cycloheximide treated cells match rates with the controls at about 16 hours. Colchicine's deleterious effect on wound healing is clearly demonstrated by this figure, exhibiting a negative rate of change at certain time points. Finally we compared the average rate of wound healing for all treatments, expressed as initial wound width divided by the total time required to fully heal (**Fig. 8C**). The average rate for anti-angiogenic treated cells was found to be statistically indistinguishable from the control rate. The rates for colchicine, cytochalasin D, cycloheximide and U0126 were

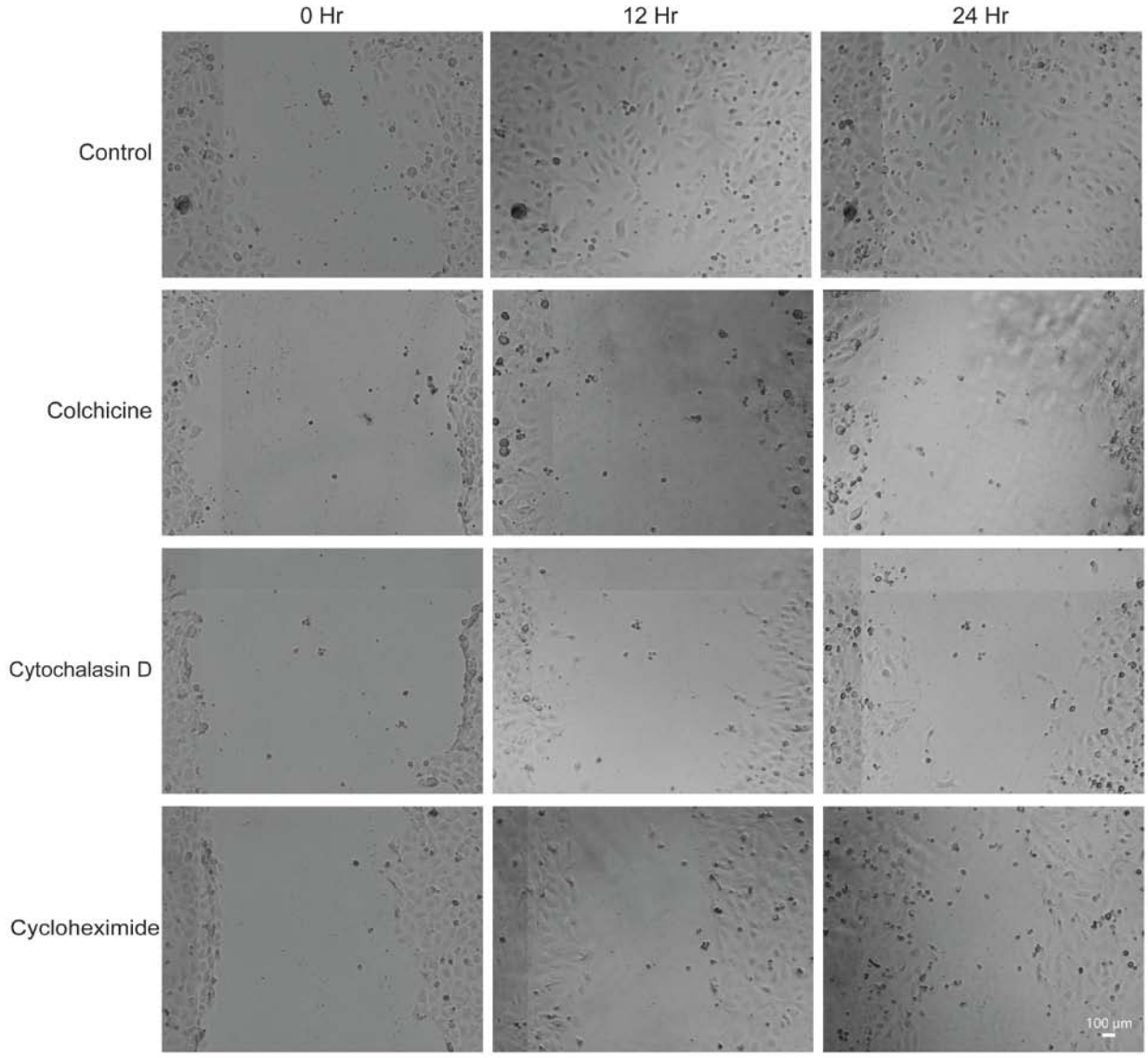

Figure 7. Phase contrast images of wounded HUVEC monolayers taken at specified time points after wounding. In contrast to colchicine, cytochalasin D and cycloheximide treated HUVECs, control HUVECs had completely repopulated the wounded area after 12 hrs. Colchicine treated cells in particular exhibited no wound healing. After 24 hrs cytochalasin D and cycloheximide-treated cells had healed further, while colchicine-treated cells had not.

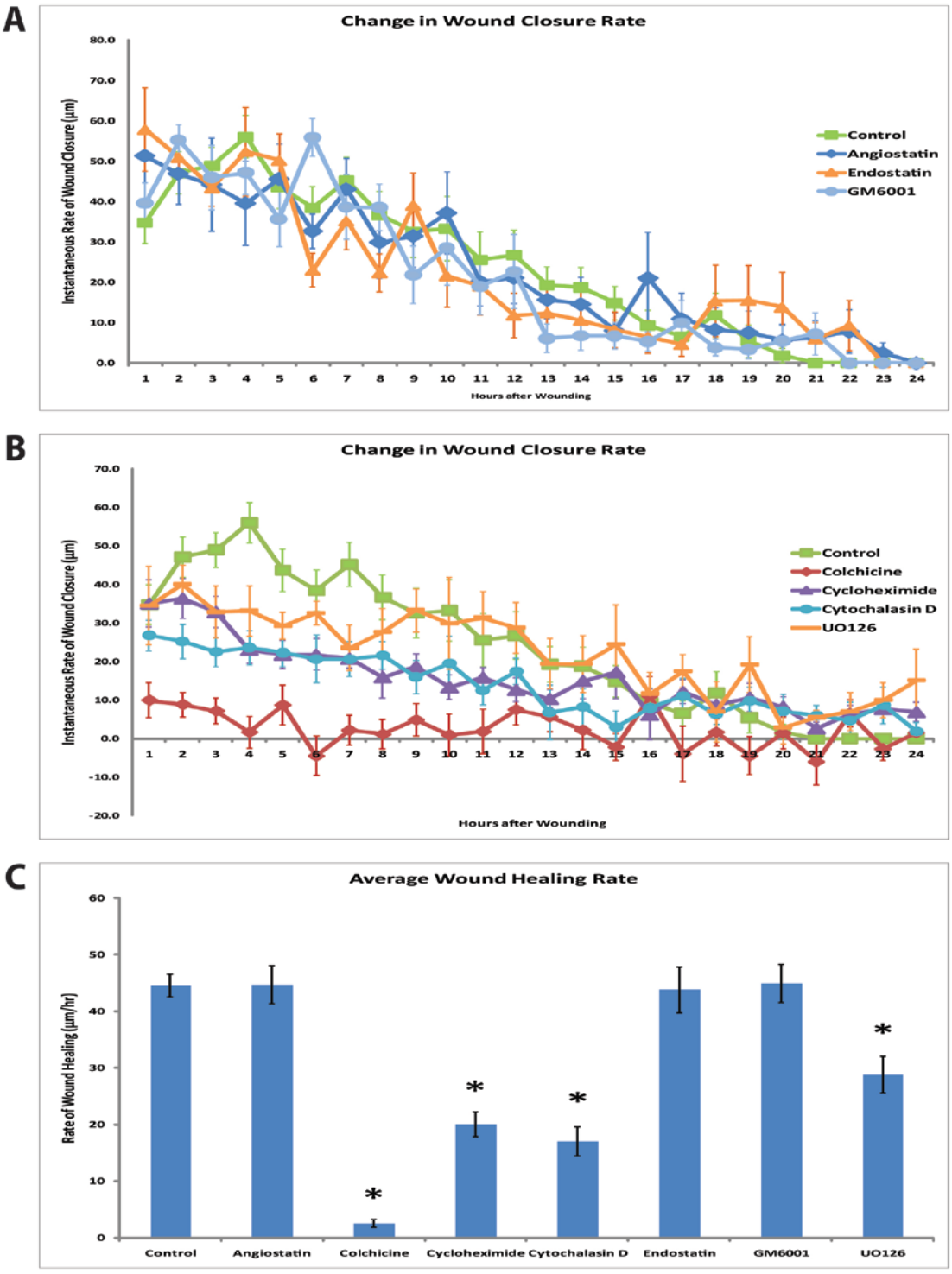

Figure 8. (**A**) The rate of wound healing decreased over time for all treatments. The anti-angiogenic drugs angiostatin, endostatin and GM6001 did not have a statistically significant effect on wound healing. Error bars represent standard error of the mean. (**B**) The drugs colchicine, cyclohexamide, cytochalasin D and UO126 all had a significant effect on rate of wound healing. Error bars represent standard error of the mean. (**C**) Comparison of overall average rate of wound healing. Error bars represent standard error of the mean. * denotes statistically significant different ($P < 0.001$).

found to be statistically different from control ($P < 0.001$), providing further quantifiable evidence that these drugs have a negative effect on wound healing.

Immunocytochemistry

We performed immunocytochemistry on the wounded cells to ascertain that the colchicine, cytochalasin D and U0126 treatments had the intended effect. It also gave further evidence that the drugs were responsible for any changes in wound healing observed during measurement if some aspect of normal cell morphology was also affected. The untreated endothelial cells in **Fig. 9A** demonstrate exhibit normal microfilament morphology. Upon treatment with cytochalasin D, the actin monomers can no longer polymerize during cytoskeletal remodeling and form globules (**Fig. 9B**). The same effect occurs when HUVECs are exposed to colchicine. Normal microtubules appear to be fine cotton-fiber like structures (**Fig. 9C**). After treatment with colchicine, the microtubules lose distinct structure (**Fig. 9D**). These images provide further evidence that these drugs disrupt the cytoskeleton. To examine how much cell proliferation affected wound healing, we looked at Ki-67 expression. Normal Ki-67 expression appears as punctate staining localized within the nuclear region (**Fig. 9E**). Ki-67 is a marker of cell proliferation and is expressed in the nucleus at all phases of the cell cycle except for G_0. Ki-67 expression was found at all time points and did not appear to change in response to drug treatment. However, expression was slightly more highly expressed at the leading edges of the wound, which means there may be a greater incidence of mitosis at those areas. We did not find any phosphorylated ERK1/2 signal in UO126-treated cells at any time point, including the 10 min peak (data not shown).

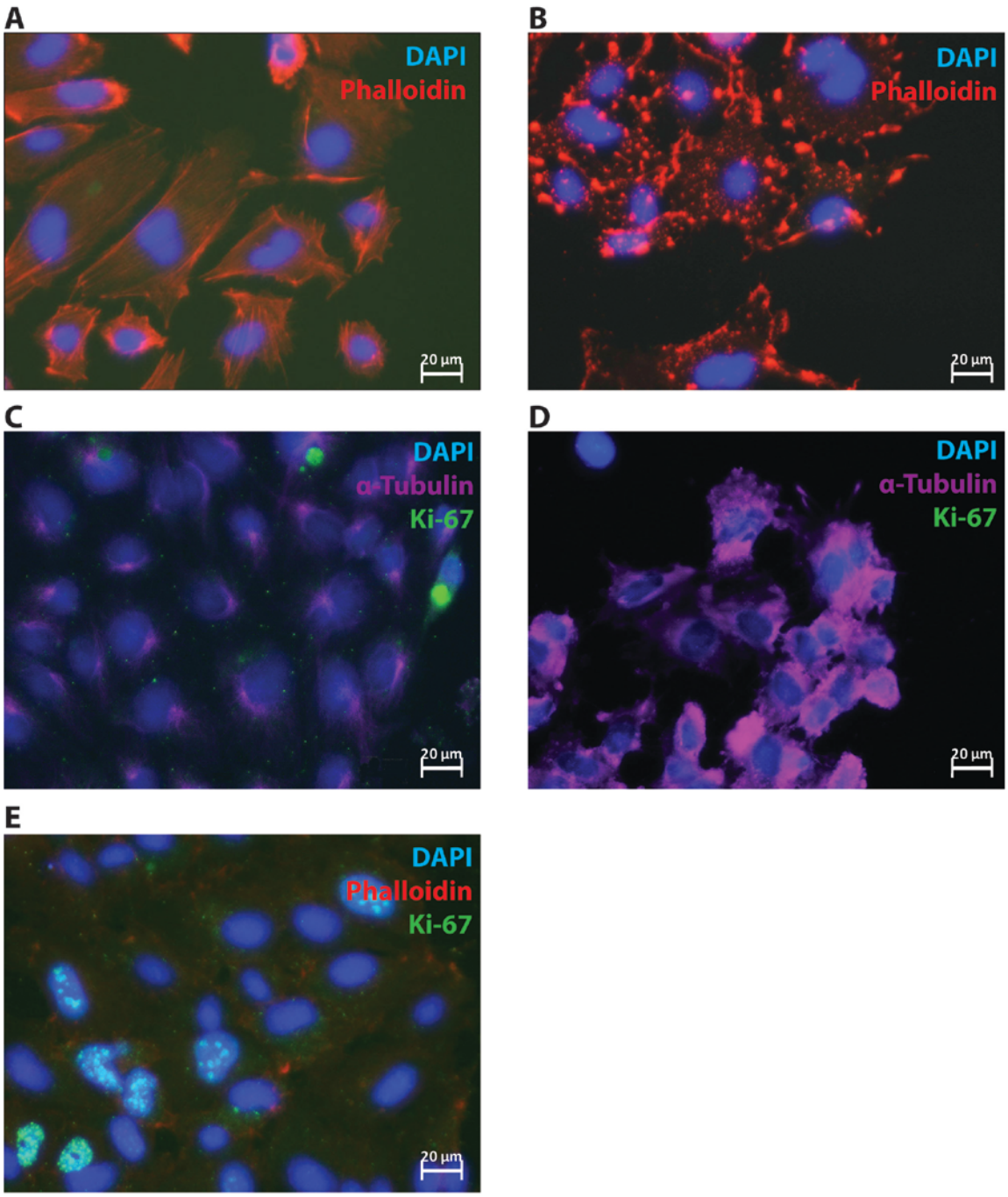

Figure 9. Immunocytochemistry images of cell structures and effects of drug treatment. (**A**) Actin microfilaments in endothelial cells are normally thin, straight structures (red signal, phalloidin bound to actin). (**B**) Upon treatment with cytochalasin D actin monomers can no longer polymerize and form indistinct globules. (**C**) α-tubulin microtubule appear as cotton-like fibers in normal endothelial cells. (**D**) After colchicine treatment they lose defined structure. (**E**) Ki-67 expression patterns localized to nuclei (blue, DAPI in nucleus). No changes in Ki-67 expressions were found in response to drug treatment.

It has been demonstrated that a wave of ERK1/2 phosphorylation occurs during wound healing in kidney epithelial cells (Nikolic et al., 2006). We stained for phospho-ERK1/2 during our immunocytochemistry protocol to examine any possible ERK1/2 activity during wound healing in endothelial cells. We compared ERK1/2 phosphorylation activity at different time points and treatments (**Fig. 10**). The ERK1/2 activation decreases in control cells shortly after the initial wounding, then increases to a peak at 10 min, then decreases through the times examined.

This pattern is carried over to colchicine treated cells, exhibiting the same peak at 10 min. The cytochalasin D treated cells do not exhibit the same extremes of activity as do control and colchicine treated cells. ERK1/2 phosphorylation remains relatively stable across the time course. U0126-treated HUVECs (**Fig. 11A**) showed no phosphorylation whatsoever at any time point, demonstrating that the previously mentioned concentration and time are sufficient to suppress ERK1/2 phosphorylation.

ERK1/2 Immunoblot Quantification

After observing changes in ERK1/2 phosphorylation during immunocytochemistry observation, we used immunoblots to get a quantifiable comparison of changes in phosphorylation levels during wound healing. Two bands are visible because the antibody used binds to two isoforms of phosphorylated ERK1/2; p44 and p42. The blot protocol also allowed us to verify that 15 minutes of U0126 treatment was sufficient to inhibit ERK1/2 activity as was also suggested by the qualitative immunocytochemistry (**Fig. 11B**). We also compared average ERK1/2 phosphorylation based on immunoblot densitometry (**Fig. 12**). ERK 1/2 phosphorylation was found to peak at 10 minutes after wounding in control HUVECs. Blot densitometry showed a

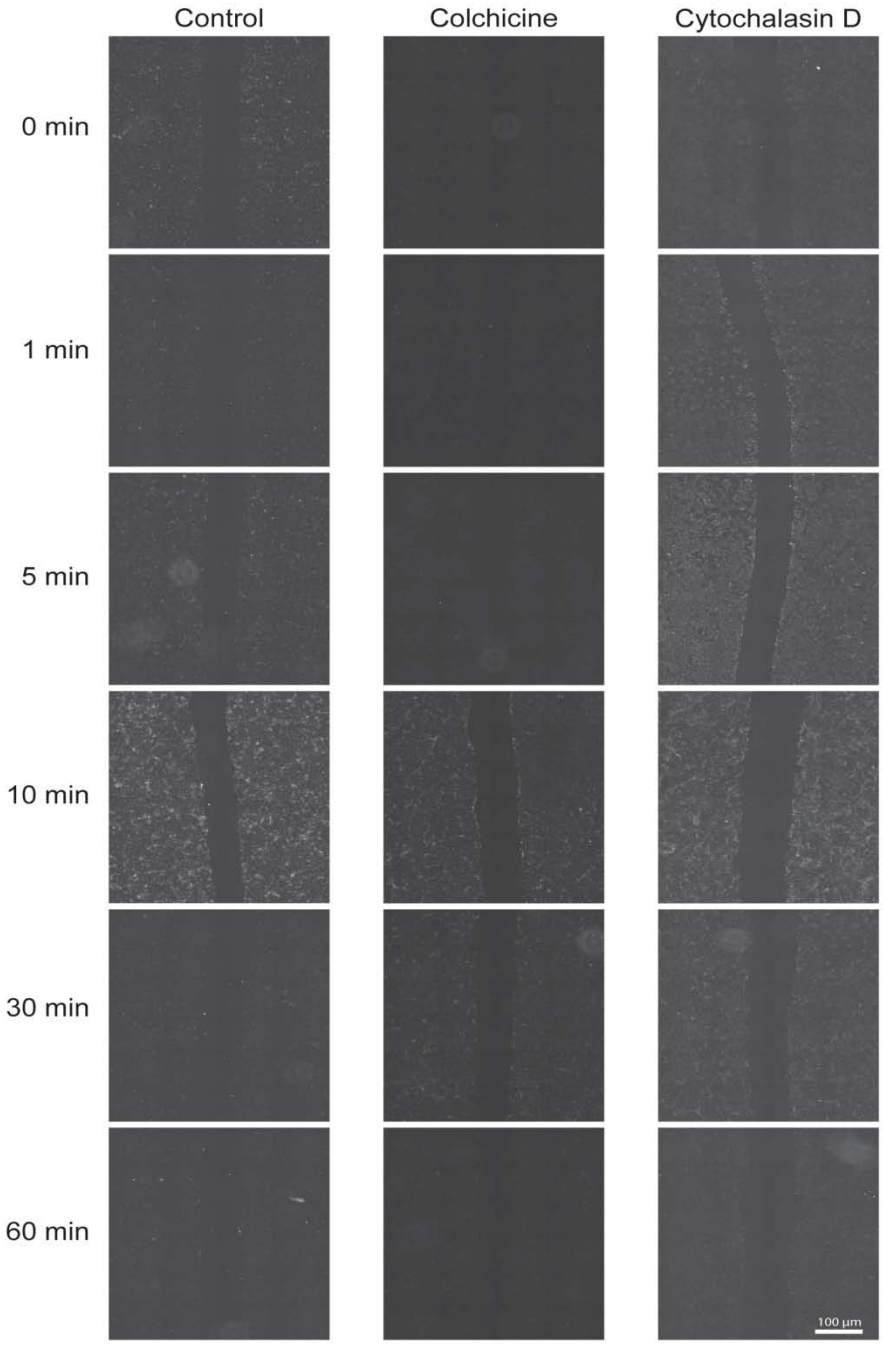

Figure 10. Immunocytochemistry of ERK1/2 phosphorylation at selected time points after wounding. Activity in control cells dropped after the initial wound, then increased to a peak at 10 min, then fell again. Colchicine-treated cells did not experience a significant drop after initial injury, but did increase in activity at 10 min, though activity did not display the same peak of phosphorylation at 10 min as the control cells. Cytochalasin D-treated cells also dropped in ERK1/2 activity shortly after wounding, and peaked at 10 min.

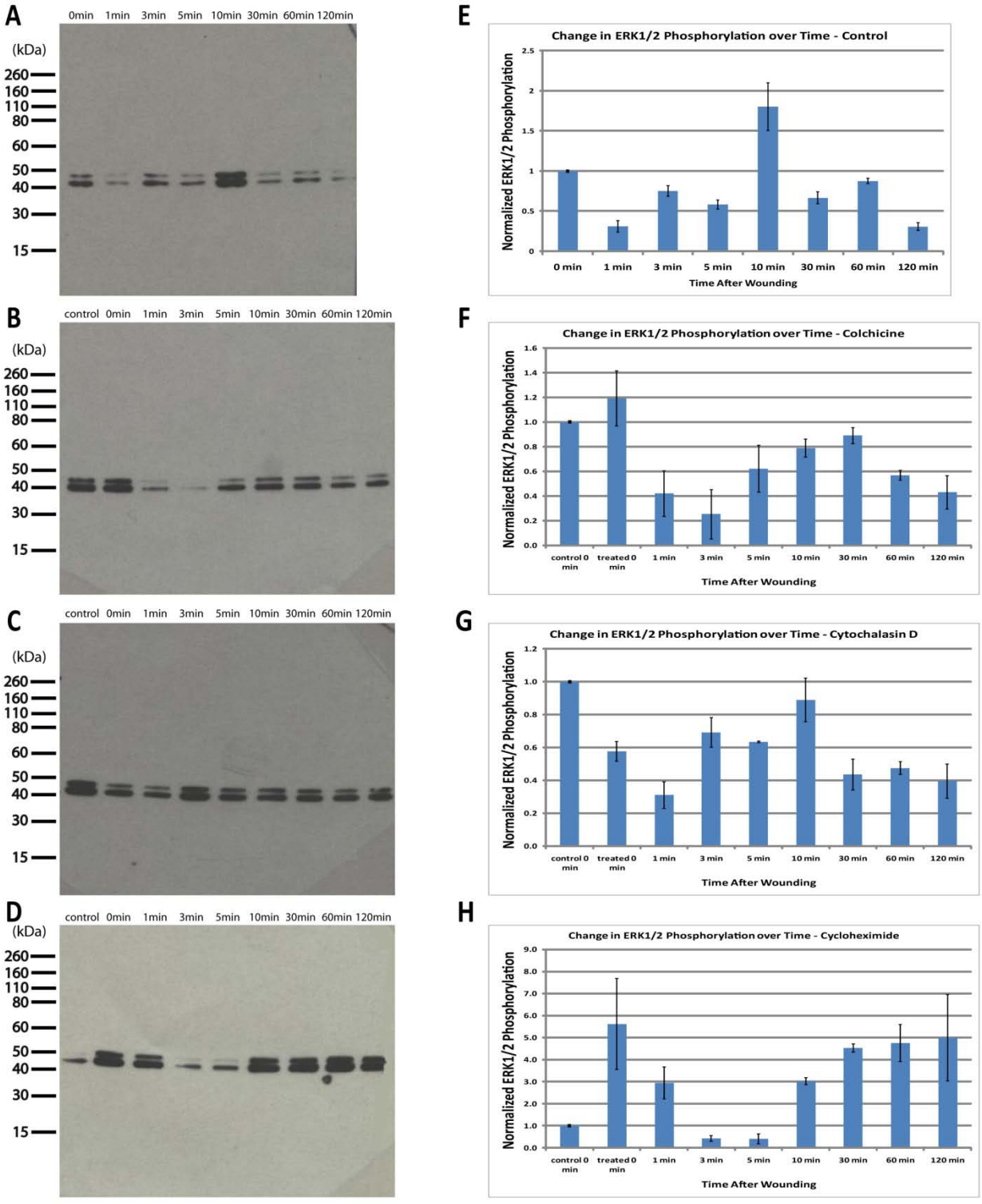

Figure 11. Immunoblot analysis of ERK 1/2 phosphorylation activity in response to drug treatments control (**A**), colchicine (**B**), cytochalasin D (**C**) and cyclohexamide (**D**). Blots were subjected to densitometric analysis using ImageJ software and normalized to ERK1/2 phosphorylation at zero minutes after wounding. Phosphorylation was normalized to non-treated controls and expressed as phosphorylation relative to zero-minute control - control (**E**), colchicine (**F**), cytochalasin D (**G**), and cycloheximide (**H**). Error bars represent standard error of the mean.

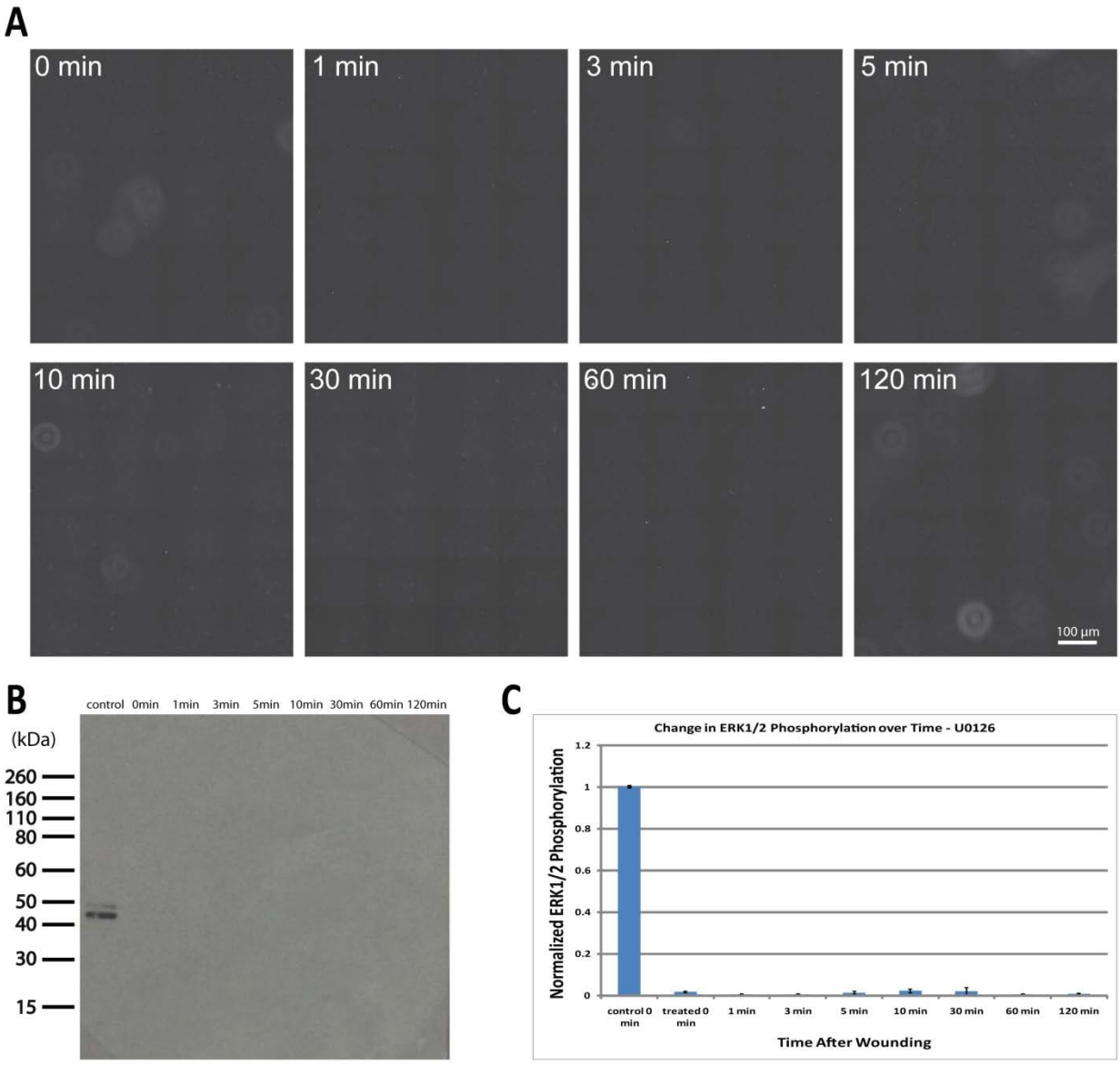

Figure 12. (**A**) UO126-treated HUVECs stained with anti-phosphorylated ERK1/2 antibodies and observed at the time points listed above. ERK phosphorylation was not observed at any time point during healing of UO126-treated cells. (**B**) Immunoblot comparing non-treated control HUVECs to UO126-treated cells. (**C**) Graph of relative ERK phosphorylation levels normalized to control HUVECs. Error bars represent standard error of the mean.

statistically significant difference between ERK1/2 activity at 10 minutes compared to the other time points (**Fig. 11**), correlating with the immunocytochemistry analysis (**Fig. 10)**. In addition, phosphorylation dropped significantly only one minute after wounding. Colchicine treatment caused ERK phosphorylation to drop significantly after the initial wound. While activity did increase after three minutes, it never rose back up to baseline. In addition, 10 minutes did not exhibit the peak of ERK phosphorylation. Cytochalasin D treatment resulted in lower ERK phosphorylation as well, showing significantly decreased activity in treated 0 min HUVECs as compared to control 0min HUVECs. Activity peaked significantly at 10 min. Cycloheximide treated HUVECs drop significantly in ERK phosphorylation shortly after the initial wound. Activity never increased back to the 0 min baseline. We saw no ERK1/2 activity in the treated protein lanes at all (**Fig. 12A**), again correlating with the previous ICC images. When compared to a non-treated control sample (**Fig. 12B**), we can see that there is virtually no ERK1/2 phosphorylation. This gives further evidence that the U0126 is suppressing ERK1/2 phosphorylation. We demonstrate the quantitative difference between non-treated control samples and U0126-treated samples (**Fig. 12C**). ERK1/2 phosphorylation in all treated samples was significantly less than in the control sample.

Discussion

The rates of wound healing were measured so as to objectively evaluate the effect of each drug on the rate of wound healing, taking into account the fact that the initial wound width for all plates would not be equal. We also measured the wound closure at each hour. This was done to determine whether the rate of wound healing remained constant throughout the time

course or if it changed over time. It also allowed us to determine if the change of rate in wound healing remained the same or changed along with the different drug treatments.

The anti-angiogenic drugs angiostatin, endostatin and GM6001 demonstrated no effect on wound healing, either on rate of change over time or on average rate. MMPIs such as GM6001 act by preventing matrix metalloproteinases from digesting the substrate (Wu et al., 2005). As the HUVECs were lying on top of the substrate, not embedded within it, it stands to reason that no digestion would be necessary, and thus GM6001 did not have an effect. Angiostatin and endostatin are more direct angiogenesis inhibitors. Angiostatin, a proteolytic fragment of plasminogen, is known to bind ATP synthase on the surface of endothelial cells (Moser et al., 1999), exerting its anti-proliferative effects on the cell directly. Endostatin, a fragment of collagen XVIII, is able to directly inhibit endothelial cell proliferation. Endostatin has been demonstrated to inhibit re-endothelialization of wounds in vivo (Hutter et al., 2003), however in our wound healing measurement data the endostatin treated cells did not differ in rate from control. It is possible that re-endothelialization in vivo occurs by a slightly different method than *in vitro*. Also of note is that the concentration of endostatin found *in vivo* (as much as 2 µg/mL) was less than our treatment (30 µg/mL). However, this in vivo concentration was due to gene alteration, not endogenous endostatin (Hutter et al., 2003). This endostatin would have been produced on a consistent basis as opposed to our one-time treatment.

We did not have a control set up for this study that ascertained whether the anti-angiogenic drugs actually had an effect on the HUVECs. An angiogenic assay could be used to determine the efficacy of the drugs. If angiogenic activity was significantly inhibited by these drugs, we

could state with confidence that these drugs demonstrated no effect on the rate of wound healing. The lack of effect we saw could be due to an insufficient dose of the drugs rather than a difference in angiogenic and wound healing pathways.

The drugs cytochalasin D and colchicine did exhibit a significant effect on wound healing, particularly colchicine. Both drugs disrupt cytoskeletal remodeling by preventing the polymerization of monomers. This disruption could play a role in the inhibition of endothelial wound healing. However, the fact that colchicine stopped wound healing, as opposed to cytochalasin D which only slowed it, implies that the microtubule system is of greater importance to endothelial migration and repopulation than the microfilament system. Microtubules are also responsible for organizing the chromosomes during the metaphase of mitosis. As remodeling is necessary for the attachment of microtubules to the kinetochores of the chromosomes, it is possible that colchicine would inhibit this process as well. Chan et al. has demonstrated that even a single unattached kinetochore can prevent the cell from proceeding to anaphase, which would thus inhibit mitosis (Chan and Yen, 2003). If wound re-endothelialization is partially dependent on endothelial proliferation, colchicine would then further inhibit the process. The microtubules are also closely associated with the protein kinase ERK1/2 (Takahashi and Berk, 1996). By disrupting the microtubule structure it is possible that ERK1/2 is unable to properly localize in order to be phosphorylated, thus decreasing overall ERK1/2 phosphorylation and cell proliferation.

It must be noted that because so many intercellular components and processes are organized and transported by microtubules, it is possible that the cessation of wound healing seen in the

colchicine-treated HUVECs is due to wide-scale disruption of the intracellular environment. If several processes are affected by breakdown of microtubule structure, we cannot say for certain that the change in rate of wound healing in colchicine-treated HUVECs is specifically due to disruption of the cytoskeleton.

Cycloheximide, which inhibits protein synthesis, slowed wound healing, demonstrating that endothelial cells need to synthesize at least some additional proteins in order to facilitate migration. It is also possible that it inhibited the synthesis of certain housekeeping proteins, which affected cell function enough that the cell was further unable to facilitate wound healing.

The ERK1/2 phosphorylation inhibitor U0126 also somewhat slowed wound healing. Our immunoblots demonstrated that ERK phosphorylation was almost completely inhibited after only 15 minutes of treatment with U0126. As this was the only difference between the treated and control cells, it provides evidence that endothelial wound healing is at least partially dependent on ERK1/2 phosphorylation. We also saw that ERK1/2 activation was overall less in colchicine and cytochalasin D-treated cells than in controls. This partial inhibition of ERK1/2 phosphorylation may have also played a role in the reduced wound healing seen in these treatments. ERK1/2 is a mitogen-activated protein kinase (MAPK), which transduces mitotic signals in the extracellular environment into intracellular responses (Meloche and Pouyssegur, 2007). Thus, it would seem that U0126 affects endothelial wound healing primarily by disrupting mitosis. We know that some proliferation does take place during the resurfacing of a wounded area, thus inhibition of ERK1/2 phosphorylation may partially suppress or completely inhibit the transduction of mitotic signals, therefore inhibiting proliferation. This also extends

to the changes in ERK1/2 phosphorylation seen in the drug-treated HUVECs. However, our antibody only binded to phosphorylated ERK1/2. We do not know for sure if the changes observed are due to less ERK1/2 being phosphorylated, or less ERK1/2 being expressed. We also cannot make any conclusions regarding the activity of the phosphorylated ERK1/2.

It must also be noted that the behaviors exhibited by our *in vitro* HUVECs may not necessarily reflect those that would be exhibited by endothelial cells in an organism. Our cell cultures were grown in static medium, while the endothelial layer experiences a constant shear stress due to blood flow. In the vascular system endothelial cells are exposed to many other cell types, including leukocytes, platelets, monocytes and smooth muscle cells, as well as extracellular matrices. Our HUVECs were exposed to tissue culture matrix only. In addition, the scratch used to wound the HUVEC monolayer does not truly mimic injury in the *in vivo* endothelium. While the wound healing assay may mimic the endothelial wound healing, we cannot be completely reproduce the actual process.

CHAPTER FOUR

The endothelial layer heals itself through two main processes. One is through the migration and differentiation of endothelial progenitor cells (EPCs). EPCs travel through the circulatory system, adhere to areas of damaged or missing endothelium, then proliferate and differentiate into mature endothelial cells, effectively replacing the lost endothelium. The second process is by migration of already-present mature endothelial cells. When an area of endothelium is removed, the remaining cells move into the damaged area to resurface the injury. Our HLS study looked at one aspect of EPC-based healing, while our second study focused on healing via migration of mature endothelial cells.

We cannot determine whether or not HLS had any effect on circulating EPC populations in rats. Our flow cytometry methodology did not allow us to specify for EPC analysis, and thus we cannot make a statement as to whether or not EPC populations change in response to HLS. We also do not know if EPCs from HLS rats are capable of functioning at the same level as those of normal rats. We had intended to culture the harvested PBMCs and use them for several functional assays in order to compare the migration and proliferative capacity of cells from HLS rats to control rats. This would have allowed us to determine if HLS leads to a decrease in EPC function instead of number. However, we were unable to grow enough cells to perform functional assays. It is unknown whether the EPCs we harvested had a low proliferative capacity or whether it is a result of the rat subjects having few EPCs to begin with. Either way, we cannot determine anything regarding the number or functionality of EPCs in HLS rats. The

evidence indicating that the physical inactivity has a detrimental effect on cardiovascular function may still point towards a correlation between EPC dysfunction and lack of exercise.

There is also the possibility that EPC levels do not change in response to HLS. While the mature endothelial layer could be damaged due to vascular dysfunction, it is possible that EPCs remain at a steady population to repair wounds too large for adjacent endothelial cells to recover. In order to remain a viable source of new endothelial cells, there is the possibility that EPCs remain unaffected by the conditions causing damage to the endothelium. There could be a host of compensatory mechanisms that keep EPC numbers steady even when mature endothelial cells are experiencing dysfunction. Future studies may include ways of assessing endothelial damage in the hind limbs, such as removing sections of arteries from the legs and performing immunohistochemical analysis. If we can confirm damage to the endothelial layer, it would give us a better idea of whether to suspect EPC dysfunction.

Another possibility is that HLS would have an effect on EPCs, but our model was insufficient to induce any changes in circulating EPC numbers. The rats were still able to bear weight on their forelimbs, which was necessary to let them obtain food and water. Therefore, the HLS model in which they were placed is an imperfect simulation of a patient confined to bed rest. Because of this limitation, we cannot fully mimic the conditions of a bedridden patient. A change in EPC number may be more indicative of a systemic problem, instead of a problem confined to the hind limbs. If only one part of the body experiences endothelial dysfunction, it may be insufficient to cause a change to the entire EPC population.

There may be a compensatory mechanism exists to maintain circulating EPCs at their normal level. Again, that number of EPCs may exhibit inferior functional capacity as compared to EPCs from a control animal. If that compensatory mechanism could be found, it could aid in future efforts to culture and increase EPC numbers.

While we were unable to perform functional assay on the cultured cells harvested from rat, our findings in the wound healing experiments did give us an idea of what to expect. We found that the rate of wound healing decreases over time regardless of treatment. This would allow us to accurately gauge the degree of dysfunction we might see in EPCs from HLS animals. We can hypothesize that cultured HLS cells would heal at a consistently lower rate than control cells.

The long-term goal of this study is to eventually find methods of treatment to attenuate the effects of peripheral arterial disease in sedentary and bedridden patients. One proposed treatment involves using the circulating EPCs of the patient's own body as a cell-based therapy. The idea is that a patient with low numbers of circulating EPCs could have those cells harvested via a simple peripheral blood draw. Those cells could then be cultured and multiplied *in vitro*, then transplanted back into the patient. Not only would the patient benefit from increased circulating EPC numbers, but there would be no risk of rejection, as the cells are those of the patient.

We found evidence that ERK1/2 activation plays at least some role in wound healing. While inhibiting ERK1/2 phosphorylation was not as deleterious as disrupting the cytoskeleton, it did slow healing somewhat. This may point to a possible treatment to patients exhibiting

endothelial dysfunction. If resurfacing of endothelial wounds can be inhibited by preventing ERK1/2 phosphorylation, it is possible that enhancing the ERK1/2 signal cascade could have a beneficial effect on wound healing.

We also found that cycloheximide slows wound healing to some degree. This may imply that endothelial cells must express a different protein set to efficiently migrate and recover wounded areas. Alternatively, it could mean the proteins expressed during the normal cell cycle are insufficient for wound healing, and that the cell must manufacture more of those proteins Additionally, we found that the microtubules are more crucial to the wound healing process than the microfilaments. Disrupting the microfilaments slowed wound healing, while disrupting microtubules stopped healing altogether. This implies that while the microfilaments are needed for efficient wound healing, the microtubules are absolutely essential to the process.

One future direction the EPC study could take is to more accurately discern EPCs using double staining. This method involves staining a cell sample with more than one antibody. During flow cytometry, cells can be separated based on the expression of their surface molecules. For example, when looking for EPCs it would be possible to only count cells that are negative for CD45 expression, but positive for CD34 expression. By purposefully screening out cells that express non-endothelial surface markers, we can get a better idea of any changes that might occur in EPC levels.

By that same note, multiple staining could also benefit our cell culture experiments. Using fluorescent-activated cells sorting (FACS), we could double-stain for EPC markers such as CD34

and VEGFR2. Like flow cytometry, FACS separates cells based on their fluorescence, and thus by their surface markers. After sorting, the cells can be harvested and used for further experiments. FACS can be used to obtain an enriched population of EPCs for cells culture experiments, with a higher probability of producing an EPC colony.

It may also be necessary to examine greater numbers of cells in future flow cytometry experiments to gain a better understanding of changes in EPC number. We only checked $1x10^5$ events per sample. If EPCs are rare in the mononuclear cell fraction, it may be necessary to examine $1x10^6$ events or more to find a few EPCs.

In regards to our *in vitro* wound healing study, we need to perform an angiogenic functional assay as a positive control for our anti-angiogenic drugs. This would allow us to make definitive statements as to whether wound healing proceeds along the same pathways as angiogenesis.

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