Regulation of the Spatial Organization of Mesenchymal Connective Tissue

Effects of Cell-Associated versus Released Isoforms of Platelet-Derived Growth Factor

Sabine A. Eming,* Martin L. Yarmush,*
Gerald G. Krueger,[†] and Jeffrey R. Morgan*

From Surgical Services,* Massachusetts General Hospital/Shriners Burns Hospital/Harvard Medical School, Boston, Massachusetts, and the Department of Dermatology,[†] University of Utah Health Sciences Center, Salt Lake City, Utah

Platelet-derived growth factor (PDGF), a mitogen and chemoattractant for mesenchymal cells, occurs as cell-associated or released isoforms. To investigate their *in vivo* role, human keratinocytes, which normally synthesize both types of PDGF, were genetically modified to overexpress either wild-type PDGF-B (cell-associated) or the truncation mutant PDGF-B211 (released). Cells expressing the mutant isoform released 20 times more PDGF (145 ng/hour/10⁷ cells) than cells expressing the wild-type isoform (6 ng/hour/10⁷ cells). When grafted as epithelial sheets onto athymic mice, modified cells formed a stratified epithelium and induced a connective tissue response that differed depending on the PDGF isoform expressed. Expression of PDGF-B211 induced a thick connective tissue with increased numbers of fibroblasts, mononuclear cells, and blood vessels evenly distributed throughout the connective tissue layer, whereas expression of PDGF-B induced a zone of fibroblasts and mononuclear cells localized to the interface of the epidermis and connective tissue, which often disrupted the continuity of the basement membrane. Immunostaining revealed that wild-type PDGF protein was deposited in the basement membrane region. These data suggest that the different binding properties of PDGF isoforms control the spatial organization of cellular events in regenerating mesenchymal tissue *in vivo*. (Am J Pathol 1999, 154:281–289)

Platelet-derived growth factor (PDGF) is a disulfide-linked dimer consisting of two related polypeptide chains (designated A and B) encoded by separate genes. Biologically active PDGF occurs as either a homodimer (AA or BB) or as a heterodimer (AB).¹ These PDGF isoforms

have overlapping biological activities but have significant differences in their matrix-binding properties. Although all PDGF isoforms have a signal sequence and are secreted to the outer side of the plasma membrane, some PDGF isoforms remain cell associated after secretion. In the case of PDGF-A, alternate splicing of exon 6 is sometimes used to switch from a released isoform to one that is cell associated.^{2–4} PDGF-B, on the other hand, is predominantly cell associated, and the amino acids responsible for cell association have also been mapped to exon 6.³ Although much is known about the *in vitro* properties of various PDGF isoforms, the role of the different isoforms *in vivo* is poorly understood.

PDGF acts predominantly on the cells of the mesenchyme. Most cells of the dermis express a receptor for PDGF, and in addition to stimulating the proliferation of fibroblasts, smooth muscle cells, and microvascular endothelial cells, PDGF is chemotactic for fibroblasts, macrophages, neutrophils, and smooth muscle cells. PDGF isoforms are synthesized by numerous cell types, including keratinocytes, the principal cell type of the epidermis, which synthesize both PDGF-A and B isoforms.⁵ Recently, we demonstrated that when keratinocytes genetically modified to overexpress the released isoform of PDGF-A were transplanted to the athymic mouse, these PDGF-A-secreting grafts formed a normal epidermal structure and induced an increase in the cellularity and vascularity of the connective tissue that formed subjacent to these grafts.⁶ These data suggest that the released isoform of PDGF-A produced by the epidermis acts as a paracrine mediator that controls distant cellular events in the adjacent dermis.

In the present study, we have sought to understand the role of PDGF-B produced by the epidermis, a PDGF

Supported by grants from the Shriners Hospitals for Children, the National Institutes of Health, and the Fritz Thyssen and German Research Foundation.

Accepted for publication September 24, 1998.

Address reprint requests to Dr. Jeffrey R. Morgan, Shriners Burns Hospital Research Center, One Kendall Square, Bldg. 1400, Cambridge, MA 02139. E-mail: jmorgan@sbi.org.

S.A. Eming's present address: Department of Dermatology, University of Cologne, Cologne, Germany.

isoform that is not released but is predominantly cell associated. Retroviral-mediated gene transfer was used to introduce into human keratinocytes the gene encoding either wild-type *PDGF-B* or a truncation mutant of *PDGF-B* that is released. *In vitro*, modified cells synthesized increased levels of PDGF and released 20-fold more of the mutant PDGF-B isoform than wild-type PDGF-B. When modified cells were grafted as epithelial sheets onto athymic mice, distinct changes occurred in the adjacent connective tissue. Grafts overexpressing the released mutant of *PDGF-B* induced an increase in the cellularity and vascularity throughout the subjacent connective tissue, similar to our previous results with *PDGF-A*.⁶ In contrast, the cellularity of the connective tissue subjacent to grafts overexpressing wild-type *PDGF-B* was also increased, but this cellularity was confined to a zone at the interface of the epidermis and the connective tissue. These data demonstrate that the released and cell-associated isoforms of PDGF both act as paracrine mediators that control cellular events in the adjacent dermis but that the released isoforms control more distal events, whereas the cell-associated isoforms control more proximal events.

Materials and Methods

Recombinant Retrovirus

A cDNA encoding human *PDGF-B* was amplified by polymerase chain reaction (PCR) using 5'-GACGATCATGAATCGCTGCTGGGCG-3' and 5'-CCGATGGATCCTAGGCTCCAAGGGTCTC-3' as forward and reverse primers, respectively. After PCR, the primers were removed and the product digested with *Bsp*HI and *Bam*HI, gel purified, and ligated into the *Nco*I/*Bam*HI sites of the retroviral vector MFG.⁷ The retroviral vector expressing the truncation mutant of *PDGF-B* with a stop codon at position 211 (*PDGF-B211*) as originally described² was prepared in a similar manner except that the sequence 5'-CTGCTGGATCCTAAATGGTCACCCGAGTTTGGG-3' was used as the reverse primer. The fidelity of the inserts were verified by DNA sequencing. To generate cell lines producing recombinant retrovirus, plasmid DNAs encoding MFG-*PDGF-B* and MFG-*PDGF-B211* were transfected into the ψ -CRIP packaging cell line.⁸ Clones of transfectants were isolated and screened for ones producing the highest titer.

Cell Culture

Human keratinocytes derived from neonatal foreskins (strains A to E) were grown on a mitomycin-C-treated (15 μ g/ml) feeder layer of mouse 3T3-J2 cells (originally obtained from H. Green, Harvard Medical School, Boston, MA) in a mixture of the Dulbecco-Vogt modification of Eagle's medium (DMEM) and Ham's F12 medium. Supplements were as described.⁹ Swiss mouse 3T3-J2 and virus-producing cells (ψ -CRIP) were grown in DMEM supplemented with 10% calf serum.

Genetic Modification

Keratinocytes were genetically modified as previously described.¹⁰ Briefly, preconfluent primary cultures were dissociated, and cells were passed to a dish containing mitomycin-C-treated virus-producing cells (2.5×10^4 cells per cm^2). After 4 to 6 days of co-cultivation, modified cells were dissociated and parallel cultures were prepared for protein analysis, grafting, and plating efficiency. Unmodified control cells were cultured in parallel on 3T3-J2 cells.

Assays for PDGF

Synthesis of PDGF proteins by transduced cells was assayed by ELISA, specific for human PDGF-BB. Briefly, 96-well plates were coated with an affinity-isolated polyclonal rabbit anti-hPDGF antibody (10 μ g/ml in 0.1 mol/L NaHCO_3 , pH 9.6; R&D Systems, Minneapolis, MN) for 16 hours at 4°C. The wells were blocked with PBS, containing 1% bovine serum albumin, 0.5% Tween 80 for 2 hours at room temperature. Test samples or known amounts of recombinant human PDGF-BB (Boehringer Mannheim, Indianapolis, IN) were added to the wells for 3 hours at room temperature. The wells were washed, and affinity-isolated polyclonal goat anti-hPDGF-BB antibody conjugated to horseradish peroxidase (R&D Systems) was added for 3 hours at room temperature, followed by tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO). The limit of detection of PDGF-BB was 30 pg/ml.

To measure the levels of PDGF proteins released into the medium, modified keratinocytes were grown to confluence (10^7 cells/10-cm dish), fresh medium was added (30 ml), and portions (1 ml) of the culture medium were removed over a 4-day period. The rate of PDGF synthesis was determined by averaging the daily rate of PDGF synthesis over a 4-day period.

To measure the amount of PDGF that was cell associated, secondary cultures of MFG-*PDGF-B* or MFG-*PDGF-B211* transduced keratinocytes were grown to confluence in serum-free medium (Clonetics MCDB 153). The cells were washed with serum-free medium and exposed three times to 1 mol/L NaCl for 30 minutes at 4°C. After each treatment, the NaCl solution was harvested, and the amount of PDGF-BB protein was measured by ELISA.

The bioactivity of PDGF released by modified keratinocytes was determined using a modification of a cell proliferation assay that measures the stimulation of [^3H]thymidine incorporation.¹¹ Quiescent Swiss 3T3 cells were incubated for 12 hours with medium (5% platelet-poor plasma; PPP) conditioned for 30 hours by modified or unmodified keratinocytes. The 3T3 cells were washed with serum-free DMEM and incubated for 20 hours with [^3H]thymidine (1 μ Ci/well with 5% PPP in DMEM). Cells were washed in serum-free DMEM, DNA was precipitated with two washes of 5% trichloroacetic acid (TCA) at 4°C, and cells were lysed with 0.6% sodium dodecyl sulfate (SDS). TCA-insoluble material was harvested on a glass fiber filter and counted in a scintillation counter (Packard Instrument Co., Meriden, CT). A standard curve

was established by incubating quiescent Swiss 3T3 cells with DMEM 5% PPP supplemented with recombinant human PDGF-BB (Boehringer Mannheim).

Grafting

Grafting was performed using 6- to 8-week-old NIH swiss *nu/nu* mice (Taconic Farms, Germantown, NY) anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (0.58 mg/g body weight; Aldrich, Milwaukee, WI).

Cultures of confluent keratinocytes (35-mm dishes) were detached as intact epithelia with Dispase II solution (1.2 U/ml for 1 hour at 37°C; Boehringer Mannheim) and grafted to athymic mice as described.¹² Briefly, the detached epithelium was placed basal side up on a small piece of Silastic membrane (thickness, 0.005 inches; Dow Corning, Arlington, TN). This graft was inserted under a full-thickness skin flap on the back of the mouse so that when the flap was closed, the basal side of the epithelium came in contact with the inner side of the mouse skin. Before inserting the epithelium, the loose connective tissue covering the inner side of the mouse skin was removed by dissection. In this way, the basal side of the epithelium was placed directly on the epimysium of the panniculus carnosus. The incision was closed with surgical clips, and grafts were harvested after 7 days.

Histology

The human epithelium and subjacent mouse connective tissue were fixed in buffered formaldehyde, and paraffin-embedded sections (5 μ m) were stained for hematoxylin and eosin (H&E; control, $n = 8$; MFG-PDGF-B, $n = 8$; MFG-PDGF-B211, $n = 7$). The thickness of the mouse connective tissue layer was determined by image analysis using the Image Analysis/Acquisition Software system (Universal Image Corp., West Chester, PA) coupled to a Nikon Diaphon 300 microscope (Nikon, Melville, NY). The edges of the epithelial grafts were avoided as these showed signs of hyperproliferation. The cross-sectional area of the mouse connective tissue layer was determined for 10 fields of view per graft, and the thickness was calculated by dividing the area by the length of the sample field of view (1.06 mm). Thickness measurements for control ($n = 8$), PDGF-B-modified ($n = 8$), and PDGF-B211-modified grafts ($n = 6$) were made from a total of 80-mm, 80-mm, and 60-mm lengths of graft, respectively. Statistical comparisons were done using analysis of variance and the Tukey honestly significant difference test.

For immunostaining, cryostat sections (6 μ m) were fixed in ice-cold acetone for 5 minutes, treated with blocking solution (3% bovine serum albumin, 1% normal goat serum, 0.02% sodium azide in PBS) for 1 hour at room temperature, and incubated with the following antibodies: affinity-isolated polyclonal rabbit anti-laminin antibody (1:400; Sigma), an affinity-isolated monoclonal mouse anti-laminin antibody (1:400; Sigma), an affinity-isolated monoclonal mouse anti-human collagen IV anti-

body (1:10; Biodesign, Kennebunk, ME), an affinity-isolated polyclonal rabbit anti-human PDGF-BB antibody (1:50; Genzyme, Cambridge, MA), adsorbed with an acetone powder of mouse skin or an affinity-isolated polyclonal rabbit anti-human PDGF-BB antibody (1:50; R&D Systems) for 2 hours at room temperature. Slides were washed four times for 15 minutes each in PBS and incubated with fluorescein-conjugated affinity-purified goat anti-rabbit IgG antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) or with lissamine rhodamine-conjugated affinity-isolated goat anti-mouse IgG antibody (1:100) for 1 hour at room temperature. When using mouse monoclonal antibodies, endogenous mouse IgG was blocked with an affinity-isolated goat anti-mouse IgG Fab fragment (1:100; Jackson ImmunoResearch Laboratories). The slides were washed four times for 15 minutes each in PBS and coverslipped using 1% *n*-propyl-gallate mounting solution (Sigma). All antibodies were diluted in blocking solution. Control sections were not exposed to primary antibody. Fluorescence due to immunoreactive PDGF-BB was viewed in a laser scanning confocal microscope (model MRC 500, BioRad Laboratories, Cambridge, MA).

Results

Genetic Modification of Human Keratinocytes

To determine the role of cell-associated *versus* released isoforms of PDGF, we prepared recombinant retroviral vectors expressing cDNAs encoding either wild-type PDGF-B, (MFG-PDGF-B), which is predominantly cell associated, or a truncation mutant of PDGF-B211 (MFG-PDGF-B211), which is predominantly released. This mutant has a stop codon inserted at position 211, and the resulting protein lacks exon 6 sequences.² La Rochelle et al showed that comparable amounts of the truncated and wild-type proteins were cell associated but that the release of the mutant protein was significantly higher than the wild-type protein.

Secondary cultures of human keratinocytes were genetically modified by co-cultivation with mitomycin-C-treated ψ -CRIP cells producing the MFG-PDGF-B or MFG-PDGF-B211 virus. After 4 days of co-cultivation, the virus-producing cells were removed and the keratinocytes were dissociated and passed onto plates containing a normal 3T3 fibroblast feeder layer. Control cultures of unmodified cells were carried in parallel. To measure the release of PDGF protein, aliquots of culture medium from modified cells were harvested over time, and PDGF levels were determined by ELISA. Cells expressing the MFG-PDGF-B211 retrovirus synthesized and released PDGF protein into the medium over a 4-day period at an average daily rate of 112 ng/10⁷ cells/hour (strain A) or 145 ng/10⁷ cells/hour (strain B) (Figure 1A). In contrast, cells modified with MFG-PDGF-B released relatively little PDGF protein into the medium, with an average daily rate of 7 ng/10⁷ cells/hour (strain A) or 6 ng/10⁷ cells/hour (strain B). The rate of PDGF release by cells modified with

Figure 1. Modified keratinocytes synthesize PDGF-B proteins. **A:** Time course of PDGF synthesis and release was determined by removing portions of the culture medium of a confluent culture of modified keratinocytes as indicated over a 4-day period, and PDGF-BB protein was measured by ELISA. **B:** Cell-associated PDGF was determined by treatment of cells with 1 mol/L NaCl in DMEM and measuring released PDGF by ELISA.

the mutant gene was 20 times higher than the cells modified with the wild-type gene. The level of PDGF-BB released by unmodified cells under these conditions was undetectable.

To measure the amount of PDGF that was cell associated, modified keratinocytes were incubated with 1 mol/L NaCl in DMEM, which releases the cell-associated PDGF-BB molecules.² Subsequently, the levels of PDGF-BB protein in the supernatants were measured by ELISA. Treatment of the cells for 30 minutes at 4°C with the high-salt solution released 80% of the cell-associated

Figure 2. Wild-type and mutant PDGF produced by keratinocytes are bioactive. Culture medium conditioned for 30 hours by unmodified and modified keratinocytes was harvested in DMEM 5% PPP, and PDGF protein levels were measured by ELISA. Various doses of keratinocyte-produced PDGF were assayed for the ability to stimulate [³H]thymidine incorporation by quiescent Swiss 3T3 cells. Triplicate samples and $\pm$ SEM are presented.

PDGF protein of MFG-PDGF-B- and MFG-PDGF-B211-modified cells (Figure 1B). Levels of cell-associated molecules were similar for cells modified with either the wild-type or mutant PDGF genes, consistent with the previous results.² Treatment of unmodified control cells did not release detectable amounts of PDGF-BB protein.

Wild-Type and Mutant PDGF Isoforms Have Comparable Biological Activity

The biological activity of the wild-type and mutant PDGF proteins produced by modified keratinocytes was measured using a bioassay. Conditioned medium from modified keratinocytes was harvested after 30 hours and tested in a bioassay that measures the incorporation of [³H]thymidine into Swiss 3T3 cells (Figure 2). As a control, conditioned medium was harvested from unmodified

cells. This control conditioned medium had significant levels of stimulatory activity, presumably due to other growth factors normally secreted by unmodified keratinocytes. As measured by ELISA, the level of PDGF-BB was only 0.25 ng/ml. When compared with this control, conditioned medium from modified cells had significantly increased levels of PDGF-BB and showed a dose-dependent stimulation of [^{3} H]thymidine uptake that was greater than control levels and correlated with PDGF-BB levels. When equated with PDGF-BB levels, conditioned medium from cells producing the wild-type molecule had comparable stimulatory activity to conditioned medium from cells producing the truncation mutant.

Wild-Type and Mutant Isoforms Induce Different Connective Tissue Responses

Keratinocytes expressing wild-type or mutant PDGF-B genes were tested for their ability to form an epidermis when transplanted to athymic mice. Normal or modified keratinocytes were grown to confluence in 35-mm dishes, detached as epithelial sheets using Dispase, and grafted to the underside of a dorsal skin flap. The basal side of the sheet was placed directly on the epimysium of the panniculus carnosus, and 7 days after grafting the human epithelium and the subjacent mouse tissue were fixed and stained.

Both control and modified keratinocytes developed a stratified epidermal structure several cell layers thick, which contained stratum granulosum and stratum corneum (Figure 3). The stratum granulosum of the epithelia expressing wild-type PDGF-B was less developed and thinner, compared with that of control and grafts expressing PDGF-B211.

The epidermal-dermal junction and the connective tissue layer subjacent to unmodified and modified grafts showed significant differences. The tissue subjacent to the control epithelium of unmodified keratinocytes contained fibroblasts and mononuclear cells distributed within a loose connective tissue with occasional blood vessels. The epidermal-dermal junction was well defined. The connective tissue layer subjacent to the graft expressing PDGF-B211 also had a well defined epidermal-dermal junction but had increased numbers of fibroblasts and mononuclear cells. Furthermore, the majority of grafts expressing PDGF-B211 (six of seven) had significantly increased numbers of blood vessels (Figure 3C). This increased cellularity and vascularization of the connective tissue subjacent to the PDGF-B211-expressing grafts was uniformly present throughout the entire connective tissue layer. These results are similar to our previous results with grafts overexpressing PDGF-A, a PDGF isoform that is also released.⁶ In contrast, the cellularity in the connective tissue subjacent to the grafts expressing wild-type PDGF-B was increased but was predominantly localized in a zone at the epidermal-dermal junction, often resulting in the disruption of the integrity and continuity of the epidermal-dermal border in wide areas of the graft (Figure 3B) and most obvious under high magnification (Figure 3E). Vascularization of the connective

tissue was also less prominent (eight of eight grafts) when compared with the connective tissue subjacent to PDGF-B211-expressing grafts.

To identify the basement membrane of the epidermal-dermal junction and blood vessels in the connective tissue, cryosections of control and modified grafts were stained for laminin (Figure 3, G–I). Laminin staining was abundant throughout the connective tissue layer subjacent to the grafts expressing PDGF-B211, identifying the presence of capillaries and/or small blood vessels. In contrast, laminin staining in the connective tissue layer subjacent to control and grafts expressing wild-type PDGF-B was nearly absent. Furthermore, laminin staining was continuous along the basement membrane zone of the epidermal-dermal junction of grafts of unmodified cells (four of four grafts; Figure 3G) and grafts expressing PDGF-B211 (four of four; Figure 3I). In contrast, grafts expressing PDGF-B (three of four) showed a rather disjointed and discontinuous staining pattern in wide areas of the epidermal-dermal region (Figure 3H). Similar staining patterns were seen with antibodies to other basement membrane components, including collagen types IV and VII (data not shown).

The cellular and vascular response of PDGF-B211-modified grafts was accompanied by a thickening of the connective tissue layer developed adjacent to these grafts. The thickness of the subjacent connective tissue of control grafts and grafts expressing PDGF-B versus grafts expressing PDGF-B211 was measured by image analysis from three different sets of grafts, using three different strains of keratinocytes (Figure 4). In each experiment, the connective tissue subjacent to the grafts expressing PDGF-B211 was significantly thicker than control grafts or grafts expressing wild-type PDGF-B, with an average increase in thickness of 54% and 50%, respectively.

Wild-Type PDGF-BB Is Localized to the Basement Membrane

To localize PDGF protein *in vivo*, cryosections of unmodified and modified epithelia 7 days after grafting were stained with an affinity-isolated polyclonal antibody against human recombinant PDGF-BB (Figures 3, J–L, and 5). Staining of endogenous PDGF-BB protein in control grafts and PDGF-B211-modified grafts was relatively weak and diffuse (Figure 3, J and L). In contrast, strong and localized staining for PDGF-BB was evident in the region of the epidermal-dermal junction in those grafts expressing wild-type PDGF-B (Figures 3K and 5, B and D). Laminin staining had shown that the basement membrane region in these grafts was often disrupted and discontinuous. Thus, to determine whether PDGF-BB protein co-localized with proteins of the basement membrane, we simultaneously stained cryosections for PDGF-BB and laminin or PDGF-BB and human collagen type IV (Figure 5). In both cases, the staining for PDGF-BB co-localized with staining for laminin and collagen type IV in short stretches where the basement membrane was contiguous. This staining pattern for

Figure 3. Wild-type and mutant PDGF induce different connective tissue responses *in vivo*. Cultures of confluent epithelia generated from unmodified (A, D, G, and J), MFG-PDGF-B (B, E, H, and K), or MFG-PDGF-B211 (C, F, I, and L) transduced keratinocytes, were grafted to athymic mice. Seven days later, grafts were fixed and sections (5 μ m) stained with H&E (A to F) and visualized under low (A to C) and high (D to F) magnification. Other grafts were frozen, and cryosections were stained for laminin (D to F) or hPDGF-BB (G to I). Note the lack of continuity of the basement membrane in grafts overexpressing wild-type PDGF-B (H) and the deposition of PDGF-BB in nondisrupted regions of the basement membrane (K) when visualized under high magnification.

PDGF-BB was unique to the grafts expressing wild-type *PDGF-B* and was confirmed using a different affinity-isolated polyclonal against human PDGF-BB (data not shown).

Discussion

We investigated the *in vivo* role of cell-associated *versus* released isoforms of PDGF-B produced by the epidermis. Human keratinocytes genetically modified to overexpress the truncation mutant *PDGF-B211* released 20 times more PDGF protein *in vitro* than keratinocytes overexpressing the wild-type *PDGF-B* gene. Grafts of modified cells formed a stratified epithelium and induced a connective tissue subjacent to the grafts that varied in orga-

nization depending on which PDGF isoform was expressed. Grafts expressing *PDGF-B211* induced a connective tissue that was thicker, more cellular, and more vascular than the connective tissue subjacent to grafts of control cells, and this increased cellularity was evenly distributed throughout the connective tissue. In contrast, the connective tissue subjacent to grafts overexpressing wild-type *PDGF-B* also contained increased numbers of fibroblasts and mononuclear cells, but the increased cellularity was confined to a zone at the interface of the epithelium and the connective tissue. By immunostaining, wild-type PDGF-B but not the released PDGF-B211 protein was shown to be deposited in the basement membrane zone. These results suggest that PDGF deposited in the basement membrane zone con-

Figure 4. Thickness of connective tissue subjacent to grafts expressing PDGF-B211 is enhanced. The thickness of the tissue subjacent to the 7-day-old grafts of three strains of keratinocytes were measured by image analysis of H&E sections. For each field of view, the area of the mouse connective tissue layer subjacent to the human epithelium was determined and the thickness computed. Measurements were taken for 10 fields of view per graft, and each point represents one graft. From each group the mean is presented. * $P < 0.05$; strain D, $n = 8$; strain C, $n = 8$; strain A, $n = 7$.

controls proximal cellular events, whereas released PDGF isoforms control more distal events and that, together, different PDGF isoforms control the spatial organization of connective tissue.

These results have similarities with our previous experiments in which PDGF-A was overexpressed by epithelial grafts and suggest that PDGF isoforms produced by the epidermis are paracrine regulators of the dermis.⁶ Grafts overexpressing PDGF-A, a released isoform, induced the formation of a thicker and more cellular connective tissue similar to the connective tissue induced by overexpression of PDGF-B211. Both grafts also induced connective tissues that were highly vascular. Any direct *in vivo* roles for either PDGF-BB or especially PDGF-AA on vascular endothelial cells remains controversial.¹³ The increased vascularity is more likely mediated by indirect actions of PDGF, such as the stimulation of other cells, probably microvascular pericytes and/or fibroblasts, to produce factors that promote angiogenesis.¹⁴⁻¹⁶

In the present study, vascularization was one significant difference between the connective tissues induced by grafts expressing wild-type PDGF-B versus PDGF-B211. Overexpression of PDGF-B211 induced a highly vascular connective tissue, whereas overexpression of wild-type PDGF-B did not. Both molecules bind the same receptor, and so the different biological responses are probably not at the receptor level, an argument that would be relevant to comparisons between PDGF-A and wild-type PDGF-B, which bind different receptors.¹ Rather, the different vascular responses are most likely due to differences in the bioavailability of the two molecules and/or the accessibility of endothelial cells to the mitogenic signal. Released PDGF-B211 molecules can

diffuse away from the graft and stimulate endothelial cell proliferation distal to the graft, whereas diffusion of wild-type PDGF-B deposited in the basement membrane zone is limited, and these molecules act on those cells proximal to the graft. Both grafts are placed on the epimysium of the panniculus carnosus, a thin layer of predominantly fibroblasts surrounding the muscle layer; thus, few endothelial cells would be accessible to PDGF-B deposited in the basement membrane.

Our experiments provide additional information on the role of exon 6 in PDGF function and suggest exon 6 is required for the deposition of PDGF-B in the basement membrane zone *in vivo*. Exon 6 of PDGF-B encodes a highly basic domain of 14 amino acids, and PDGF-B211 lacks only this domain. PDGF-B211 was originally described by LaRochelle et al, who transfected 3T3 fibroblasts with a series of truncation mutants and showed that both wild-type and PDGF-B211 were cell associated and could be released by high-salt treatment. However, very little wild-type PDGF-B was released, whereas most of PDGF-B211 was released.² Using keratinocytes, we have similar results *in vitro* that show comparable cell-associated levels of wild-type and mutant molecules and high-level release of PDGF-B211. However, when these cells were transplanted, we detected only wild-type PDGF-B in the basement membrane zone *in vivo*. Thus, exon 6 is required for deposition of PDGF-B in the basement membrane zone. Exon 6 has been shown to be necessary for binding of PDGF isoforms to heparan-sulfate proteoglycans and SPARC (secreted protein, rich in cysteine) *in vitro* and both are components of the basement membrane.¹⁷ The removal of the cationic domain in PDGF-B211 results in a significant decrease in the theoretical isoelectric point of the secreted molecule and potentially its binding properties. Wild-type PDGF-B (residues 21 to 241) has a pI of 9.42, whereas PDGF-B-211 (residues 21 to 211) has a pI of 8.21.

PDGF-A also has two natural isoforms that arise by alternate splicing of exon 6. PDGF-A_S, the predominant form that lacks exon 6 is released, and PDGF-A_L, which contains exon 6, is cell associated.³ In our previous studies, we overexpressed PDGF-A_S, which was released and promoted vascularization and formation of a thicker connective tissue.³ Exon 6 in PDGF-A might also serve a similar function and direct the deposition of PDGF-A_L to the basement membrane zone. It would be interesting to use our system to determine the *in vivo* tissue response to overexpression of PDGF-A with and without exon 6 and compare this response to our results with PDGF-B.

Vascular endothelial growth factor (VEGF), an endothelial cell mitogen and chemoattractant for monocytes, shares significant homology with PDGF and also occurs as multiple isoforms through alternate splicing.¹⁸ Two short forms, VEGF₁₂₁ and VEGF₁₆₅, lack exon 6 and are released, whereas two longer forms, VEGF₁₈₉ and VEGF₂₀₆, contain exon 6 and are cell associated. Like PDGF, exon 6 of VEGF also encodes a domain of basic amino acids. Keratinocytes have been shown to synthesize at least three of the splice variants, VEGF_{121,165,189},^{19,20} raising the possibility that the bio-

Figure 5. Wild-type PDGF-BB is deposited in the basement membrane zone. Cryosections of 7-day grafts of cells expressing wild-type PDGF-B were simultaneously stained with a rabbit antibody to hPDGF-BB (B and D) and mouse antibodies to either collagen IV (A) or laminin (C). Staining was separately visualized with a fluorescein-conjugated affinity-purified goat-anti-rabbit IgG antibody or lissamine-rhodamine-conjugated affinity-purified goat anti-mouse IgG antibody.

availability of VEGF produced by the epidermis is controlled in a manner similar to PDGF isoforms.

Other growth factors have been shown to be associated with the extracellular matrix, including granulocyte/macrophage colony-stimulating factor,²¹ transforming growth factor- β ,²² and differentiation-inhibiting activity.²³ A notable example is basic fibroblast growth factor (bFGF), an endothelial cell mitogen that binds glycosaminoglycans in the basement membrane of cornea.²⁴ It has been suggested that sequestered bFGF serves to control angiogenesis and can act as a signal for repair when released after injury.²⁴ In normal skin, such a role is also possible for sequestered PDGF-B in the basement membrane.

Conversely, over-accumulation of growth factors in the matrix and/or basement membrane could underlie some pathologies. It has been widely suggested that PDGF, a potent mitogen for smooth muscle cells, is involved in the neointimal hyperplasia associated with atherosclerosis.²⁵ The association of PDGF-B isoforms with the basement membrane zone as seen in our studies could be one mechanism by which PDGF contributes to localized hyperproliferative responses.

In our grafting model, overexpression of wild-type PDGF-B resulted in disruption of the basement membrane zone. Lichenoid reactions of the skin and mucosa are also characterized by a degeneration and disruption of the basement membrane zone. Growth factors, such

as PDGF-B, that are associated with components of the basement membrane zone could play a role in the pathogenesis. Disruption of the basement membrane might also be an important event in the multistep process of tumorigenesis and metastasis. PDGF-B is structurally related to the *v-sis* transforming gene of simian sarcoma virus, and like wild-type PDGF-B, the *v-sis* gene product is also cell associated after secretion.¹ Typically, autocrine pathways have been implicated in oncogenesis, and several tumors, such as sarcomas and gliomas, express PDGF and receptors for PDGF.^{26,27} However, some tumors expressing PDGF do not express receptors for PDGF, suggesting an alternative role for PDGF in oncogenesis.²⁸ Overproduction by a tumor cell of a matrix-associated growth factor, such as PDGF-B, might stimulate the proliferation of neighboring nontransformed cells that disrupt the extracellular matrix and/or basement membrane and allow further proliferation and migration of the tumorigenic cells.

Lastly, our experiments demonstrate that changes in the concentration/bioavailability of a growth factor such as PDGF can profoundly influence the type of biological response *in vivo* as well as its spatial organization. For example, PDGF-BB deposited in the basement membrane promoted a proximal cellular response but failed to elicit either a distal or proximal vascular response, whereas the same growth factor released as PDGF-B211 promoted distal vascularization but failed to promote a

proximal cellular response. Such compartments or gradients of growth factors could be a means by which cellular events are spatially controlled during tissue regeneration as well as during development.

Acknowledgments

We gratefully acknowledge the assistance of Rick Snow and Blanca Lusetti and Drs. Robert Ezzell, Prabhas Moghe (laser confocal microscopy), and Charles Hart (PDGF immunostaining).

References

- Raines EW, Bowen-Pope DE, Ross R: Platelet-derived growth factor. Peptide Growth Factors and Their Receptors I. Edited by Sporn MB, Roberts AB. Berlin, Springer 1991, pp 173–242
- LaRoche WJ, May-Siroff M, Robbins KC, Aaronson SA: A novel mechanism regulating growth factor association of a PDGF retention domain. *Genes Dev* 1991, 5:1191–1199
- Raines EW, Ross R: Compartmentalization of PDGF on extracellular binding sites dependent on exon-6-encoded sequences. *J Cell Biol* 1992, 116:533–543
- Kelly JL, Sanchez A, Brown GS, Chetsreman CN, Sleight MJ: Accumulation of PDGF-B and cell-binding forms of PDGF-A in the extracellular matrix. *J Cell Biol* 1993, 121:1153–1163
- Ansel JC, Tiesman JP, Olerud JE, Krueger JG, Krane JF, Tara DC, Shipley GD, Gilbertson D, Usui ML, Hart CE: Human keratinocytes are a major source of cutaneous platelet-derived growth factor. *J Clin Invest* 1993, 92:671–678
- Eming SA, Lee J, Snow RG, Tompkins RG, Yarmush ML, Morgan JR: Genetically modified human epidermis overexpressing PDGF-A directs the formation of a cellular and vascular connective tissue stroma when transplanted to athymic mice: implications for the use of genetically modified keratinocytes to modulate dermal regeneration. *J Invest Dermatol* 1995, 105:756–763
- Ohashi T, Boggs S, Robbins P, Bahnson A, Patrene K, Wei F, Wei J, Li J, Lucht L, Fei Y, Clark S, Kimak M, He F, Mowery-Rushton P, Barranger JA: Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by a retroviral vector. *Proc Natl Acad Sci USA* 1992, 89:11332–11336
- Danos O, Mulligan RC: Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA* 1988, 85:6460–6464
- Allen-Hoffman BL, Rheinwald JG: Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture. *Proc Natl Acad Sci USA* 1984, 81:7802–7806
- Morgan JR, Barrandon Y, Green H, Mulligan RC: Expression of an exogenous growth hormone gene by transplantable human epidermal cells. *Science* 1987, 237:1476–1479
- Pledger WJ, Stiles CD, Antoniades HN, Scher CD: Induction of DNA synthesis in BALB/c 3T3 cells by serum components: reevaluation of the commitment process. *Proc Natl Acad Sci USA* 1977, 74:4485–4489
- Barrandon Y, Li V, Green H: Two techniques for grafting of cultured human epidermal cells onto athymic animals. *J Invest Dermatol* 1988, 91:315–318
- Marx M, Perlmutter RA, Madri JA: Modulation of platelet-derived growth factor receptor expression in microvascular endothelial cells during in vitro angiogenesis. *J Clin Invest* 1994, 93:131–139
- Cochran BH, Reffel AC, Stiles CD: Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* 1983, 33:939–947
- Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, Pekna M, Hellstrom M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Tornell J, Heath JK, Betsholtz C: PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 1996, 85:863–873
- Lindahl P, Johansson BR, Leveen P, Betsholtz C: Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 1997, 277:242–245
- Timpl R: Structure and biological activity of basement membrane proteins. *Eur J Biochem* 1989, 180:487–502
- Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternate exon splicing. *J Biol Chem* 1991, 266:11947–11954
- Ballaun C, Weninger W, Uthman A, Weich H, Tschachler E: Human keratinocytes express the three major splice forms of vascular endothelial growth factor. *J Invest Dermatol* 1995, 104:7–10
- Detmar M, Yeo K-T, Nagy JA, Van De Water L, Brown LF, Berse B, Elicker BM, Ledbetter S, Dvorak HF: Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J Invest Dermatol* 1995, 105:44–50
- Gordon MY: Hemopoietic growth factors and receptors: bound and free. *Cancer Cells* 1991, 3:127–133
- Dallas SL, Miyazono K, Skerry TM, Mundy GR, Bonewald LF: Dual role for the latent transforming growth factor- β binding protein in storage of latent TGF- β in the extracellular matrix and as a structural matrix protein. *J Cell Biol* 1995, 131:539–549
- Robertson M, Chambers I, Rathjen P, Nichols J, Smith A: Expression of alternative forms of differentiation inhibiting activity (DIA/LIF) during murine embryogenesis and in neonatal and adult tissues. *Dev Genet* 1993, 141:165–173
- Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I: A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. *Am J Pathol* 1988, 130:393–400
- Abedi H, Zachary I: Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc Res* 1995, 30:544–556
- Leveen P, Claesson-Welsh L, Heldin CH, Westermark B, Betsholtz C: Expression of messenger RNAs for platelet-derived growth factor and its receptors in human sarcoma cell lines. *Int J Cancer* 1990, 46:1066–1070
- Nister M, Claesson-Welsh L, Eriksson A, Heldin CH, Westermark B: Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines. *J Biol Chem* 1991, 266:16755–16763
- Sariban E, Sitaras NM, Antoniades HN, Kufe DW, Pantazis P: Expression of platelet-derived growth factor (PDGF)-related transcripts and synthesis of biologically active PDGF-like proteins by human malignant epithelial cell lines. *J Clin Invest* 1988, 82:1157–1164