$\alpha 5$ and $\alpha 2$ Integrin Gene Transfers Mimic the PDGF-B–Induced Transformed Phenotype of Fibroblasts in Human Skin

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SUMMARY: Platelet-derived growth factor (PDGF)-B is a proto-oncogene capable of transforming fibroblasts. Using adenoviral vectors, we tested whether endogenous PDGF-B expression in human skin xenotransplants leads to changes in the expression of $\alpha 5$ and $\alpha 2$ integrin subunits and whether integrin overexpression leads to PDGF-related changes in the skin. In vitro, transduction of fibroblasts with PDGF-B or the integrin $\alpha 5$ subunit stimulated multilayered growth and spindle-type morphology, both markers of mesenchymal cell transformation. In vivo, PDGF-B transduction of the human dermis was associated with up-regulation of collagen and fibronectin synthesis, increases in $\alpha 5$ and $\alpha 2$ integrin subunit expression, vessel formation, and proliferation of fibroblasts, keratinocytes, and pericytes. A similar stromal response was induced when $\alpha 5$ and $\alpha 2$ integrin subunits were overexpressed in the human dermis, suggesting that integrins play a major role in the induction of a transformed phenotype of fibroblasts by PDGF-B. (*Lab Invest* 2001, 81:1263–1274).

Platelet-derived growth factor (PDGF) is a normal constituent of platelets from which it is released by degranulation of α granules at sites of injury (Ross et al, 1986). PDGF-A and PDGF-B can function as homo- or heterodimers of 28 to 35 kDa (Kohler and Lipton, 1974; Ross et al, 1974; Westermarck and Wasteson, 1976). PDGF-BB and PDGF-AB bind to the PDGF β receptor and, to a lesser extent, the PDGF α receptor, both present predominantly on mesenchymal cells. However, PDGF-AA homodimers bind only to the PDGF α receptor (Hart et al, 1988; Heldin et al, 1988). PDGF-B exerts a mitogenic effect on fibroblasts (Kohler and Lipton, 1974) and smooth muscle cells (Ross et al, 1974), which express the PDGF β receptor and normally do not secrete PDGF-B. Secretion of PDGF-B can be induced with peptide regulatory factors, such as transforming growth factor- β (Soma and Grotendorst, 1989). Binding of PDGF-B to PDGF β induces receptor dimerization and is associated with autophosphorylation and binding of downstream modulators to the cytoplasmic domain of the receptor (Benito and Lorenzo, 1993; Bornfeldt et al,

1995). Malignant cells often express PDGF-B constitutively (Fahrer et al, 1989; Ito et al, 1990; Leveen et al, 1990; Mauro and Bulfone, 1990), and in glioma, PDGF-B is an autocrine growth factor (Mauro and Bulfone, 1990). Other tumors, such as melanomas, lack the receptor and produce the factor for stimulating normal cells in the tumor stroma (Forsberg et al, 1993).

In fibroblasts, activation of the PDGF β receptor leads to transformation (Gazit et al, 1984; Fleming et al, 1992), defined as loss of contact inhibition in monolayer cultures and anchorage-independent growth in soft agar (Fry et al, 1990). Immortalized, but nontumorigenic murine fibroblasts can form tumors in mice when transduced with the proto-oncogene c-sis (Kim et al, 1994); the PDGF-B gene shares 94% sequence homology with the avian viral oncogene, v-sis (Waterfield et al, 1983), further underscoring the potential for fibroblast transformation by PDGF-B (Benito and Lorenzo, 1993; Huang et al, 1984; Leal et al, 1985; Valgeirsdottir et al, 1995).

Besides its growth-stimulating activity, PDGF-B up-regulates expression of $\alpha 5$ and $\alpha 2$ integrin subunits, which heterodimerize with the $\beta 1$ integrin subunit (Ahlen and Rubin, 1994; Gailit et al, 1996). The $\alpha 5\beta 1$ integrin is the major fibronectin receptor present on most cells; it mediates adhesion, migration, assembly of extracellular matrix, and signal transduction (Greiling and Clark, 1997; Wu et al, 1993), which can induce gene expression and affect transit through the cell cycle (Juliano and Varner, 1993). The induction of $\alpha 5$

Received June 6, 2001.

MN and HS contributed equally to the study. This study was supported in part by National Institutes of Health (NIH) Grants CA25874, CA76674, CA74294, DK50306, and CA10815. HS was supported by the Austrian Science Foundation (FWF-1652-Med) and the Max Kade Foundation. Address reprint requests to: Dr. Meenhard Herlyn, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. E-mail: HerlynM@Wistar.Upern.edu

integrin monomer expression may be involved in the regulation of tumor cell growth and differentiation, provided that $\beta 1$ integrin subunit is available. In colon carcinomas, overexpression of $\alpha 5$ integrin subunit leads to increased tumorigenicity (Gong et al, 1997) when fibronectin is present. In the absence of fibronectin, $\alpha 5\beta 1$ integrin expression induces a negative growth signal that can only be overcome when cells are provided with fibronectin (Allen-Hoffmann et al, 1990; Juliano and Varner, 1993; Varner et al, 1995).

PDGF-B increases production of fibronectin in cells (Allen-Hoffmann et al, 1990) and enhances collagen synthesis as part of the wound-healing response (Pierce et al, 1995). We hypothesized that the phenotype induced in vivo by PDGF-B in fibroblasts is due to the up-regulation of integrin $\alpha 5$ and $\alpha 2$ subunits, which, in turn, increase fibronectin and collagen production. We further questioned whether the induced changes by PDGF-B in human skin can be partially mimicked by infection with integrin $\alpha 5$ or $\alpha 2$ subunits. To study the effects of locally overproduced human PDGF-B and integrins $\alpha 5$ and $\alpha 2$, we transfected human fibroblasts with adenoviral vectors carrying the PDGF-B, $\alpha 5$, or $\alpha 2$ genes (PDGF-B/Ad5, $\alpha 5$ /Ad5, and $\alpha 2$ /Ad5, respectively). Replication-defective adenoviruses infect proliferating and nonproliferating cells and induce gene expression with high efficiency (Kozarsky and Wilson, 1993). The biological effects of PDGF-B, $\alpha 5$, and $\alpha 2$ integrin subunit overexpression were analyzed in human skin grafted to immunodeficient mice. The grafted skin maintains its human-specific architecture throughout the lifetime of the animals and is well-suited for studies of human skin cell transformation (Juhász et al, 1993a; Soballe et al, 1996). Our results demonstrate that PDGF-B transduction of fibroblasts induces an increase in fibronectin and collagen, leads to up-regulation of $\alpha 5$ and $\alpha 2$ integrin subunits, increases vessel formation and the numbers of undifferentiated keratinocytes in the epidermis as well as pericytes. A transformed phenotype, ie, loss of contact inhibited growth, was also observed in fibroblasts overexpressing $\alpha 5$ in culture. Those cells showed up-regulated production of fibronectin, which was mostly pronounced in human skin grafts compared to cultured cells. Infection of fibroblasts in human skin xenotransplants with either integrin $\alpha 5$ or $\alpha 2$ subunits also enhanced fibronectin and collagen production. These changes resembled the transformed phenotype after PDGF-B infection, except that there was less growth stimulation. Our in vitro experiments with transduced fibroblasts demonstrate that both PDGF-B and $\alpha 5$ integrin enhance survival of fibroblasts.

Results

In Vitro and In Vivo Expression of PDGF after Transduction with PDGF-B/Ad5

Western blot analysis of lysates from primary human fibroblasts transduced with a replication-deficient adenoviral vector carrying the PDGF-B gene (PDGF-B/

Ad5) revealed a 36 kDa band representing the PDGF-BB homodimer and several bands of precursor proteins, whereas *LacZ* transduced or nontransduced fibroblasts showed neither the mature nor precursor bands (Fig. 1A). Western analysis of lysates of fibroblasts transduced with PDGF, $\alpha 5$, or *LacZ* revealed high levels of $\alpha 5$ integrin in $\alpha 5$ -transduced cells, lower levels in PDGF-B-transduced cells, and very little $\alpha 5$ in *LacZ*- or nontransduced cells (data not shown). Beta1 integrin was present at high levels independent of adenoviral transduction. Mitogenic activity in the culture supernatant of mouse fibroblasts was abrogated by addition of anti-PDGF-BB antibodies, suggesting that most of the activity in the supernatant was due to PDGF-BB. Overexpression of PDGF-B in human fibroblasts was mitogenic and stimulated [3 H]-thymidine incorporation significantly, demonstrating the biological activity of adenovirus-induced PDGF-BB secretion (data not shown). Extractions of human skin injected 3 days earlier with PDGF-B/Ad5 revealed that two isoforms of PDGF, PDGF-AA and PDGF-AB/BB, were more highly expressed compared with skin injected with PBS or *LacZ*/Ad5 (Fig. 1B). Series of bands of precursor proteins, down to 30 kDa, were detected for both PDGF-AA and PDGF-AB/BB in the lysates. The lowest bands for both isoforms are shown in Figure 1B, accounting for about 32 kDa for PDGF-AA and 34 kDa for PDGF-AB/BB. Seven days after injection there was still a higher production of PDGF-AA and PDGF-AB/BB after PDGF-B/Ad5 injection compared with *LacZ*/Ad5. The increase in expression of PDGF-BB was confirmed by ELISA, in which a significant increase after 3 and 7 days was observed ($p < 0.05$; Fig. 1C).

Human Skin Remodeling by Intradermal PDGF-B Transduction

Human skin xenografts injected once with PDGF-B/Ad5 became raised, with increased soft tissue growth after 3 days (Fig. 2A, top), compared with their appearance after injection with *LacZ*/Ad5 (Fig. 2A, bottom) or before injection (Fig. 2B). Histological analysis of hematoxylin and eosin (H&E)-stained sections revealed dermal hypercellularity and irregular epidermal hyperplasia 3 days after PDGF-B/Ad5 injection (Fig. 2C), which was minimal in sections from the *LacZ*/Ad5 control group (Fig. 2D). After 3 days, PDGF-B/Ad5-injected grafts showed intense expression of PDGF-B in the dermis, with no expression in the epidermis (Fig. 2E). In contrast, PDGF-B could not be detected in *LacZ*/Ad5 (Fig. 2F) or in $\alpha 5$ /Ad5-injected skin (data not shown). Injection with $\alpha 5$ /Ad5 integrin led to phenotypically similar changes as PDGF-B/Ad5. Alpha 5 integrin was found to be expressed in the dermis (Fig. 3F).

Comparison of Changes in Human Skin after Transduction with Adenoviral Vectors for $\alpha 5$ and $\alpha 2$ Integrin Subunits and Those Resulting from PDGF-B Overexpression

Seven days after transduction, immunostaining of human skin grafts confirmed the morphological re-

Figure 1.

Production of platelet-derived growth factor (PDGF) isoforms in vitro and in vivo after transduction with PDGF-B using an adenoviral vector. **A**, Western blot analysis of PDGF-BB protein in lysates of fibroblasts (FF2030) transduced with either PDGF-B, *LacZ*, or nontransduced PBS. Equal quantities of lysate (100 μ g) were electrophoresed in 15% polyacrylamide gels under nonreducing conditions and incubated with polyclonal goat anti-human PDGF-BB antibody. **B**, Western blot analysis of extracts of human skin injected 3 or 7 days earlier with PDGF-B/Ad5, *LacZ*/Ad5 at 5×10^8 plaque-forming units (pfu)/cell, or PBS. Equal quantities of protein (50 μ g) were loaded onto 15% gels under nonreducing conditions and incubated with a monoclonal mouse anti-human PDGF-BB or polyclonal goat anti-human PDGF-AA antibody at 2 μ g/ml. Blots were reprobed with anti- β -actin antibody. Lane 1, Lysates of fibroblasts (FF2030) transduced with PDGF-B at Day 2; Lane 2, human skin injected with PBS at Day 3; Lane 3, human skin injected with *LacZ* at Day 3; Lane 4, human skin injected with PDGF-B at Day 3; Lane 5, human skin injected with *LacZ* at Day 7; Lane 6, human skin injected with PDGF-B at Day 7. **C**, PDGF-BB levels in human skin extracts after injection with PDGF-B/Ad5, *LacZ*/Ad5 at 5×10^8 pfu/cell, or PBS. Levels were determined by ELISA. Data are given as mean $\pm$ so. * $p < 0.05$ by Student's *t* test.

modeling induced by PDGF-B, $\alpha 5$, or $\alpha 2$ integrin subunits, compared with *LacZ* injected skin (Fig. 3). The dermis showed strong and diffuse expression of fibronectin after PDGF-B and $\alpha 5$ integrin injection (Fig. 3, A and B). A slight increase in fibronectin was also found in skin after $\alpha 2$ integrin injection (Fig. 3C), which was in contrast to sections of skin injected with *LacZ*/Ad5 (Fig. 3D). Expression of the $\alpha 5$ integrin subunit was up-regulated by PDGF-B (Fig. 3E), and a strong, but more focal expression of $\alpha 5$ integrin was found after injection with $\alpha 5$ (Fig. 3F). Injection with

either $\alpha 2$ integrin or *LacZ* did not show a substantial expression of $\alpha 5$ (Fig. 3, G and H).

Three days after intradermal injection with either PDGF-B or $\alpha 2$ integrin adenoviral vectors, massive collagen deposition was visualized by Masson's trichrome (Fig. 4, A and C, respectively), but only a slight increase in production of collagen was detected after injection with $\alpha 5$ (Fig. 4B) compared with *LacZ* (Fig. 4D). Strong positive, focal staining for $\alpha 2$ integrin subunit was observed in the dermis after injection of PDGF-B and $\alpha 2$ integrin adenoviral vectors (Fig. 4, E

Figure 2.

Changes in human skin grafted to recombination activating gene 1 (RAG-1) mice after PDGF-B/Ad5 injection. A, Human skin grafts in mice 3 days after intradermal injection of PDGF-B/Ad5 (top) or *LacZ*/Ad5 (bottom); B, human skin graft before injection; C, hematoxylin and eosin (H&E)-stained section of human skin 3 days after injection of PDGF-B/Ad5 showing epidermal hyperplasia and a mixed infiltrate of neutrophils and mononuclear cells ($\times 10$, bar = 200 μm); D, *LacZ*/Ad5-injected human skin showing minimal epidermal hyperplasia and sparse inflammation ($\times 10$, bar = 200 μm); E, immunohistochemical staining using rabbit anti-PDGF-BB antibodies 3 days after PDGF-B/Ad5 injection of human skin graft shows widespread expression in dermis but not in epidermis ($\times 20$); F, staining for PDGF-B in human skin graft section after injection of *LacZ*/Ad5 reveals no PDGF-BB expression ($\times 20$).

and G, respectively), compared with *LacZ* (Fig. 4H). No increase in positive staining for $\alpha 2$ integrin was found after $\alpha 5$ integrin injection (Fig. 4F).

Further changes in skin morphology after PDGF-B, $\alpha 5$, and $\alpha 2$ integrin subunit transduction are shown in Figure 5. Staining for chondroitin sulfate proteoglycan, a marker of vascular smooth muscle cells and pericytes, showed perivascular positivity and dermal focal positive staining. Compared with *LacZ* (Fig. 5D), an increase in positive staining was observed after injection with PDGF-B (Fig. 5A), $\alpha 5$ (Fig. 5B), and $\alpha 2$ (Fig. 5C) integrin subunits ($\times 80$). Staining for CD31 (PECAM-1), an endothelial cell marker, demonstrated the strong angiogenic effect of PDGF-B-injected (Fig. 5E) compared with *LacZ*-injected skin (Fig. 5F). PDGF-B transduction also led to an increase in undifferentiated keratinocytes (Fig. 4, G and H). Staining for cytokeratins 5 and 8 showed multiple layers of undifferentiated keratinocytes in the basal layer in PDGF-B-injected skin (Fig. 5G) compared with *LacZ*/Ad5-transduced human skin, which had a single layer of undifferentiated keratinocytes in the basal layer (Fig. 4H). Staining for Mac-1 (mouse CD11b), a marker for mouse macrophages, did not show any differences in infiltration between *LacZ*/Ad5- and PDGF-B/Ad5-, $\alpha 5$ /Ad5-, or $\alpha 2$ /Ad5-injected skin. There was no overall significant increase in inflammatory cells compared with PBS-injected human skin.

Changes in Morphology In Vitro after Transduction with PDGF-B/Ad5 or $\alpha 5$ /Ad5 and Enhanced Survival of Human Fibroblasts In Vivo

Normal human fibroblasts transduced with PDGF-B had spindle morphology and lost contact inhibition by growing in multiple layers (Fig. 6A), whereas control cultures transduced with *LacZ* (Fig. 6B) or nontransduced (Fig. 6D) showed a flat morphology and remained contact-inhibited. Like PDGF-B-transduced cultures, fibroblasts transduced with $\alpha 5$ integrin subunit grew in multiple layers (Fig. 6C). Fibroblasts overexpressing PDGF-B grew anchorage independently in soft agar at up to 10% colony-forming efficiency ($p < 0.05$); colonies contained 20 or more cells (not shown). In contrast nontransduced or $\alpha 5$ -transduced fibroblasts showed no significant colony formation. Small colonies of four cells at 1% to 2% efficiency were seen in cells transduced with *LacZ*. PDGF-B-induced proliferation of transduced fibroblasts was significantly inhibited by using a monoclonal antibody (MAb) against $\alpha 5$ (10 $\mu\text{g}/\text{ml}$) (not shown), as measured by [^3H]-thymidine incorporation. Marginal inhibition was achieved in fibroblasts transduced with *LacZ* or nontransduced. No inhibition of proliferation was observed in PDGF-B-transduced fibroblasts when incubated with control IgG (100 $\mu\text{g}/\text{ml}$) (not shown).

Figure 3.

Injection of PDGF-B, $\alpha 5$, and $\alpha 2$ integrin subunits with adenoviral vectors induces similar changes in expression of fibronectin and its receptor. Human skin grafts were injected with either PDGF-B (A and E), $\alpha 5$ integrin (B and F), $\alpha 2$ integrin subunit (C and G), or *LacZ* (D and H). The skin grafts were stained 3 days later for fibronectin (A to D) or $\alpha 5$ integrin (E to H). Magnification, $\times 80$.

Figure 4.

Injection of PDGF-B, $\alpha 5$, and $\alpha 2$ integrin subunits with adenoviral vectors induces similar changes in expression of collagen and its receptor. Human skin grafts were injected with either PDGF-B (A and E), $\alpha 5$ integrin (B and F), $\alpha 2$ integrin (C and G), or *LacZ* (D and H). The skin grafts were stained 3 days later for collagen visualized with Masson's trichrome (A to D) or $\alpha 2$ integrin (E to H). Magnification, $\times 80$.

Human dermal fibroblasts, overexpressing PDGF-B and injected into severe combined immunodeficiency (SCID) mice, remained viable for at least 21 days, and lesions developed their own vasculature (Fig. 6E). After 35 days, subcutaneous lesions were no longer palpable, and after 60 days, all human cells were gone at the injection site. In contrast, fibroblasts that were transduced with *LacZ* 24 hours earlier or nontransduced underwent massive necrosis within 3 days (Fig. 6F), and by Day 5, only cell debris was detected. A similar prolonged survival as for PDGF-B was observed for fibroblasts transduced before injection with $\alpha 5$ /Ad5 (shown in Fig. 6G after 10 days). The lesions were surrounded by stroma and started to disappear after 2 to 3 weeks. In both types of lesions, nuclear staining with Hoechst dye showed that the center of the lesion contained human cells mixed with a few

murine cells, as indicated by a uniform staining pattern of human rather than mouse cells.

Discussion

Endogenous overexpression of PDGF-B by adenoviral vectors induces multiple biological responses, both in vitro in human fibroblasts and in vivo in human skin. These effects include stimulation of fibroblast growth in vitro and in vivo, induction of matrix protein production, paracrine stimulation of epidermal keratinocytes, and prolonged survival of fibroblasts after in vivo injection.

PDGF-B/Ad5 transduction of human fibroblasts stimulated anchorage-independent growth in soft agar, loss of contact inhibition, and a spindle-type morphology, which are all known markers of mesen-

Figure 5.

Immunohistochemical staining for chondroitin sulfate proteoglycan (HMW-MAA), CD31 (PECAM-1), and cytokeratins 5 and 8 in human skin 7 days after injection with adenoviral vectors. Immunostaining of pericytes and vascular smooth muscle cells using an antibody against chondroitin sulfate proteoglycan after overexpression with PDGF-B (A), $\alpha 5$ integrin subunit (B), $\alpha 2$ integrin subunit (C), or *LacZ*/Ad5 (D). Staining for endothelial cells with an antibody against human CD31 (PECAM-1) after PDGF-B (E) or *LacZ* (F) overexpression; staining for undifferentiated keratinocytes with antibody against cytokeratins 5 and 8 after PDGF (G) or *LacZ* (H) overexpression. Note the multilayered expression of cytokeratins 5 and 8 indicating undifferentiated keratinocytes in the suprabasal layers. Magnification, $\times 80$.

chymal cell transformation. Loss of contact inhibition in vitro and spindle-type morphology was also achieved by overexpression of $\alpha 5$ integrin, whereas in soft agar no anchorage-independent growth was observed. The loss of $\alpha 1$ integrin and the increase in $\alpha 5$ integrin expression have been demonstrated in vitro in response to exogenous PDGF-B (Gailit et al, 1996).

Mitogenic stimulation of smooth muscle cells by PDGF-B further suggests that $\alpha 5\beta 1$ integrins regulate growth through a pathway that is distinct from the growth factor-activated MAP kinase pathway (Hedin et al, 1997).

The remodeling of human dermis after PDGF-B transduction was similar to changes seen in the early

Figure 6.

Morphologic changes in human fibroblasts overexpressing PDGF-B or $\alpha 5$ integrin and histology of lesions in immunodeficient mice injected subcutaneously with PDGF-B or $\alpha 5$ -transduced human fibroblasts. A, Changes in growth of human fibroblasts FF2030 after transduction with PDGF-B or $\alpha 5$. Cells were transduced with PDGF-B (20 pfu/cell) and maintained for 10 days before fixation and staining with Diff-Quick, or transduced with *LacZ* (B), $\alpha 5$ integrin subunit (C), or not transduced (D). For in vivo experiments, FF2030 cells were transduced at 20 pfu/cell with adenoviral vectors and 24 hours later injected sc at 1×10^7 /mouse. E, H&E staining of a tissue section through the area of PDGF-B-transduced fibroblasts 21 days after injection; F, *LacZ*-transduced fibroblasts 3 days postinjection; G, $\alpha 5$ /Ad5-transduced fibroblasts 10 days postinjection. Magnification, $\times 10$.

stages of wound healing, which are also associated with $\alpha 5$ integrin and fibronectin up-regulation (Juhász et al, 1993b). The increased production of matrix proteins and $\alpha 5$ integrin could be due to the release of PDGF during the initial stages of wound healing to accelerate re-epithelization and scar tissue formation (Juhász et al, 1993b). Expression of $\alpha 5$ integrin after adenoviral injection with $\alpha 5$ /Ad5 did not induce $\alpha 2$ integrin expression or vice versa, whereas PDGF-B induced both integrin subunits. Concomitant expression and, subsequently, changes in extracellular matrices may be the reason for the strong effects

observed with PDGF-B. In a recent study, the induction of vascular endothelial growth factor (VEGF) in pericyte-like cells after PDGF-BB stimulation has been described (Reinmuth et al, 2001). VEGF, in turn, is beneficial for the survival of endothelial cells. In this study, we found, by immunostaining for CD31 (PECAM-1), an increase in expression of chondroitin-sulfate proteoglycan, a marker for pericytes and vascular smooth muscle cells, as well as a dramatic increase in vessel formation, thus pinpointing the importance of PDGF-BB for modulating angiogenesis. Paracrine PDGF may also contribute to angiogenesis

by increasing PDGFR- β mRNA in proliferating endothelial cells (Battagay et al, 1994).

In a recent study, we used the adenoviral-mediated gene transfer of PDGF-B to investigate the potential usefulness of this approach in wounds of diabetic mice (Y Kitano, M Nesbit, M Herlyn, A Radu, KG Sylvester, NS Adzick, and TM Crombleholme, unpublished data). We found a significant enhancement in re-epithelialization, which was due to an induced accumulation of fibroblasts and endothelial cells. Extraction of PDGF from human skin after injection with PDGF-B/Ad5 in this study demonstrated that PDGF isoforms are indeed expressed at higher levels than in *LacZ*/Ad5-transduced samples. Further, we have shown that the adenoviral transfer of PDGF-B not only induced PDGF-A, but also PDGF-R α expression in fibroblasts, which is in accordance with a previous report about the induction of native PDGF-A by exogenous PDGF-B (Pierce et al, 1995). Therefore, fibroblasts may amplify the effects of PDGF-B through PDGF receptors expressed on these cells. This process of receptor induction through continuous PDGF-B expression may also contribute to the in vivo changes observed in this study.

PDGF-B gene transfer in human skin led to a strong stimulatory effect of epidermal keratinocytes, which was already seen after 3 days. Keratinocytes do not express the receptor for PDGF-B and must be stimulated by other growth factors, possibly by the mitogen keratinocyte growth factor (Marchese et al, 1995) produced by PDGF-B-activated fibroblasts. In a preliminary study, where wounds of diabetic mice were treated with a PDGF-B adenoviral vector, we observed an increase in TGF- α , which stimulates keratinocyte proliferation and migration. In this study, staining for undifferentiated keratinocytes with cytokeratins 5 and 8 at the Stratum basale revealed a dramatic increase in PDGF/Ad5-transduced human skin. PDGF-B transduction also leads to up-regulation of inflammatory factors. For example, monocyte chemoattractant protein-1 is up-regulated (Yoshimura and Leonard, 1990), explaining the increased infiltration of mononuclear cells in grafts injected with PDGF-B/Ad5 when compared with grafts injected with the control vector. Also in this study, we found accumulation of murine macrophages in lesions of fibroblasts transduced with either PDGF-B or $\alpha 5$. However, in human skin grafts injected with the virus there were no changes regarding the infiltration of perilesional murine macrophages.

The paracrine stimulatory effects observed after PDGF-B transduction were not detected when $\alpha 5$ integrin/Ad5 was injected into the grafts, suggesting that the up-regulation of the adhesion receptor does not lead to increased production of growth factors. However, like PDGF-B, $\alpha 5$ injection led to an increase in fibronectin production, which was more clearly detectable in vivo in human skin than in cultured fibroblasts where serum-induced fibronectin production (not shown) obscured significant construct-induced increases. Likewise, $\alpha 2$ injection led to an increase in collagen synthesis. Up-regulation of collagen gene transcription by integrin $\alpha 2\beta 1$ has been

shown recently (Ivaska et al, 1999) and involved the $\alpha 2$ cytoplasmic tail. The increased survival of fibroblasts after injection in vivo could be due to the expression of $\alpha 5\beta 1$ integrin in fibroblasts through PDGF-B or $\alpha 5$ transduction and the increased production of fibronectin. Thus, fibronectin may promote survival of fibroblasts in vivo and contribute to a lack of contact inhibition in vitro.

Long-term transforming effects of PDGF-B on human fibroblasts were not evident when the viral vector was directly injected into human skin, or when human fibroblasts were transduced in vivo followed by injection into SCID mice, suggesting that PDGF-B expression from an adenovirus does not lead to malignant transformation. The episomal nature of the adenovirus-mediated gene transfer does not allow for stable integration of the PDGF-B gene into the fibroblast genome (Kozarsky and Wilson, 1993). This temporal expression of PDGF-B from an adenovirus and the effects elicited in vivo reflect the transient nature of the transformed phenotype observed in this study in vitro and in vivo.

Materials and Methods

Construction of Adenoviral Vectors for PDGF-B and $\alpha 5$ Integrin Subunit

A 1.2 kbp *Pst*I/*Eco*RI cDNA fragment, encoding the entire open reading frame of PDGF-B (Dr. B. Westermark, University Hospital, Uppsala, Sweden), was subcloned into the *Not*I-modified plasmid pSL301 (Vector Core, University of Pennsylvania, Philadelphia, Pennsylvania). The *Not*I fragments containing the PDGF-B open reading frame were cloned into the unique *Not*I site of the pAdCMV vector pSL301. A 3.7 kbp *Sal*I-*Xba*I cDNA fragment of $\alpha 5$ integrin was cut from pECE (Dr. Erkki Ruoslahti, Burnham Institute, LaJolla, California) and ligated into *Xho*I-*Spe*I-cut pAdCMV-Link.1. Plasmids containing the PDGF-B or $\alpha 5$ integrin gene were cotransfected separately into human 293 cells (Rosenfeld et al, 1991) along with *Cla*I-digested dL7001 adenoviral genome (Ranheim et al, 1993), in which both the E1 and E3 genes were deleted. Plaques were expanded in 293 cells and adenoviral DNA was isolated (Hirt, 1967) and digested with *Bam*HI to determine the presence of the PDGF-B or $\alpha 5$ integrin gene by Southern blotting. Positive plaques were purified by two rounds of limited dilution cloning. Production of PDGF-B and $\alpha 5$ integrin proteins by fibroblasts transduced with PDGF-B/Ad5 and $\alpha 5$ /Ad5, respectively, was confirmed by Western blotting. After bulk production in 293 cells, viral vectors were purified over cesium chloride step gradients by ultracentrifugation (Rosenfeld et al, 1991), dialyzed against PBS, titrated for plaque-forming units (pfu), and stored in 10% glycerol at -80°C . For gene transduction, cells were infected at a multiplicity of infection of 20:1. *LacZ*/Ad5 (Vector Core, University of Pennsylvania) induces expression of the reporter gene β -galactosidase and was used as control in each experiment.

Cell Lines and Growth Assays

Normal diploid human fibroblast cell lines were established from the dermis of human newborn foreskin (Cornil et al, 1991) and maintained in DMEM (Gibco/BRL, St. Louis, Missouri) supplemented with 10% heat-inactivated FCS. NIH3T3 murine fibroblasts for growth stimulation assays and human 293 cells for adenovirus replication were also cultured in DMEM with 10% FCS. WM239A melanoma cells were maintained in MCDB 153 with 20% L-15 medium and supplemented with 2 mM Ca^{2+} , 2% FCS, and 5 $\mu\text{g}/\text{ml}$ insulin.

[^3H]Thymidine incorporation into murine and human fibroblasts was measured by incubating cells in 96-well microtiter plates for 24 hours with 1 $\mu\text{Ci}/\text{well}$. All data represent the mean of quadruplicate wells, and all assays were repeated twice. [^3H]-Thymidine incorporation was performed the same way as mentioned above to demonstrate inhibition of PDGF-B-transduced fibroblast growth, except that 96-well microtiter plates were coated with fibronectin dissolved in PBS (pH 7.5) at 10 $\mu\text{g}/\text{ml}$ for 15 hours at 4° C. Fibroblasts were seeded and incubated with a human MAb against $\alpha 5$ (10 $\mu\text{g}/\text{ml}$, PharMingen, San Diego, California) or unspecific human IgG MAb (100 $\mu\text{g}/\text{ml}$) for 6 hours. To test for anchorage-independent growth in soft agar, 3×10^4 cells were resuspended in 2 ml medium containing 0.3% Noble agar in 2% FCS and DMEM and seeded in 6-well plates coated with 0.5% agar in growth medium. Fresh overlay was added after 5 days, and the colony-forming efficiency was calculated based on the number of colonies of eight or more cells after 10 days. For in vivo growth studies, 1×10^7 cells were injected subcutaneously into SCID mice per group. Mice were sacrificed 3, 10, 14, 21, or 60 days later to determine viability of the injected cells.

Immunoblotting and ELISA

Cell lysates (100 μg) from transduced fibroblasts or WM239A cells were subjected to electrophoresis in 15% polyacrylamide gels, loading equal amounts of lysates onto gels (50 μg per slot). Gels were electroblotted to polyvinylidene fluoride (PVDF) membranes, and PDGF-BB or $\alpha 5$ integrin subunit was detected using goat anti-PDGF-BB (R&D Systems, Minneapolis, Minnesota) or anti- $\alpha 5$ integrin subunit MAbs (Transduction Labs., Lexington, Kentucky), respectively. Human skin, previously grafted onto SCID mice, was excised, placed on ice, and minced in ice-cold modified RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 5 mM EGTA; 0.1% SDS; 1% sodium deoxycholate; 1% NP-40; 0.5 mM sodium fluoride; and 100 μM sodium orthovanadate). Equal amounts of lysates were loaded onto 15% polyacrylamide gels. After protein transfer to PVDF membranes, PDGF-AA or PDGF-AB/BB were detected using rabbit anti-human PDGF-AA (Santa Cruz, Biotechnology, Inc., Santa Cruz, California) or anti-human PDGF-AB/BB (PharMingen), respectively. Membranes were probed for β -actin expression with anti- β -actin (SIGMA, St.

Louis, Missouri). Levels of PDGF-BB in human skin lysates were measured using an ELISA kit (Quantikine; R&D Systems).

Human Skin Grafting and Immunohistochemistry

Human neonatal foreskins were used for grafting within 48 hours after removal. At ages 8 to 12 weeks, recombination-activating gene 1 (RAG-1) (RAG-1/RAG-1) or SCID mice, bred at the Wistar Institute Animal Facility (Philadelphia, Pennsylvania), were anesthetized and 1- to 3- cm^2 full-thickness cutaneous defects were created on the dorsal torso. Full-thickness human skin was sutured in place, and mice were used for experiments after 4 to 6 weeks when grafts were well healed. Xenografts were then injected intradermally with adenoviral vector at 5×10^8 pfu in a total volume of 100 μl PBS. Six mice were used for each group and killed at Days 3 and 7 for histological, histochemical, and immunohistochemical analyses of grafts.

Skin grafts were dissected free from surrounding murine tissue and bisected perpendicular to the long axis of the graft. Half of the graft was fixed overnight in 10% neutral buffered formalin at room temperature and paraffin-embedded. Serial sections were stained with H&E for pathohistological evaluation. Hoechst dye was used for nuclear staining to distinguish murine from human cells. The other half of each graft was embedded in OCT medium (Miles, Elkhart, Indiana), snap-frozen in liquid nitrogen, and cryosectioned at 5 μm .

Immunohistochemistry was performed on serial cryosections using an avidin-biotin-peroxidase complex system (Vector Lab., Burlingame, California) and 3,3'-diaminobenzidine as chromagen. Tissue sections were fixed in acetone for 10 minutes at 4° C and incubated with primary antibodies in a humidified chamber overnight at 4° C. Antibodies used in the study include goat anti-human PDGF-BB (R&D Systems), anti-human $\alpha 2$ integrin subunit (PharMingen), anti-human CD31 (PECAM-1) (PharMingen), anti-human cytokeratins 5 and 8 (Zymed, San Francisco, California), anti-mouse CD11b (Mac-1 α chain) (PharMingen), anti-human $\alpha 5$ integrin subunit (Transduction Labs), anti-human fibronectin (Novacastra, Newcastle, United Kingdom), and mouse antibodies IgG1, which recognize a chondroitin sulfate proteoglycan found on collagenous connective tissue, vascular smooth muscle cells, and pericytes (Ross et al, 1983). After rinsing with PBS, sections were overlaid with biotinylated anti-mouse, -rabbit, or -goat IgG for 30 minutes at room temperature followed by staining.

Acknowledgement

We thank the Editorial Department at the Wistar Institute (Philadelphia, Pennsylvania) for their help and assistance in preparing this manuscript.

References

- Ahlen K and Rubin K (1994). Platelet-derived growth factor-BB stimulates synthesis of the integrin $\alpha 2$ -subunit in human diploid fibroblasts. *Exp Cell Res* 215:347–353.
- Allen-Hoffmann BL, Schlosser SJ, Brondyk WH, and Fahl WE (1990). Fibronectin levels are enhanced in human fibroblasts overexpressing the c-sis proto-oncogene. *J Biol Chem* 265: 5219–5225.
- Battegay EJ, Rupp J, Iruela-Arispe L, Sage EH, and Pech M (1994). PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *J Cell Biol* 125:917–928.
- Benito M and Lorenzo M (1993). Platelet derived growth factor/tyrosine kinase receptor mediated proliferation. *Growth Regul* 3:172–179.
- Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG, and Ross R (1995). Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. *Ann NY Acad Sci* 766:416–430.
- Cornil I, Theodorescu D, Man S, Herlyn M, Jambrosic J, and Kerbel RS (1991). Fibroblast cell interactions with human melanoma cells affect tumor cell growth as a function of tumor progression. *Proc Natl Acad Sci USA* 88:6028–6032.
- Fahrer C, Brachmann R, and von der Helm K (1989) Expression of c-sis and other cellular proto-oncogenes in human sarcoma cell lines and biopsies. *Int J Cancer* 44:652–657.
- Fleming TP, Matsui T, and Aaronson SA (1992). Platelet-derived growth factor (PDGF) receptor activation in cell transformation and human malignancy. *Exp Gerontol* 27: 523–532.
- Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M, and Westermark B (1993). Platelet-derived growth factor (PDGF) in oncogenesis: Development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proc Natl Acad Sci USA* 90:393–397.
- Fry DG, Milam LD, Dillberger JE, Maher VM, and McCormick JJ (1990). Malignant transformation of an infinite life span human fibroblast cell strain by transfection with v-Ki-ras. *Oncogene* 5:1415–1418.
- Gailit J, Xu J, Bueller H, and Clark RA (1996). Platelet-derived growth factor and inflammatory cytokines have differential effects on the expression of integrins $\alpha 1 \beta 1$ and $\alpha 5 \beta 1$ by human dermal fibroblasts in vitro. *J Cell Physiol* 169:281–289.
- Gazit A, Igarashi H, Chiu IM, Srinivasan A, Yaniv A, Tronick SR, Robbins KC, and Aaronson SA (1984). Expression of the normal human sis/PDGF-2 coding sequence induces cellular transformation. *Cell* 39:89–97.
- Gong J, Wang D, Sun L, Zborowska E, Willson JK, and Brattain MG (1997). Role of $\alpha 5 \beta 1$ integrin in determining malignant properties of colon carcinoma cells. *Cell Growth Differ* 8:83–90.
- Greiling D and Clark RA (1997). Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *J Cell Sci* 110:861–870.
- Hart CE, Forstrom JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, and Bowen-Pope DF (1988). Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* 240:1529–1531.
- Hedin UL, Daum G, and Clowes AW (1997). Disruption of integrin $\alpha 5 \beta 1$ signaling does not impair PDGF-BB-mediated stimulation of the extracellular signal-regulated kinase pathway in smooth muscle cells. *J Cell Physiol* 172:109–116.
- Heldin CH, Backstrom G, Ostman A, Hammacher A, Ronnstrand L, Rubin K, Nister M, and Westermark B (1988). Binding of different dimeric forms of PDGF to human fibroblasts: Evidence for two separate receptor types. *EMBO J* 7:1387–1393.
- Hirt B (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* 26:365–369.
- Huang JS, Huang SS, and Deuel TF (1984). Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. *Cell* 39:79–87.
- Ito M, Yoshida K, Kyo E, Ayhan A, Nakayama H, Yasui W, Ito H, and Tahara E (1990). Expression of several growth factors and their receptor genes in human colon carcinomas. *Virchows Archiv B Cell Pathol Incl Mol Pathol* 59:173–178.
- Ivaska J, Reunanen H, Westermarck J, Koivisto L, Kahari VM, and Heino J (1999). Integrin $\alpha 2 \beta 1$ mediates isoform-specific activation of p38 and upregulation of collagen gene transcription by a mechanism involving the $\alpha 2$ cytoplasmic tail. *J Cell Biol* 147:401–416.
- Juhasz I, Albelda SM, Elder DE, Murphy GF, Adachi K, Herlyn D, Valyi-Nagy IT, and Herlyn M (1993a). Growth and invasion of human melanomas in human skin grafted to immunodeficient mice. *Am J Pathol* 143:528–537.
- Juhasz I, Murphy GF, Yan HC, Herlyn M, and Albelda SM (1993b). Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous human wound healing in vivo. *Am J Pathol* 143: 1458–1469.
- Juliano RL and Varner JA (1993). Adhesion molecules in cancer: The role of integrins. *Curr Opin Cell Biol* 5:812–818.
- Kim HR, Upadhyay S, Korsmeyer S, and Deuel TF (1994). Platelet-derived growth factor (PDGF) B and A homodimers transform murine fibroblasts depending on the genetic background of the cell. *J Biol Chem* 269:30604–30608.
- Kohler N and Lipton A (1974). Platelets as a source of fibroblast growth-promoting activity. *Exp Cell Res* 87:297–301.
- Kozarsky KF and Wilson JM (1993). Gene therapy: Adenovirus vectors. *Curr Opin Genet Dev* 3:499–503.
- Leal F, Williams LT, Robbins KC, and Aaronson SA (1985). Evidence that the v-sis gene product transforms by interaction with the receptor for platelet-derived growth factor. *Science* 230:327–330.
- Leveen P, Claesson-Welsh L, Heldin CH, Westermark B, and Betsholtz C (1990). Expression of messenger RNAs for platelet-derived growth factor and its receptors in human sarcoma cell lines. *Int J Cancer* 46:1066–1070.
- Marchese C, Chedid M, Dirsch OR, Csaky KG, Santanelli F, Latini C, LaRochelle WJ, Torrisi MR, and Aaronson SA (1995). Modulation of keratinocyte growth factor and its receptor in reepithelializing human skin. *J Exp Med* 182: 1369–1376.
- Mauro A and Bulfone A (1990). Oncogenes and growth factors in gliomas. *J Neurosurg Sci* 34:171–173.

- Pierce GF, Tarpley JE, Tseng J, Bready J, Chang D, Kenney WC, Rudolph R, Robson MC, Berg JV, Reid P, Kaufman S, and Farrell CL (1995). Detection of platelet-derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic nonhealing wounds. *J Clin Invest* 96:1336–1350.
- Ranheim TS, Shisler J, Horton TM, Wold LJ, Gooding LR, and Wold WS (1993). Characterization of mutants within the gene for the adenovirus E3 14.7-kilodalton protein which prevents cytolysis by tumor necrosis factor. *J Virol* 67:2159–2167.
- Reinmuth N, Liu W, Jung YD, Ahmad SA, Shaheen RM, Fan F, Bucana CD, McMahon G, Gallick GE, and Ellis LM (2001). Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. *FASEB J* 15:1239–1241.
- Rosenfeld MA, Siegfried W, Yoshimura K, Yoneyama K, Fukayama M, Stier LE, Pääkkö PK, Gilardi P, Stratford-Perricaudet LD, Perricaudet M, Jallat S, Pavirani A, Lecocq JP, and Crystal RG (1991). Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo (see comments). *Science* 252:431–434.
- Ross AH, Herlyn M, Ernst CS, Guerry D, Bannicelli J, Ghrist BF, Atkinson B, and Koprowski H (1983). Immunoassay for melanoma-associated proteoglycan in the sera of patients using monoclonal and polyclonal antibodies. *Cancer Res* 44:4642–4647.
- Ross R, Glomset J, Kariya B, and Harker L (1974). A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci USA* 71:1207–1210.
- Ross R, Raines EW, and Bowen-Pope DF (1986). The biology of platelet-derived growth factor. *Cell* 46:155–169.
- Soballe PW, Montone KT, Satyamoorthy K, Nesbit M, and Herlyn M (1996). Carcinogenesis in human skin grafted to SCID mice. *Cancer Res* 56:757–764.
- Soma Y and Grotendorst GR (1989). TGF-beta stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. *J Cell Physiol* 140:246–253.
- Valgeirsdottir S, Eriksson A, Nister M, Heldin CH, Westermarck B, and Claesson-Welsh L (1995). Compartmentalization of autocrine signal transduction pathways in *Sis*-transformed NIH 3T3 cells. *J Biol Chem* 270:10161–10170.
- Varner JA, Emerson DA, and Juliano RL (1995). Integrin alpha 5 beta 1 expression negatively regulates cell growth: Reversal by attachment to fibronectin. *Mol Biol Cell* 6:725–740.
- Waterfield MD, Scrase GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermarck B, Heldin CH, Huang JS, and Deuel TF (1983). Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature* 304:35–39.
- Westermarck B and Wasteson A (1976). A platelet factor stimulating human normal glial cells. *Exp Cell Res* 98:170–174.
- Wu C, Bauer JS, Juliano RL, and McDonald JA (1993). The alpha 5 beta 1 integrin fibronectin receptor, but not the alpha 5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. *J Biol Chem* 268:21883–21888.
- Yoshimura T and Leonard EJ (1990). Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. *J Immunol* 144:2377–2383.