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DIFFERENTIAL EFFECTS OF PLATELET-DERIVED GROWTH FACTOR ISOFORMS ON LARGE AND SMALL VESSEL ENDOTHELIAL CELLS AND VASCULAR SMOOTH MUSCLE CELLS

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3/4/92

Date
Differential Effects of Platelet-Derived Growth Factor Isoforms on Large and Small Vessel Endothelial Cells and Vascular Smooth Muscle Cells

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Robin Alexandra Perlmutter

1992
Acknowledgements

I wish to thank Ms. Adeline Tucker for her unending patience and good humor. For the past three years, Addie has proven to be a wonderful and willing teacher who demonstrated extreme bravery when she agreed to take this laboratory neophyte under her wing.

I am grateful to Dr. Marc Basson for his enormous help with the $^3$H-thymidine incorporation assay.

I wish to thank Dr. Patrick Buckley for his thoughtful review of this thesis.

I am indebted to Dr. Martin Marx for serving as an invaluable source of both data and critical insight into this project. His careful attention to detail focused this project on many occasions.

For the past four years Dr. Leonard Bell has served as a sounding board for my research, clinical, and personal concerns. Part mentor, part big brother, Lenny has been an unwavering source of support.

And finally, Dr. Joseph Madri who constantly demonstrates the importance of personal and professional integrity. Joe is an amazing role model and only a fraction of what I've learned from him has anything to do with science.
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ABSTRACT

Platelet-Derived Growth Factor (PDGF) is a dimeric protein which exists in PDGF-AA, PDGF-AB, and PDGF-BB isoforms. PDGF has been shown to play a major role in wound healing and the migration and the proliferation of vascular cells. The PDGF isoforms interact with a dimeric PDGF receptor, αα, αβ, or ββ, which exhibit isoform binding specificities. We identified the proliferative and migratory influences of the PDGF isoforms on bovine aortic endothelial cells (BAECs), rat epididymal fat pad capillary endothelial cells (RFCs), and bovine aortic smooth muscle cells (BASMCs).

In cells grown on Type I collagen (2D culture), treatment of BAECs with the isoforms of PDGF did not affect migration or proliferation. Treatment of RFCs and BASMCs with PDGF-AB (10 ng/ml) resulted in 27% and 13% increases in proliferation (p=0.006, p=0.048). BASMCs given PDGF-AB (10 ng/ml) had an 11% increase in migration (p=0.035). RFCs and BASMCs treated with PDGF-BB (10 ng/ml) had a 100% and 50% increases in proliferation (p<0.001, p=0.002). There was a 22% increase in migration of the BASMCs treated with PDGF-BB (p<0.001).

In cells grown in a Type I collagen gel (3D culture), treatment of BAECs and RFCs with the PDGF isoforms did not result in a change in proliferation. Conversely, proliferation increased 13% in BASMCs given PDGF-AA, 23% in cells given PDGF-AB, and 42% in BASMCs treated with PDGF-BB.

We conclude that the PDGF isoforms have similar effects in RFCs and BASMCs in 2D culture in that PDGF-AB and PDGF-BB are promoters of five day proliferation which may be regulated through the β subunit of PDGF receptors. Conversely, the PDGF isoforms do not affect the behavior of BAECs. In addition, RFCs grown in 3D culture do not respond to PDGF treatment while BASMCs do, suggesting that RFCs are a cell population capable of modulating their
phenotype in response to changes in the organization of the surrounding extracellular matrix.
INTRODUCTION

Platelet-Derived Growth Factor

Platelet-Derived Growth Factor (PDGF) is a 30-34k mw heat stable, basic, dimeric protein whose two polypeptide chains are linked by disulfide bonds. The two chains, A chain and B chain (both 17k mw), are coded for by different genes located on chromosomes 7 and 22, respectively but share 60% amino acid homology including eight cysteine residues (1-4). Waterfield, et al. and Doolittle, et al. have demonstrated that the B chain is the cellular proto-oncogene (c-sis) homologous to transforming simian sarcoma virus protein (v-sis) (5,6). The A and the B chains can associate in any dimeric combination giving rise to three possible PDGF isoforms: PDGF-AA, PDGF-AB, and PDGF-BB.

Even before the identification of the individual isoforms it was found that PDGF is differentially secreted by a variety of cells including platelets, macrophages, vascular endothelial and smooth muscle cells, fibroblasts, and neoplastic cells including malignant gliomas, mammary carcinomas, colonic adenocarcinomas, adrenal carcinomas, non-small cell lung carcinomas, melanomas, fibrosarcomas, and osteosarcomas (7-16). PDGF is a potent mitogen for mesenchymal cells and has therefore been associated with many actions in different cell types including the ability to affect migration and proliferation of vascular cells, promote wound healing, induction of cytoskeletal changes, and the ability to affect the differentiation of progenitor cells along specific paths during normal development.

Through some of the above mechanisms, PDGF has been linked extensively to atherogenesis and ischemia. Specifically, Bell and Madri
found that PDGF increases both the migration and proliferation of bovine aortic smooth muscle cells but PDGF inhibited both proliferation and migration in bovine aortic endothelial cells, suggesting a role for neointimal development by smooth muscle migration and subsequent atheroma formation which may also serve as a nidus of platelet aggregation (17). Furthermore, not only has PDGF been shown to be a mitogen for vascular smooth muscle cells in vitro (and therefore without the benefit of a non-thrombogenic surface) but Golden, et al. have demonstrated that vascular smooth muscle cells are capable of secreting PDGF even under an intact endothelium (18). Evidence for the cytoskeletal manifestations of PDGF treatment on migration comes from Mallestrom, et al. who have demonstrated membrane ruffling (also shown to be associated with actin reorganization) in human foreskin fibroblasts. This was felt to be a reaction specific for PDGF in that it was reversible with anti-PDGF antibodies (19). In addition to PDGF's effect on migration and proliferation and their role in vascular pathology, PDGF has been shown to be a potent vasoconstrictor causing contraction when added to fresh isolates of rat aorta (PDGF is more potent than Angiotensin II in this model), thus adding the possibility of increased vascular tone and vasospasm to underlying atherosclerosis with their resulting contribution to ischemia (20).

With regard to the beneficial effects of PDGF, Greenhalgh, et al. demonstrated that PDGF improved wound healing in diabetic mice who received large full-thickness skin wounds compared to those who received similar wounds but were not treated with PDGF. Wound healing was considered to be improved given that there was an increased number of fibroblasts and capillaries seen at 10 and 21 days after injury and improved wound closure seen at 21 days (21). PDGF is also implicated in normal
developmental pathways. Noble, et al. and Richardson, et al. have elucidated the role that PDGF plays in differentiation of bipotential oligodentrocyte/type-2 astrocyte progenitor cells (22,23). In this system, type-1 astrocytes secrete growth factors (largely PDGF) which act on the bipotential progenitor cells causing them to differentiate into oligodendrocytes (which will myelinate neurons in the central nervous system) or into type-2 astrocytes (which are associated with nodes of Ranvier). In addition, it has been demonstrated that PDGF promoted progenitor cell motility and inhibits the premature differentiation of progenitor cells into oligodendrocytes.

The identification of the PDGF isoforms has led to the finding that the secretion of individual isoforms is specific for a given cell type. For instance, 80% and 90% of mammary carcinoma cell lines tested by Peres, et al. produced both PDGF-A chain and PDGF-B chain respectively (11) but many other cells secrete only one type of chain. Vascular smooth muscle cells have been shown to secrete only PDGF-A chain whether the adjacent intima was intact or had been subjected to denudation injury (18,24). Fingerle, et al. showed an increased secretion of only the A chain after denudation. Some vascular endothelial cells, conversely, produce large amounts of B chain but very little A chain (25,26). Mendoza, et al. have demonstrated the increased production of PDGF-A chain mRNA (but not B chain mRNA) by smooth muscle cells in the gravid versus non-gravid uterus and have proposed that PDGF-A chain is intimately linked with uterine expansion during pregnancy (27). O'Donnell, et al. found that human embryonal cell carcinomas secrete PDGF-A chain (13).

PDGF-B chain has been localized in macrophages during all stages of atherogenesis (8) and the homodimer of PDGF-B chain (PDGF-BB)
improved healing of full thickness skin wounds in diabetic mice (21).
Beckmann, et al. suggest that the PDGF-B chain has a greater ability to transform cells than PDGF-A chain which is consistent with the finding that the PDGF-B chain is homologous to the c-sis proto-oncogene (28).
Some investigators have suggested that the actions of PDGF-BB and the PDGF heterodimer, PDGF-AB, are similar, differing only with respect to intensity of response, whereas the actions of PDGF-AA are often disparate from those of the PDGF isoforms which contain a B chain. Siegbahn, et al. demonstrated that PDGF-AB and PDGF-BB have chemotactic effects on monocytes and fibroblasts whereas PDGF-AA does not (29). Similarly, Hammacher, et al. have demonstrated that only PDGF-AB and PDGF-BB are capable of causing the membrane ruffling and actin reorganization seen in human foreskin fibroblasts (30). The PDGF receptor has been implicated in generating this vast number of actions that PDGF has on effector cells.

**Platelet-Derived Growth Factor Receptor**

The PDGF receptor is composed of a dimer of two 170-180 kDa transmembrane proteins in the split tyrosine kinase family which uses this activity as its intracellular signalling mechanism. The receptor is a dimer consisting of α or β subunits and associate as either homo- or heterodimers: αα, αβ, ββ. The receptor subunits share 44% homology, both contain five immunoglobulin-like domains extracellularly, and both contain intracellular split tyrosine kinase domains (31,32). Because each receptor subunit possesses a specific PDGF chain preference, the receptor dimers differentially bind the PDGF isoforms (Figure 1). Specifically, the α receptor subunit will bind either PDGF-A chain or PDGF-B chain whereas the β receptor subunit will only bind the PDGF-B chain. Based on these
specificities, a ββ receptor will bind PDGF-BB with the greatest affinity, PDGF-AB to a slight degree, and PDGF-AA not at all. An αα PDGF receptor, conversely, will bind all three isoforms of PDGF (33,34).

Once the PDGF molecule is bound to the receptor, there is internalization of the receptor-ligand complex resulting in a phosphorylation-mediated conformational change in the PDGF receptor (which only takes place in the presence of ATP) which induces the tyrosine kinase activity (35,36). Sorkin, et al. demonstrated that while the intrinsic tyrosine kinase enhances receptor-ligand internalization, it was not an essential for this action (35). Westermark, et al. found that tyrosine kinase activity was necessary for membrane ruffling, cytoskeletal reorganization, and migration. Porcine aortic endothelial cells transfected with tyrosine kinase negative ββ receptors did not exhibit these actions while those with the wild-type ββ receptor did (36). While the exact sequence of events remains elusive, it is generally held that ligand binding stimulates the breakdown of phosphatidylinositol bisphosphate followed by the generation of inositol trisphosphate and diacylglycerol as second messengers. Inositol trisphosphate promotes the release of intracellular calcium stores while diacylglycerol leads to the activation of protein kinase C (37,38). Sachinidis, et al., found a detectable increase in diacylglycerol within 20 seconds after the addition of recombinant PDGF-AB or PDGF-BB to rat aortic smooth muscle cells. In these cells, PDGF-AA had a slower and highly variable ability to stimulate diacylglycerol production. This production peaked at one minute with a six-fold increase in diacylglycerol and a fall off to its stable and half-maximal concentration six minutes after stimulation. Reasonably low doses of PDGF-AB or PDGF-BB (1-30 ng/ml) caused a seven to twelve-fold increase in inositol trisphosphate production
in rat aortic smooth muscle cells while PDGF-AA had no effect on inositol trisphosphate production in these cells (39).

Given that there are only three PDGF receptor configurations and a multitude of cell responses to PDGF, Gronwald, et al. have postulated that the multitudinous effects of PDGF are a reflection of differing absolute and relative numbers of receptor types expressed on cells (40). Lee, et al. have identified the α receptor subunit which possesses tyrosine kinase activity in the cDNA library of rat olfactory epithelium (41). Werner, et al. have evidence that both α and β receptor subunits exist on the surface of cultured Kaposi sarcoma cells (42). Hermansson, et al. have identified α but not β receptor subunits in human glioma cells, whereas Wang, et al. have identified functional PDGF receptors containing the β subunit in cultured human meningioma cells (9,43). Similarly, Heldin, et al. demonstrated β receptor subunits in an anaplastic thyroid carcinoma (10). Given that PDGF-AB and PDGF-BB were the only isoforms to cause membrane ruffling and actin reorganization, Hammacher, et al. deduced that PDGF interaction with the β receptor subunit was essential for this action (30). Recently, Yablonka-Reuveni, et al. demonstrated that mouse C-2 myoblasts (derived from adult skeletal muscle) express ββ receptors. When stimulated with the different isoforms of PDGF, PDGF-BB causes a five-fold increase in cell proliferation with a decrease in differentiation (as measured by the amount of myosin in cells) compared to the cells treated with PDGF-AA and PDGF-AB (44). Expanding on Gronwald's idea that the actions of PDGF in different cell types are a reflection of the absolute and relative differences in receptor expression, the addition of independent regulation of A and B chains can explain the multitudinous effects of PDGF.
**PDGF and PDGF Receptor Interaction**

Mercola, et al. illustrated that both the A and B chains of PDGF and the α and β receptor subunits are expressed differentially and may be under independent control (45). These investigators found that only PDGF-A chain was expressed in mouse embryonic and extraembryonic tissue at 6.5 to 8.5 days and that while both α and β receptor subunits are expressed in the mouse embryo, the α subunit is expressed both earlier and to a greater degree than the β subunit. Several neoplastic cell lines have been shown to contain both PDGF receptors and the ability to secrete PDGF. Hermansson, et al. and Nister, et al. illustrated this point in malignant glioma cell lines suggesting the possibility of an autocrine loop as a mechanism for neoplastic growth (9,46). This interaction is not confined to embryogenesis and neoplasia, however. Majesky, et al. proposed a two phase process in arterial repair after balloon catheter denudation injury to rat carotid arteries (47). In the acute phase (first six hours) they observed a significant decrease in the amount of β subunit expressed on smooth muscle cells while seeing a 10-12-fold increase in the amount of PDGF-AA expressed and no change in either PDGF-B chain or α subunit expression. In the chronic phase of repair (two weeks after injury) the smooth muscle cells expressed a smaller amount of α subunit mRNA but an increased amount of β subunit mRNA expression. In addition there may be differential expression of PDGF receptor subunits and secretion of specific PDGF chains in the same cell type in this model. Specifically, the smooth muscle cells which were located on the luminal side, i.e., close to the site of the denudation injury, expressed a larger amount of PDGF-A chain and β receptor subunit than smooth muscle cells located farther away from the site of intimal damage.
Expression of PDGF receptor subunits may also be affected by other growth factors. Gronwald, et al. describe a phenomenon they term "transmodulation" in which Transforming Growth Factor-β (TGF-β) regulates the expression of PDGF receptor subunits (48). These investigators found that quiescent 3T3 cells which were treated with 24 hours of TGF-β bound almost no PDGF-AA and PDGF-AB, but had only a 40% reduction in their ability to bind PDGF-BB compared to controls. As expected, these cells also expressed a diminished number of α receptor subunits while the number of β receptor subunits was increased.

Research Questions

Investigators have identified the structure of PDGF and its function in many different cell types. Much of the work elucidating PDGF's function, however, was performed before the specific isoforms and the receptor binding specificities were recognized. In addition, while the mitogenic effect of PDGF on vascular smooth muscle cells is well characterized, the effect of PDGF on vascular endothelial cells is still under investigation. Bar, et al. found that PDGF did not affect ³H-thymidine incorporation (a measure of DNA synthesis and therefore an indirect measure of proliferation) in large vessel endothelial cells (bovine aorta and pulmonary artery), whereas there was increased ³H-thymidine in small vessel endothelial cells (bovine fat and mouse brain capillaries) (49). Conversely, Bell and Madri did identify an effect in large vessel endothelial cells. Specifically, these authors found that PDGF decreased the migration and proliferation of bovine aortic endothelial cells (17). Both of these studies, however, were performed before the isolation of the different PDGF isoforms and the advent of recombinant technology. In the present study,
I will use functional assays which measure migration and proliferation to determine whether the specific PDGF isoforms have differing actions on endothelial cells and vascular smooth muscle cells and whether different types of endothelial cells (namely small and large vessel endothelial cells) react differently to PDGF treatment.

Secondly, Madri, et al. have demonstrated some cell populations can modulate their phenotype depending on the environment in which they are cultured. Environmental changes which have been shown to influence cell morphology include extracellular matrix components, soluble factors, and culturing cells in a three-dimensional collagen gel (50) (versus the traditional two-dimensional culture system). I seek to compare the phenotypic responses of endothelial and vascular smooth muscle cells grown in two dimensional and three dimensional cultures.
METHODS

Cells and Culture

Microvascular endothelial cells were cultured by Ms. Adeline Tucker from the fat pad capillaries of rat epididymides (RFCs) as described by Madri and Williams (51). Briefly, the epididymal fat pad of 400 g Sprague-Dawley rats were removed from recently sacrificed animal and placed in sterile Williams HEPES buffer (NaCl 140 mM, HEPES 10 mM, KCl 10 mM, CaCl₂ 0.1 mM, MgCl₂ 0.2 mM, NaHCO₃ 11.9 g/l, glucose 5.0 g/l, pH 7.4). Pieces of fat which appeared to be free of large blood vessels were minced and placed in a solution of HEPES buffer and collagenase (Type II 250 mg/50 ml HEPES buffer, Sigma). After incubation at 37°C with agitation (for approximately one hour), the suspension was centrifuged at 200 g for seven minutes. The pellet was washed in HEPES buffer and 5% bovine serum albumin twice and then centrifuged at 200 g for five minutes. The pellet was then resuspended in 45% Percoll (Pharmacia Fine Chemicals) and recentrifuged at 15,000 g for 20 minutes resulting in identifiable capillary tufts in the tissue isolate. The tufts were then washed with HEPES-bovine serum albumin buffer and then plated on 60-mm Petri dishes previously coated with 12.5 mg/ml Type I and Type III collagen in medium (199 E, Gibco) with 20% fetal bovine serum, penicillin, streptomycin, and glutamine. To this medium was added an equal amount of 10% fetal bovine serum medium conditioned by confluent bovine aortic endothelial cells. This medium was used to maintain the RFC population (RFC medium). The cells were maintained in an incubator containing 5% CO₂, 95% humidified atmosphere at 37°C.
The cells were passaged to 1:3 split ratio when confluent (approximately every seven days) in 0.2% trypsin with EDTA. The endothelial cell origin of these cells was confirmed by the presence of angiotensin-converting enzyme activity and reactivity with anti-factor VIII (data not shown, see ref 51). Passages four through ten were used for experiments.

The large vessel endothelial cells, i.e., vessels which contain both intima and media, used in the following experiments were bovine aortic endothelial cells (BAECs) and were isolated from calf aorta by Ms. Adeline Tucker (52). The vessel was obtained from a slaughterhouse and was washed in iced phosphate buffered saline (PBS) with penicillin, streptomycin, and fungisone added. The aortic adventitia was dissected from the vessel and both ends of the aorta were cannulated. The intima was washed with a PBS/penicillin/streptomycin solution and then with a solution of 20% 199E medium (Gibco) with penicillin and streptomycin added. The distal end of the aorta was then clamped and the lumen was filled 199E medium with collagenase 1 mg/ml (Worthington) and was incubated for 30 minutes.

The media/collagenase solution was removed and the lumen was then washed with 20% heat-inactivated fetal bovine serum 199E medium containing glutamine, penicillin, and streptomycin. The aorta was then massaged and the contents of the lumen were plated on a 75 cc tissue culture flask and were incubated at 37°C with 5% CO₂.

The cells were fed 10% fetal bovine serum medium (DM, Gibco) supplemented with glutamine, penicillin, and streptomycin twice weekly. This medium containing 10% fetal bovine serum was used to maintain the BAEC and bovine aortic smooth muscle cell (BASMC) populations (10% FBS
medium). The cells were passaged at a 1:3 split ratio when confluent (about every seven days) in 0.2% trypsin with EDTA. The endothelial cell origin of these cells was confirmed by the presence of angiotensin-converting enzyme activity and reactivity with anti-factor VIII (data not shown, see ref 51). Passages four through ten were used for experiments.

Bovine aortic smooth muscle cells (BASMCs) were isolated by Ms. Adeline Tucker from fresh calf aorta obtained from a slaughterhouse (52). Using sterile conditions, each aorta was cut lengthwise to expose the intima. The intimal surface was rubbed with sterile gauze to denude the endothelium. The most intimal layer of smooth muscle cells was teased away in strips from the remaining media using sterile forceps and was then placed in a Petri dish. These strips were then minced to a very fine consistency and were then covered with a small amount of 10% FBS medium so that the minced strips remain in contact with the Petri dish. After several days, the smooth muscle cells which were later used for culture migrated away from the minced strips. The cells were fed 10% fetal bovine serum medium (DM, Gibco) supplemented with glutamine, penicillin, and streptomycin twice weekly. The cells were passaged at a 1:3 split ratio when confluent (about every seven days) in 0.2% trypsin with EDTA. Passages two through five were used for experiments.

**Proliferation of Cells in Two-Dimensional Cultures**

Petri dishes were coated with Type I collagen (12.5 µl/ml dissolved in a sodium carbonate buffer at pH 9.3), stored overnight at 4°C, and washed three times with warm PBS and 2 ml of medium was placed in each dish. The cells, whether BAECs, RFCs, or BASMCs, were prepared by trypsinizing a 75 mm tissue culture flask and resuspending the cells in medium. A
suspension of $1.4 \times 10^4$ cells in 1.5 ml medium was added to each of the dishes and the dishes were incubated for six hours at 4°C in a 5% CO$_2$ incubator. After six hours, the cells were treated with either RFC or 10% FBS medium (depending on which cell type was used) containing no PDGF (control group) or medium containing a specific PDGF isoform and were reincubated. The cells were fed medium containing their treatment at three days. After one, two, or five days the dishes were treated with 0.2% trypsin to lift the cells from the dish and the absolute number of cells/ml was determined using a Coulter Counter.

$^3$H Thymidine Incorporation

Quantitative $^3$H-thymidine uptake has been used as an indication of DNA synthesis and therefore as an indirect measure of cell proliferation. Approximately 12,000 BASMCs were plated onto a 24-well bacteriologic culture dish that had been coated with Type I collagen (12.5 μg/ml). The BASMCs were allowed to attach to the matrix for six hours and were then treated with 10% FBS medium containing a specific PDGF isoform (10 ng/ml) or without any growth factor (control) and were incubated for 24 or 48 hours at 37°C. In a radioactive hood, the cells were treated with a 1% solution of $^3$H-thymidine in 10% FBS medium and were incubated at 37°C for 4 hours. The cells were washed with PBS and were treated with a methanol/acetic acid solution (3:1) for ten minutes. The cells were then treated with a chilled 20% trichloroacetic acid solution (in ddH$_2$O) and were then washed with PBS. The cells were left in a 0.2M NaOH solution at 37°C overnight. The cell/NaOH solution was added to 3.5 ml of Optifluor solution (Packard) and placed in a scintillation vial. The radioactivity was measured using a Bechman LS 300 scintillation counter.
Proliferation of Cells in Three Dimensional Cultures

Evaluation of cell proliferation in three dimensions was performed by suspending and culturing cells in a Type I collagen gel as described by Madri, et al. (53). To prepare the gel, lyophilized bovine calf dermis was suspended in sterile 10 mM acetic acid (5 mg/ml) and was stirred overnight at 4°C overnight. A known amount of the Type I collagen solution was placed in a cold, sterile 50 cc tube and ten percent of this volume of HBSS was added. Approximately 50 μl of 1N NaOH were added in 10 μl aliquots to the solution, vortexing after each addition, until the pH indicator changed from yellow to pink which indicated a pH of 7.0. BAECs, RFCs, or BASMCs were added to the Type I collagen suspension until a concentration of 10^6 cells/ml was reached.

The cells were added to millicells (0.5 ml/millicell) placed in a 24-well culture dish and were incubated at 37°C for several minutes until the gel solidified. RFC or 10% FBS medium (with or without PDGF) was added to the 24-well culture dish and the gel was incubated at 37°C. The cells were fed with media with or without PDGF the following day and on day three.

After five days, the collagen gel was washed five times with PBS and were digested in 3 ml of a collagenase solution consisting of PBS, type II collagenase at 1 mg/ml (Sigma), CaCl₂ 1mM, and MgCl₂ 1mM. The gels were digested in this solution for approximately an hour in a 37°C shaking water bath. An equal volume of medium was added to the digested gel solution, and an aliquot of this new solution was used for counting the cells in either a Coulter Counter or using a hemocytometer.
Migration Assay

Cell migration was determined using a radial migration assay described by Pratt, et al. (54). Prior to the migration assay, 35 mm bacteriologic Petri dishes (Falcon) were coated with 2 ml of Type I collagen (12.5 μl/ml) dissolved in a sodium carbonate buffer at pH 9.3. The dishes were kept overnight at 4°C. The dishes were washed three times with warm sterile PBS and 2 ml of 10% FBS medium was added to each dish.

Stainless steel fences (approximately 4 cm in diameter, 1 cm high, with a 1 cm hole cut in the center; see ref 52) were added to each of the Petri dishes and the media contained in the center well was removed. A BAEC or BASMC cell suspension which was made by trypsinizing a 75 mm tissue culture flask and resuspending the lifted cells in 10% FBS medium and 0.4 ml of this cell suspension was placed in the center well of each of the fences. The dishes were incubated in a 37°C, 5% CO₂ incubator for six hours to allow attachment and confluence of the suspended cells.

After six hours, the fences were removed thus releasing the cells from contact inhibition and allowing the cells to migrate radially. At the time the fences were removed, the cells were washed with PBS to remove any nonadherent cells and were then treated with the specific experimental treatment being investigated. In the case of cells treated with Platelet-Derived Growth Factor (PDGF), whether the given treatment was PDGF-AA, PDGF-AB, or PDGF-BB, 1.5 ml of medium containing 10 ng/ml PDGF was added to each dish. The cells treated with 10% FBS medium alone served as controls. New medium containing the same treatment was added to each dish after three days.
After five days, the cells were fixed in 10% buffered formalin and stained with hematoxylin. The areas covered by the radially migrating cells were traced using MacMeasure (Yale Shareware).

**Data Analysis**

Data were analyzed using CLINFO, an NIH-sponsored statistical analysis program. Data were analyzed using Student's t-Test (unpaired, 2 tailed). For comparisons of more than two conditions, the data were analyzed using Analysis of Variance with Newman-Keuls Multiple Range Test to test for significant differences between individual treatment groups. Data are expressed as mean ± standard error and significance was defined as probability less than 0.05.
RESULTS

Proliferation in Two Dimensional Cultures: Dosage in RFCs

The dose response that each PDGF isoform had on proliferation was measured in RFCs. The effects were measured after five days based on cells grown on collagen Type I in 0.5% fetal bovine serum medium and the doses of each PDGF isoform used were 0.625 ng/ml, 1.25 ng/ml, 2.5 ng/ml, 5.0 ng/ml, and 10.0 ng/ml (Figure 2).

When the proliferation of RFCs was measured in cells receiving different doses of PDGF-AA, there was no significant change in proliferation compared to control treatment regardless of the dose of PDGF-AA used (Figure 2). Specifically, the change in proliferation ranged from a 9% decrease in those cells treated with PDGF-AA at 0.625 ng/ml to a decrease in proliferation of 38% in those cells treated with PDGF-AA at 1.25 ng/ml. There was no increase in proliferation in any of the groups treated with PDGF-AA and all of the treatment groups were not significantly different from those cells treated with 10% fetal bovine serum alone.

Some of the doses of PDGF-AB were associated with an increase in proliferation in RFCs as seen in Figure 2. Treatment with PDGF-AB at 0.625 ng/ml resulted in a 6% increase in proliferation (p=NS) while cells treated with PDGF-AB at 1.25 showed a 24% increase in proliferation (p=NS). Cells treated with PDGF-AB at a dose of 2.5 ng/ml had a 33% increase in proliferation compared to control (p=0.010). Those RFCs treated with PDGF-AB at 5.0 ng/ml showed a 60% increase in proliferation compared to control (p=0.001 vs control, p<0.05 vs 0.625 ng/ml, 1.25 ng/ml, and 2.5 ng/ml). The RFCs treated with PDGF-AB at 10.0 ng/ml demonstrated a 63% increase in
proliferation compared to control cells (p=0.004, also p<0.05 vs 1.25 ng/ml, 2.5 ng/ml, 5.0 ng/ml).

RFCs treated with some doses of PDGF-BB also showed an increase in proliferation compared to cells treated with 0.5% fetal bovine serum medium alone at some doses (Figure 2). Cells treated with 0.625 ng/ml showed a 45% increase (p=NS) while RFCs treated with PDGF-BB at 1.25 ng/ml had a 79% increase in proliferation (p=0.027). Cells treated with PDGF-BB at 2.5 ng/ml demonstrated a 118% increase in proliferation compared to control (p=0.001, p<0.05 compared to 0.625 ng/ml). The group treated with 5 ng/ml had a 163% increase in proliferation compared to the cells treated with medium alone (p<0.001, and p<0.05 compared to 0.625 ng/ml and 1.25 ng/ml). Finally, the RFCs that were treated with PDGF-BB at a dose of 10.0 ng/ml had a 195% increase in proliferation compared to control (p=0.002, p<0.05 compared to 5 ng/ml, 2.5 ng/ml, and 1.25 ng/ml).

### Proliferation of Two Dimensional Cultures: Dosage in BASMCs

The dose response of the PDGF isoforms were examined by Dr. Martin Marx in BASMCs in 0% fetal bovine serum medium on Type I collagen (Figure 3). These cells were grown in two dimensions and absolute cell number was measured after five days. The doses of each isoform used were 1.25 ng/ml, 2.5 ng/ml, 5.0 ng/ml, 10.0 ng/ml, and 20.0 ng/ml.

As in RFCs, treatment with PDGF-AA was not associated with significant changes in proliferation of BASMCs regardless of the dose tested as seen in Figure 3. Specifically, the responses ranged from a 12% increase in proliferation compared to control with PDGF-AA at 1.25 ng/ml to a 6% decrease in proliferation with PDGF-AA at 20.0 ng/ml.
When BASMCs were treated with PDGF-AB, some of the doses tested were associated with significantly increased proliferation compared to cells treated with 0% fetal bovine serum medium alone (Figure 3). PDGF-AB at a dose of 1.25 ng/ml resulted in a 6% increase in proliferation (p=NS) while cells treated with 2.5 ng/ml had a 32% increase in proliferation (p=0.016, p<0.05 vs 1.25 ng/ml). Those BASMCs treated with PDGF-AB 5.0 ng/ml had a 43% increase in proliferation compared to the control group (p=0.007, p<0.05 vs 1.25 ng/ml). Treatment with PDGF-AB at 10.0 ng/ml was associated with a 60% increase in cell number compared to control cells (p<0.001, p<0.05 compared to 1.25 ng/ml and 2.5 ng/ml). This proliferation was somewhat diminished when PDGF-AB 20 ng/ml was the treatment, with the BASMCs having a 56% increased proliferation over the control cells (p=0.001, p<0.05 compared to 1.25 ng/ml and 2.5 ng/ml).

As in the RFCs, BASMCs which were treated with increasing doses of PDGF-BB demonstrated increasing degree of proliferation compared to BASMCs treated with 0% fetal bovine serum medium alone as illustrated in Figure 3. Those BASMCs given PDGF-BB 1.25 ng/ml had a 1.3% decrease in proliferation compared to control (p=NS) while giving PDGF-BB 2.5 ng/ml to these cells was associated with a 12% increase in proliferation (p=NS). The BASMCs treated with PDGF-BB at 5.0 ng/ml proliferated 26% more than control cells (p=0.024), while cells which received PDGF-BB 10.0 ng/ml had a 56% increased proliferation (p=0.011, p<0.05 compared to 1.25 ng/ml, 2.5 ng/ml, 5.0 ng/ml). Those BASMCs which received PDGF-BB 20.0 ng/ml had a 129% increased proliferation compared to control cells (p<0.001, p<0.05 compared to 1.25 ng/ml, 2.5 ng/ml, 5.0 ng/ml, 10.0 ng/ml).

Based on the above data in both RFCs and BASMCs, the dosage used to compare the differing effects of the PDGF isoforms was 10.0 ng/ml in that
this dose fell well above the dose needed to generate increased proliferation in both RFCs and BASMCs for both PDGF-AB and PDGF-BB.

Proliferation of Cells in Two Dimensional Cultures: Five Day Data (10 ng/ml)

The treatment of BAECs with PDGF-AA, -AB, and -BB at 10 ng/ml did not significantly affect five day cell proliferation in two dimensional cultures (13%, 9%, and 23% increase compared to control, respectively; Figure 4).

When RFCs grown in two dimensional cultures were treated with the PDGF isoforms at 10 ng/ml, each isoform had a different effect on cell proliferation after five days (Figure 5). When treated with PDGF-AA, the RFCs had only a 6% increase in proliferation compared to controls (those treated with RFC medium with no added PDGF, p=NS), while those RFCs that were treated with PDGF-AB had a 27% increase in proliferation (p=0.006, p<0.05 compared to PDGF-AA group). When RFCs were treated with PDGF-BB, there was 100% increase in proliferation compared to controls (p<0.001, p<0.05 compared to PDGF-AA, and PDGF-AB groups).

When BASMCs grown in two dimensional culture were treated with 10 ng/ml PDGF isoforms in 10% FBS medium, those cells treated with PDGF-AA showed a 1% decrease in proliferation compared to controls after five days (p=NS; Figure 6). The BASMCs treated with PDGF-AB showed a 13% increase in proliferation (p=0.048), and those cells treated with PDGF-BB showed a 50% increase in proliferation compared to control cells (p=0.002, p<0.05 compared to PDGF-AA, PDGF-AB).
Early Proliferation of BASMCs in Two Dimensional Culture: Cell Number

In order to determine whether the PDGF isoforms exhibit their actions at different points in time, the 24 and 48 hour proliferation was measured by counting the number of BASMCs grown in two dimensional culture (Figure 7).

After 24 hours, those BASMCs treated with PDGF-AA (10 ng/ml) showed a 14% increase in cell number (p=NS). Those cells given PDGF-AB had a 24% increase in cell number (p=0.012), and those BASMCs treated with PDGF-BB demonstrated a 54% increase in cell number (p=0.007).

After 48 hours, the BASMCs treatment with PDGF-AA was associated with a 29% increase in cell number (p=0.003), while treatment with PDGF-AB resulted in a 15% increase in the number of cells (p=0.028). Treatment of BASMCs with PDGF-BB was associated with a 65% increase in cell number at this point in time (p<0.001).

Early Proliferation of BASMCs in Two Dimensional Culture: $^3$H-Thymidine Incorporation

DNA synthesis in BASMCs grown in two dimensional cultures measured by $^3$H-thymidine incorporation has traditionally been used as an indirect measure of cell proliferation. In the present study, the $^3$H-thymidine incorporation after PDGF treatment was compared to changes in proliferation as measured by cell counts (Figure 8).

After 24 hour treatment with specific PDGF isoforms (10 ng/ml in 10% FBS medium), those BASMCs treated with PDGF-AA expressed a 28% increase in scintillation counts compared to control (p=0.004) (Figure 7). Those cells treated with PDGF-AB showed a 19% increase (p=0.014), and those cells treated with PDGF-BB had a 4% decrease in scintillation counts.
After treatment for 48 hours, the BASMCs which received PDGF-AA showed a 91% increase in radioactivity (p<0.001) (Figure 8), while those treated with PDGF-AB had a 31% increase (p=0.023). Those BASMCs receiving PDGF-BB for 48 hours had a 53% increase in radioactivity compared to control (p=0.001).

**Proliferation of Cells in Three Dimensional Cultures**

When the five day proliferative effects of PDGF-AA, -AB, and -BB (10 ng/ml) were measured in BAECs grown in a Type I collagen gel, there was no significant difference between the treated cells and the control cells (5% increase, 4% decrease, and 6% increase, respectively) (Figure 9). Similarly, Dr. Martin Marx found that the proliferation of RFCs grown in three dimensional culture and treated with PDGF-AA, -AB, -BB (10 ng/ml) did not differ significantly from control (1%, 6%, and 7% decrease respectively) (Figure 10).

When the effects of the different PDGF isoforms were measured in BASMCs, there was a strong trend toward significance which could not be analyzed statistically because of small sample sizes (Figure 11). Specifically, those BASMCs treated with PDGF-AA (10 ng/ml) had a 13% increase in cell number after four days, while those which were treated with PDGF-AB had a 23% increase in cell number. Those BASMCs which received PDGF-BB demonstrated a 42% increase in cell number.

**Migration**

PDGF did not affect the degree of radial migration in BAECs grown on Type I collagen (Figure 12). Specifically, treatment with PDGF-AA, -AB,
and -BB (10 ng/ml) had a 0.3%, 1%, and 2.8% increase in migration, respectively. These differences were not statistically significant.

BASMCs grown in 10% FBS medium had different reactions to treatment with the different isoforms of PDGF (Figure 13). The BASMCs that were treated with PDGF-AA at 10 ng/ml demonstrated a 1% increase in migration compared to those cells grown in 10% FBS medium alone (p=NS). The cells treated with PDGF-AB showed an 11% increase in migration compared to control (p=0.035, p<0.05 compared to PDGF-AA), and those BASMCs treated with PDGF-BB had a 22% increase in migration compared to control (p<0.001, p<0.05 compared to PDGF-AA, and PDGF-AB).

Previous experiments have demonstrated that RFCs will not migrate under these conditions (data not shown).
DISCUSSION

The Effect of PDGF Isoforms

Platelet-derived Growth Factor (PDGF) is a ubiquitous dimeric protein whose actions play a major role in normal development, neoplasia and both vascular injury and repair. In an attempt to further clarify the interaction between PDGF and vascular cells, large and small vessel endothelial cells and vascular smooth muscle cells were treated with the three isoforms of PDGF and proliferation and migration of these cells were measured. Previous work by Bell and Madri in bovine aortic smooth muscle cells (BASMCs) and bovine aortic endothelial cells (BAECs) treated with Mitomycin C illustrated that these two processes are independent (17).

After five days of treatment with PDGF, a consistent relationship emerged with respect to the two dimensional proliferative and migratory responses of rat epididymal fat pad capillary endothelial cells (RFCs) and BASMCs to the specific isoforms of PDGF. Namely, when proliferation (i.e., absolute number of cells) was measured in RFCs and BASMCs treated with the different isoforms of PDGF grown in two dimensions, PDGF-AA did not induce a significant change compared to control cells. PDGF-AB was associated with a significant increase in proliferation in RFCs and BASMCs while the cells treated with PDGF-BB demonstrated a significant increase over control or cells treated with PDGF-AB. The same relationship held true for the radial migration of BASMCs. Specifically, the migration of those cells treated with PDGF-AA did not differ from control while those given PDGF-AB or PDGF-BB did. In addition, the cells treated with PDGF-BB migrated significantly more than those cells given PDGF-AB.
The relationship identified between the three isoforms of PDGF and increasing proliferation and migration is consistent with that identified by other investigations. The doses of PDGF used in these studies, however, varied somewhat. In general, the PDGF doses examined in the present study are within the range examined in other investigations. Siegbahn, et al. found that PDGF-AB and PDGF-BB were chemotactic for both monocytes and fibroblasts, but that PDGF-AA had no such effect (29). Sachinidis, et al. found that treatment with recombinant PDGF-AB and PDGF-BB (at doses between 3 and 10 ng/ml) was associated with increased $^3$H-thymidine incorporation, inositol trisphosphate release, increased intracellular calcium, and diacylglycerol production in rat aortic smooth muscle cells (39). In a second study examining the effects of PDGF isoforms on rat aortic smooth muscle cells, Sachinidis, et al. found that PDGF-AB and PDGF-BB were also associated with an increase in intracellular pH, activation of the Na$^+$/H$^+$ exchanger, production of thromboxane A$_2$, and caused contraction of smooth muscle cells (55). Westermark, et al. found that both membrane ruffling and actin reorganization took place in the presence of PDGF-AB or PDGF-BB (at 10 ng/ml to 100 ng/ml) and not in cells treated with PDGF-AA (56). These results are consistent with those found by Hammacher, et al. in which human foreskin fibroblasts treated with PDGF-AB and PDGF-BB demonstrated actin reorganization while those treated with PDGF-AA did not (30). Based on the relationship between specific chains of PDGF and its receptor subunit binding specificity (Figure 1), it is reasonable to presume that the proliferative response observed at five days in the present study is mediated by the interaction of PDGF-AB and PDGF-BB with the β receptor subunit. These isoforms will both bind to receptors containing a β subunit (PDGF-AB can bind to αα or an αβ receptors and PDGF-BB can bind to αα, αβ,
or ββ receptors). Given that PDGF-AA is the only isoform which cannot bind to a receptor containing a β subunit and is the only isoform which did not elicit a migratory or proliferative response after five days, it is reasonable to infer that the β subunit of the PDGF receptor is intimately involved in these two actions. This conclusion, however, appears to be valid only when considering proliferation or possibly migration after several days.

Two studies have identified a proliferative effect of PDGF-AA. Interestingly, this association was identified after a time interval substantially shorter than the initial experiments in this study, namely after 16 to 24 hours, by investigators using $^3$H-thymidine incorporation as their means of assessing proliferation. Sachinidis, et al. found that there was a variable degree of DNA synthesis in rat vascular smooth muscle cells after 20 hours of PDGF-AA exposure but this required doses in excess of 10 ng/ml (52). Majak, et al. found that recombinant PDGF-AA used at a greater dose (50 ng/ml) was associated with significant $^3$H-thymidine uptake in rat aortic smooth muscle cells (57). In that study, incorporation was measured at 16 hours for sparsely grown cells and after 24 hours for confluent cells. Two of the experiments in the present investigation which demonstrated a proliferative response to PDGF-AA did so when $^3$H-thymidine incorporation was measured in BASMCs after 24 and 48 hours of PDGF-AA exposure (this response was generated at 10 ng/ml in contrast to the studies cited).

Specifically, PDGF-AA generated a highly significant increase in DNA synthesis at 24 hours, while PDGF-AB treatment was associated with a more modest, but significant, increase. Given the receptor subunit binding specificities, it is likely that the early increase in $^3$H-thymidine incorporation in the BASMCs treated with PDGF-AA (which can bind only to
αα receptors) and PDGF-AB (which can bind to αα or αβ receptors) represents interaction of the isoform with receptors containing an α subunit. After 48 hours of treatment with the PDGF isoforms, however, the BASMCs treated with each isoform had a significant increase in 3H-thymidine incorporation compared to control, with PDGF-BB showing the greatest increase in incorporation over the second 24 hour period.

The observation that all of the isoforms of PDGF are associated with increased 3H-thymidine incorporation raises several interesting issues. First, several investigations cited used 3H-thymidine incorporation as an indirect measure of cell proliferation (50,57). While it is true that DNA synthesis (as measured by 3H-thymidine incorporation) is necessary for cell proliferation, an increase in 3H-thymidine uptake may not be an adequate surrogate for cell number as a measure of proliferation. Using cell number as the gold standard for cell proliferation in BASMCs 24 and 48 hours after treatment with PDGF reflects essentially the same relationship between the isoforms identified in the five day proliferation experiments. These findings warrant further investigation with regard to the time course of proliferation after PDGF treatment. The observation that PDGF-AA treatment results in a dramatic increase in 3H-thymidine incorporation while only inducing a modest increase in cell number, while the opposite is true of PDGF-BB suggests that the α and β PDGF receptor subunits may be linked to different intracellular actions. Dr. Martin Marx has demonstrated that treatment of RFCs with PDGF results in phosphorylation of the membrane associated proto-oncogene c-src within 10 minutes regardless of which isoform is used (data not shown) (58). Phosphorylation of c-src has been associated with cytoskeletal reorganization in epithelial cells and decreased intercellular molecular transport (56,60). Bell, et al. have
recently demonstrated that BAECs transfected to overexpress c-src have an increased migratory rate compared to sham-transfected BAECs (61). After the phosphorylation of c-src, however, the specific intracellular events triggered by interaction of PDGF with the α and β subunit remain elusive. While some investigations have suggested that the actions which PDGF has on different cell types is a reflection of absolute and relative differences in PDGF receptor expression (45,47), the observations generated by Dr. Marx and Bell, et al. illustrate what may be one of many intracellular steps needed to generate the behavioral differences seen as a result of treatment with the PDGF isoforms.

The Effect of PDGF on Specific Vascular Cell Types

This study sought to illustrate some of the differences between large and small vessel endothelial cells by virtue of the way in which they react to treatment with PDGF. Specifically, treating microvascular capillary endothelial cells, RFCs in this case, with PDGF elicited different responses than treating large vessel endothelial cells, e.g., BAECs, with PDGF.

The differential effects that PDGF has on the two dimensional proliferation of BAECs and RFCs illustrates that, while both are endothelial cell cultures, these cells represent very different populations. Specifically, RFCs treated with PDGF isoforms demonstrated increased proliferation while BAECs did not. This observation that capillary and arteriolar endothelial cells respond differently to PDGF is consistent with the findings of Bar, et al. (49). In their study, bovine fat and mouse brain capillary endothelial cells treated with PDGF showed increased \(^{3}\)H-thymidine incorporation, α-amino isobutyric acid and 2-deoxyglucose uptake, while these events did not occur in bovine aortic and pulmonary artery
endothelial cells. In addition, $^{125}$I-PDGF bound to the microvascular endothelial cells but not the large vessel endothelial cells. Conversely, Bell and Madri found that PDGF inhibited both proliferation and migration of bovine aortic endothelial cells (17). Both of these studies, however, were conducted before the isolation of the individual PDGF isoforms and before recombinant forms of the isoforms were generated. Given the disparity between the observation found in investigations using "PDGF" (before the isolation of specific isoforms), with isoforms which were isolated, and now with recombinant PDGF isoforms, it is likely that the early PDGF preparations were contaminated with other growth factors. Despite their earlier findings, Bell and Madri now think that the inhibition of migration and proliferation of BAECs they noted may have reflected contamination of the early PDGF preparations with TGF-β (62). While several studies have evaluated the effects of nonrecombinant PDGF on endothelial cells and the effects of recombinant PDGF on vascular smooth muscle cells, it appears that this is the first investigation which compares the effects of the recombinant PDGF isoforms in large and small vessel endothelial cells.

Interestingly, when BASMCs were treated with the PDGF isoforms, these cells behaved similarly to the RFCs cultured in two dimensions. Specifically, treatment with PDGF-BB and, to a lesser degree, PDGF-AB were associated with increased five day proliferation in both of these cell types. These isoforms did not affect proliferation in BAECs. Further evidence illustrating the similarities between these two populations comes from a western blot performed by Ms. Adeline Tucker (Figure 14) (63). This blot demonstrates that RFCs and BASMCs grown on Type I collagen (i.e., in two dimensions) express PDGF β receptor subunits whereas BAECs cultured in the same manner did not. While this finding does not preclude the
possibility of αα receptor expression on BAECs, antibodies to the α receptor subunit were not commercially available at the time the blot was done. However the lack of functional effects of PDGF in these cells make it unlikely that these cells express any PDGF receptors. The observation that RFCs cultured in two dimensions on Type I collagen are phenotypically similar to BASMCs and differ from large vessel endothelial cells is not a new one. In 1989, Kocher and Madri found that microvascular endothelial cells (RFCs) grown on specific extracellular matrix components including Type I collagen expressed a phenotype with features common to both endothelial cells and smooth muscle cells (64). Specifically, RFCs grown in two dimensions stained positively for the expression of Factor VIII and angiotensin-converting enzyme (both markers for endothelial cell differentiation and seen in BAECs) and also expressed α-smooth muscle-specific actin, a marker for smooth muscle differentiation. The list of phenotypic similarities between BASMCs and RFCs now includes the increase in proliferation associated with PDGF treatment and the common expression of PDGF β receptor receptor subunits in cells cultured in two dimensions. These findings support the hypothesis put forth by Kocher and Madri that RFCs and BASMCs may be closely related and may even be the descendents of a common progenitor cell (64).

This hypothesis that BASMCs and RFCs have a common ancestor could be further strengthened with the tools available currently by examining α-smooth muscle-specific actin expression after stimulation with the different isoforms of PDGF. Several investigators have found that smooth muscle cells which are actively proliferating do not express α-smooth muscle-specific actin the way quiescent smooth muscle cells do. Studies by Bjorkerud, and Blank and Owens found that smooth muscle cells
treated with PDGF had an increase in DNA synthesis (i.e., they were actively proliferating) and a concomitant decrease in $\alpha$-smooth muscle-specific actin (65,66). Both of these studies support the finding that PDGF promotes smooth muscle cells to enter the cell cycle while decreasing expression of $\alpha$-smooth muscle-specific actin. No study, however, has yet addressed the idea that the different PDGF isoforms may result in differential effects on $\alpha$-smooth muscle-specific actin expression. Given the findings of the present study, it would be expected that all the isoforms would induce these changes, though at different points in time. In addition, if the same relationship between the PDGF isoforms and $\alpha$-smooth muscle-specific actin held true in RFCs, it would strengthen the contention that BASMCs and RFCs are closely related.

**Modulation of Cell Phenotype in Two and Three Dimensional Cultures**

The similarities between RFCs and BASMCs, however, only hold true in two dimensional cultures. There was no increase in proliferation found when the effect of the PDGF isoforms on RFCs grown in three dimensional Type I collagen gels was measured. Conversely, BASMCs grown in three dimensions and treated with PDGF continued to demonstrate the same relationship between the isoforms identified in cells grown in two dimensions. Finally, BAECs grown in three dimensional culture failed to respond to PDGF treatment, consistent with the findings generated in two dimensions. This disparity in RFC proliferation in two and three dimensions is paralleled by the findings of Ms. Adeline Tucker who did not identify PDGF $\beta$ receptor subunits on RFCs grown in three dimensions while present when RFCs were grown in two dimensions (Figure 14) (63).

Further evidence of the phenotypic differences generated by culturing
RFCs in two and three dimensions comes from Kocher and Madri (64). These investigators found that RFCs cultured in a three dimensional Type I collagen gel did not express α-smooth muscle-specific actin as they had when cultured in two dimensions nor did they identify α-smooth muscle-specific actin in RFCs grown in vivo. This disparity between RFC morphology in two and three dimensional cultures was further described by Madri, et al. who reported that RFCs in three dimensions formed multicellular capillary-like tubes with lumina, form tight junctions, and develop distinct cell polarity (51). In contrast, RFCs cultured in two dimensions fail to form tubes, tight junctions, or specialized regions of the cell membrane. These findings raise the question whether assessing RFC behavior based on two dimensional cultures is appropriate. Despite this, the finding that RFCs are cells capable of modulating their phenotype based on culture conditions while other types of endothelial cells (e.g., BAECs) cannot is not diminished and raises questions concerning the ancestry of RFCs as well as the pericyte.

This ontologic question is pertinent when discussing the origin of the pericyte, the cell responsible for providing vascular tone to capillaries in the same fashion that smooth muscle cells provide tone for larger vessels. Because the pericyte expresses α-smooth muscle-specific actin, some contend that these cells are the descendants of smooth muscle cells which have migrated into capillary walls and redifferentiated to their pericytic morphology. The evidence generated by Madri and coworkers (50,54,64) and supported by the findings of the present investigation raise the possibility that capillary endothelial cells may be the progenitor of pericytes. Given that RFCs are plastic cells in that they behave like smooth muscle cells (including expressing markers of smooth muscle
differentiation) when cultured in two dimensions and like endothelial cells when cultured in three dimensions, this cell population seems a likely candidate as the progenitor of the pericyte.

Conclusion

The present study has demonstrated that the isoforms of PDGF have differing effects on selected vascular cells. Specifically, PDGF-AA is associated with increased DNA synthesis in BASMCs (an indirect measurement of cell proliferation) within the first 24 to 48 hours after stimulation and is diminished thereafter. This effect is probably mediated through the α receptor subunit of the PDGF receptor. Treatment with PDGF-AB and PDGF-BB was associated with an increase in cell proliferation and migration after several days. This effect is likely regulated through the β subunit of the PDGF receptor.

This investigation also presented data which supported the argument that RFCs are a plastic cell population, capable of modulating its morphology based on external conditions. Specifically, it was demonstrated that RFCs, when cultured in two dimensions, expressed PDGF β receptor subunits and exhibited two dimensional proliferation which is similar to BASMCs. Conversely, when cultured in a three dimensional Type I collagen gel, RFCs lost their BASMC-like phenotype and behaved in a fashion similar to BAECs, large vessel endothelial cells.

These findings point to several roles PDGF may play in clinical circumstances, including having a beneficial impact on wound healing. Greenhalgh, et al., in their study of skin wounds in diabetic mice found a dramatic increase in both the rate in strength of wounds treated with PDGF (21). In the first human trial of PDGF, Robson, et al. sprayed recombinant
PDGF-BB (100 µg/ml) on chronic non-healing pressure sores (up to 165 months old) for one month and found 96% decrease pressure sore volume compared to pretreatment (67). Histological examination of these wounds revealed an increased number of fibroblasts and endothelial cells in the healing wounds and there was no long-term evidence of hypertrophic scarring or keloid formation. Clinical settings such as this in which PDGF-generated increases in the cell migration and proliferation of the cells involved in wound healing is beneficial represent one possible use for exogenous forms of this growth factor.

Settings in which migration and proliferation of these cells is detrimental represent another venue in PDGF research. To some degree, migration and proliferation of smooth muscle cells at the site of vascular injury is maladaptive in that it leads to neointima formation and perpetuates the cycle of vascular injury. Circumventing this process is considered to be one of the main challenges facing cardiovascular medicine especially with regard to the use of percutaneous transluminal coronary angioplasty. In this setting, endothelial denudation and exposure of the basement membrane to circulating platelets initiates the cascade which leads to stimulation of smooth muscle cell proliferation, migration, and neointima formation by factors such as PDGF. Given this chain of events, it is understandable why restenosis after angioplasty remains a major problem in the long term success of this procedure. Recent investigations have turned toward the possible regulation of growth factor-induced smooth muscle cell migration and proliferation. Naftilan, et al. have identified a dramatic up-regulation of PDGF-A chain mRNA expressed in quiescent rat aortic smooth muscle cells when treated with Angiotensin II (68). Araki, et al. demonstrated that Angiotensin II
did not induce a mitogenic response in rabbit vascular smooth muscle cells, but that the combination of Angiotensin II and PDGF was associated with DNA synthesis to almost twice the degree induced by PDGF alone (69). Recent findings from Bell and Madri demonstrated both the Angiotensin Converting Enzyme Inhibitor, Lisinopril, and the Angiotensin II antagonist, Saralasin, were associated with 41% increase in bovine aortic endothelial cell migration (70). Conversely, the treatment of bovine aortic smooth muscle cells with these factors did not significantly affect migration. Treatment of the smooth muscle cells with Angiotensin II resulted in a highly significant increase in migration while the treatment of the endothelial cells with this factor had no effect. These observations have led to the belief that the PDGF and Angiotensin systems are intimately linked and it seems likely that the ability to regulate PDGF effects may come through manipulation of the Angiotensin system. It is clear that this line of research will have major implications in the treatment of atherosclerotic disease.
Figure 1. Platelet-Derived Growth Factor Binding Specificity.
Figure 2. Effects of the PDGF Isoforms at Differing Doses on the Five Day Proliferation of RFCs Cultured in Two Dimensions. * = Dose at which PDGF-BB differed from Control (1.25 ng/ml; p=0.027). ** = Dose at which PDGF-AB differed from Control (2.5 ng/ml; p=0.010). N=3 for each condition.
Figure 3. Effects of the PDGF Isoforms at Differing Doses on the Five Day Proliferation of BASMCs Cultured in Two Dimensions. *=Dose at which PDGF-BB differed from Control (5.0 ng/ml; p=0.024). **=Dose at which PDGF-AB differed from Control (2.5 ng/ml; p=0.016). N=4 for each condition. Data courtesy of Dr. Martin Marx.
Figure 4. Effects of the PDGF Isoforms at 10 ng/ml on the Five Day Proliferation of BAECs Cultured in Two Dimensions. N=5 for each condition.
Figure 5. Effects of the PDGF Isoforms at 10 ng/ml on the Five Day Proliferation of RFCs Cultured in Two Dimensions. N=4 for each condition.
Figure 6. Effects of the PDGF Isoforms at 10 ng/ml on the Five Day Proliferation of BASMCs Cultured in Two Dimensions. N=4 for each condition.
Figure 7. Effects of the PDGF Isoforms at 10 ng/ml on the 24 and 48 Hour Proliferation of BASMCs Cultured in Two Dimensions as measured by Cell Counts. N=4 for each condition.
Figure 8. Effects of the PDGF Isoforms at 10 ng/ml on the 24 and 48 Hour $^3$H-Thymidine Incorporation of BASMCs Cultured in Two Dimensions. N=6 for each condition.
Figure 9. Effects of the PDGF Isoforms at 10 ng/ml on Five Day Proliferation of BAECs in Three Dimensional Culture. N=3 for each condition.
Figure 10. Effects of the PDGF Isoforms at 10 ng/ml on Five Day Proliferation of RFCs in Three Dimensional Culture. N=3 for each condition. Data courtesy of Dr. Martin Marx.
Figure 11. Effects of the PDGF Isoforms at 10 ng/ml on Five Day Proliferation of BASMCs in Three Dimensional Culture. N=2 for each condition.
Figure 12. Effects of the PDGF Isoforms at 10 ng/ml on Five Day Radial Migration of BAECs. N=10 for each condition.
Figure 13. Effects of the PDGF Isoforms at 10 ng/ml on Five Day Radial Migration of BASMCs. N=5 for each condition.
Figure 14. Western Blot Illustrating PDGF Receptor Expression in BAECs (in two dimensions: 170kDa band absent), RFCs (in two dimensions: approx. 170kDa bands present), RFCs (in three dimensions: 170kDa band absent), and BASMCs (in two dimensions: approx. 170kDa bands present). Blot courtesy of Ms. Adeline Tucker.
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