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Platelet-derived growth factor BB induces functional vascular anastomoses *in vivo*

(angiogenesis/arteries/growth factors/ischemia/wound healing)

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ABSTRACT **Neovascularization that generates collateral blood flow can limit the extent of tissue damage after acute ischemia caused by occlusion of the primary blood supply. The neovascular response stimulated by the BB homodimeric form of recombinant platelet-derived growth factor (PDGF-BB) was evaluated for its capacity to protect tissue from necrosis in a rat skin flap model of acutely induced ischemia. Complete survival of the tissue ensued, when the original nutritive blood supply was occluded, as early as 5 days after local PDGF-BB application, and the presence of a patent vasculature was evident compared to control flaps. To further evaluate the vascular regenerative response, PDGF-BB was injected into the muscle/connective tissue bed between the separated ends of a divided femoral artery in rats. A patent new vessel that functionally reconnected the ends of the divided artery within the original 3- to 4-mm gap was regenerated 3 weeks later in all PDGF-BB-treated limbs. In contrast, none of the paired control limbs, which received vehicle with an inactive variant of PDGF-BB, had vessel regrowth ($P < 0.001$). The absence of a sustained inflammatory response and granulation tissue suggests locally delivered PDGF-BB may directly stimulate the angiogenic phenotype in endothelial cells. These findings indicate that PDGF-BB can generate functional new blood vessels and nonsurgically anastomose severed vessels *in vivo*. This study supports the possibility of a therapeutic modality for the salvage of ischemic tissue through exogenous cytokine-induced vascular reconnection.**

Ischemia resulting from progressive atherosclerotic narrowing of the major nutritive blood supply to tissue stimulates an angiogenic response that can increase blood flow to the ischemic region by generating new collateral vascular channels and/or augmenting existing ones (1). This natural response is limited in its ability to salvage tissue from myocardial infarction, stroke, or extremity gangrene (2). Hypoxic gradients in ischemic tissue induce secretion of angiogenic factors by macrophages and other cell types and concomitant endothelial cell responses (3–7). Thus, local stimulation by cytokines and growth factors likely plays a vital role in establishing collateral nutritive blood flow to ischemic tissue. Candidate growth factors mediating ischemic-induced angiogenesis *in vivo* include basic and acidic fibroblast growth factors, vascular endothelial growth factor/vascular permeability factor (reviewed in refs. 8–11), and possibly platelet-derived growth factor (PDGF).

PDGF is a 28-kDa dimeric protein that is released from activated platelets and is mitogenic for most mesenchymally derived cells including fibroblasts and smooth muscle cells (refs. 12 and 13; reviewed in ref. 14). PDGF occurs in homo- and heterodimeric isoforms from two gene products designated A and B, which differentially activate at least two separate receptors (15, 16). It is present in several physiologic and pathologic processes that involve the formation of new blood vessels, including wound repair, embryogenesis, atherosclerosis, and tumor growth (14, 17–19). Secreted PDGF-like protein (20) and both PDGF transcripts are produced by endothelial cells (21–24) and arterial smooth muscle cells (25, 26), and receptors for PDGF are expressed by many microvascular endothelial cells and arterial smooth muscle cells (25–27). Recently, PDGF has been found to stimulate angiogenesis *in vivo*, in the chicken chorioallantoic membrane assay, in dermal repair under both ischemic and nonischemic conditions, and within the stroma of PDGF-BB-secreting tumors (28–32). *In vivo*, it is not clear whether the PDGF effect on microvascular endothelial cells is direct or indirect in these systems. Although some conflicting results have been obtained (27, 33) in several *in vitro* angiogenesis assays, PDGF-BB has been shown to directly stimulate endothelial cells (34, 35). In most assays, the BB homodimer displays stronger activity than the other two dimeric forms of PDGF. The present study sought to further evaluate the angiogenic potential of PDGF-BB using two different models that challenge the capacity for generating functional neovascularization.

MATERIALS AND METHODS

Growth Factor. The cDNA for PDGF-B was transfected into *Escherichia coli*, and PDGF-B monomers were produced by fermentation, purified to homogeneity by conventional metal ion chromatography techniques, and refolded to form dimers 119 amino acids in length (29). No endotoxin was detectable, and each lot was bioassayed prior to use (36). For the experiments using collagen films, PDGF-BB was incorporated into soluble type I collagen films as described (36, 37). *In vivo*, the collagen film degraded within 24 hr and provides a relative low-dose, long-term delivery of the growth factor. For the experiments using the PDGF-BB in solution, the factor was diluted in PBS to a concentration of 500 μg/ml. This formulation was used for the administration of a relatively high single-dose administration. The mutagenized PDGF-BB contained Ala-35 → His-35, and had a deletion of Arg-28. The resultant dimer had no detectable biological activity (37).

Skin Flap Model. In adult, male Lewis rats (Harlan, Indianapolis), bilateral island skin flaps (3 × 6 cm each) were elevated on the ventral surface, severing all tissue connections except for the main nourishing epigastric artery and vein to each flap (Fig. 1). In rats where the biodegradable collagen film (2 × 5 cm) containing either PDGF-BB (360 μg per film) or inactive mutagenized PDGF-BB was used, films were placed between the flap of tissue and the wound bed. The skin margins were

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Abbreviations: PDGF-BB, BB homodimeric platelet-derived growth factor.

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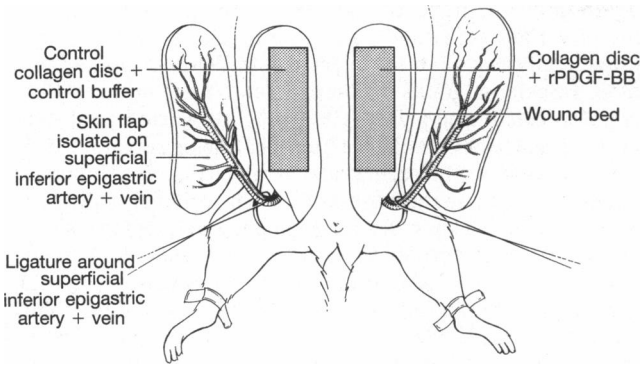

FIG. 1. The rat skin-flap model of acute ischemic injury. Bilateral abdominal skin flaps (3 × 6 cm) were elevated, isolating vascular flow to one pair of nutritive vessels on each flap (superficial epigastric artery and vein). Prior to wound closure, a 2 × 5 cm collagen disc containing 360 μg of PDGF-BB or an inactive mutant variant was placed between the flap and the wound bed. The nutritive vessels were ligated 1–10 days after initially raising the flap.

then sutured back to their original positions. In experiments where aqueous PDGF was used, the flap was infiltrated with multiple subcutaneous injections after suturing.

Blood Flow Measurements. Rats that had collagen film implants were used for measurement of blood flow. The primary blood flow to each flap was acutely terminated 1, 2, 3, 4, 5, 7, or 10 days later by tying closed the original nourishing vessels. Vascular flow to the skin through new collateral channels was determined by injecting sodium fluorescein intravenously and measuring UV-stimulated skin fluorescence (Fluoroscan, Boulder, CO). Readings were made over each flap from 18 points at 1-cm intervals using a superimposed orthogonal grid overlap; readings were also made from the adjacent nonflap skin. Average values were compared to create a percentage index of flap skin fluorescence relative to control skin using the formula

$$\text{DFI} = (\text{DF of flap skin}/\text{DF of control skin}) \times 100,$$

where DF is the dermatofluorometric skin reading and DFI is the dermatofluorometry index (38).

The area of surviving skin was determined 3 days later (39). At that time, necrotic tissue appeared grossly black. On transparent polystyrene sheets, the perimeter of the flaps was outlined, and the areas of necrotic tissue were marked. These areas were then measured using a digital image analysis system linked to morphometry software (Leco 2005, Leco Co., St. Joseph, MO), and the percentage of surviving tissue was calculated.

Microangiography. At sacrifice, the aorta was cannulated, the vasculature was flushed with 20 ml of warm heparinized saline, and a mixture of lead oxide, saline, and gelatin was injected (40). The abdominal tissues were resected and then radiographed after overnight refrigeration.

Histological Analysis. Rats were euthanized, and the abdominal flap and underlying muscle were removed and fixed in 4% (vol/vol) paraformaldehyde in PBS. These specimens were processed into paraffin blocks, sectioned, and stained with hematoxylin/eosin or with antibodies to von Willebrand factor (Dako) using the TechMate immunostainer (BioTek Solutions, Santa Barbara, CA) (G.F.P. and C.L.F., unpublished results).

Arterial Resection Model. To explore whether PDGF-BB could induce the regeneration of a single large-caliber vessel between two vessel ends, a segment of the rat superficial femoral artery was cauterized and resected, creating a 3- to 4-mm gap. A 0.2-ml volume of saline containing either active or inactive PDGF-BB (1 mg/ml) was injected into the muscle and connective tissue between the divided ends of the vessel. Each animal underwent femoral artery resection on both legs; the active PDGF-BB was injected in the gap on one leg and the mutant variant was injected on the other leg of each animal, in a random fashion. Vascular continuity and patency were evaluated at 2 weeks (n = 12) and 3 weeks (n = 18) by direct observation, latex injection, microangiography, and serial histologic sectioning. In addition, histological analysis was performed at 1 and 2 weeks on arteries from rats that had been injected with BrdUrd (50 mg/kg i.v., 1 hr before sacrifice) that were fixed and processed as above and stained with antibodies to von Willebrand factor (Dako), smooth muscle cell actin (Sigma), and BrdUrd (Dako) (G.F.P. and C.L.F., unpublished results).

Statistical Analyses. All data were collected by individuals blinded to the treatments. Results are presented as the mean ± SE. Paired two-tailed Student's t tests were performed to assess differences in skin flap blood flow and survival. Fisher's exact test was utilized to analyze differences in the formation of patent new vessels.

RESULTS

Skin Flap Model. Perfusion of the ischemic skin flaps through newly developed neovascular channels, as assessed by the dermatofluorometry index, was higher in flaps receiving PDGF-BB in comparison to the inactive variant (Fig. 2). The flow was significantly higher in the PDGF-BB-treated flaps when the flap vessels were ligated 3, 4, 5, 7, and 10 days after flap creation and collagen disk placement. No differences were detected in flaps ligated 1 or 2 days after creation and growth factor treatment. The area of surviving tissue was significantly greater in the PDGF-BB-treated flaps when the flap vessels were ligated at 2, 3, 4, or 5 days (Fig. 3). By 7 and 10 days, the entire flap survived in both groups after ligation. The amount of collateral vessel formation visible with microangiography correlated directly with both tissue perfusion and surviving area (Fig. 4). In dose–response studies using aqueous PDGF-BB, flow was higher at 3 and 5 days in rats that had been injected with 1 mg compared to those treated with 500 μg (data not shown).

At 2 and 4 days after flaps were raised, the feeding vessel was ligated, and animals were sacrificed 24 hr later (days 3 and 5

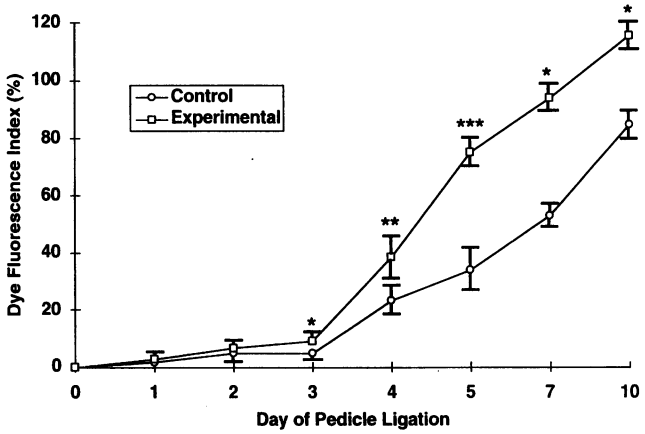

FIG. 2. The average dermatofluorometry index for flaps 20 min after ligation of the nutritive vessels. Each flap had 18 readings taken from points based on a rectangular coordinate grid. The readings were averaged and converted to a proportion of fluorescence relative to the adjacent normal skin. Each point on the graph represents the average of 9 or 10 flaps. Each animal served as its own control; collagen discs inserted on one side were treated with the active form of PDGF-BB (experimental) and with the inactive mutant variant (control) on the other flap. Data at each time point (postoperative day when the nutritive vessels were ligated) were compared using a paired Student's t test. *, $P < 0.0005$; **, $P < 0.02$; ***, $P < 0.005$.

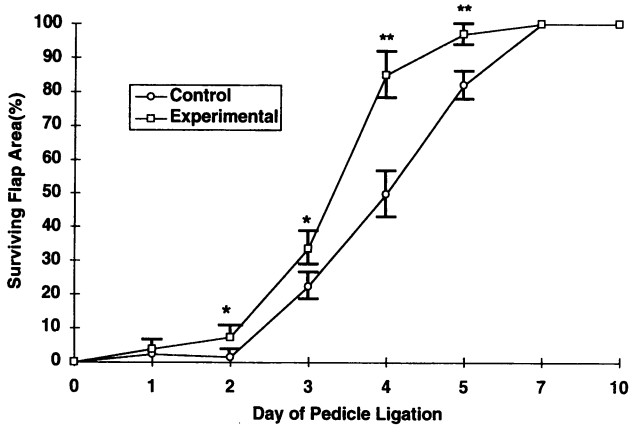

FIG. 3. The proportion of surviving flap skin relative to the initial flap skin area. Each data point represents the average of 9 or 10 flaps. Each rat served as its own control (see Fig. 2); data were compared using a paired Student's t test. *, $P < 0.05$; **, $P < 0.005$.

postflap creation). Histological analysis revealed that there was a transient dose-dependent increase in granulation tissue formation in the PDGF-BB-treated flaps compared to controls. In the flaps treated with PDGF-BB, the response at day 5 was characterized by a mononuclear cell infiltration into the dermis and throughout the subpannicular adipose tissue. No evidence of necrotic tissue was detected in the flap skin and the tissue appeared well perfused (Fig. 5 *A* and *C*). In contrast, in untreated flaps, there was extensive vascular stasis, occlusion, thrombosis, and hemorrhage in the dermis and underlying adipose tissue with accompanying inflammatory cells. Epidermal hypoplasia and focal necrosis was accompanied by focal exudation, and dermal collagen was disorganized and showed evidence of collagenolysis. A mixed cell infiltrate containing neutrophils was detected in control flaps, accompanied by cellular degeneration and fragmentation, suggesting a response to ongoing hypoxia (Fig. 5 *B* and *D*). None of the flaps were connected directly to the underlying muscle layer except at the flap margins where there were focal adhesions underlying the incision site. Detection of endothelial cells using von Willebrand factor confirmed the existence of a patent, well-perfused vasculature in the PDGF-treated rats, in contrast to controls (Fig. 5 *B* and *D*).

Femoral Artery Resection. New vessels were generated 2 weeks after creation of the femoral artery gap in 33% (4 of 12) of the legs that were injected with the active form of PDGF-BB; in contrast, none of the vessel sites injected with the mutant variant generated new arteries. Three weeks postoperatively, all sites (18 of 18) injected with PDGF-BB generated patent vascular conduits in comparison to none in the mutant variant-injected group ($P < 0.001$) (Fig. 6). Histological evaluation revealed a complete endothelial layer surrounded by smooth muscle in the regenerated artery (Fig. 7). The vessel diameter was comparable in size to the original superficial femoral artery. The new arterial segment was found in some of the specimens to sprout off as a branch vessel close to the thrombosed end of the proximal severed vessel (Fig. 7). Surprisingly, histological analysis at 1- and 2-week time points revealed that there were no apparent differences in degree of proliferation of cells in either arteries or surrounding connective tissue or musculature. There was no evidence of inflammation or increased granulation tissue formation in the PDGF-BB-treated rats, suggesting PDGF-BB's well-established transient induction of inflammation was short-lived. In all rats there was no loss of extremity function, likely due to the presence of extensive collateral circulation in this species.

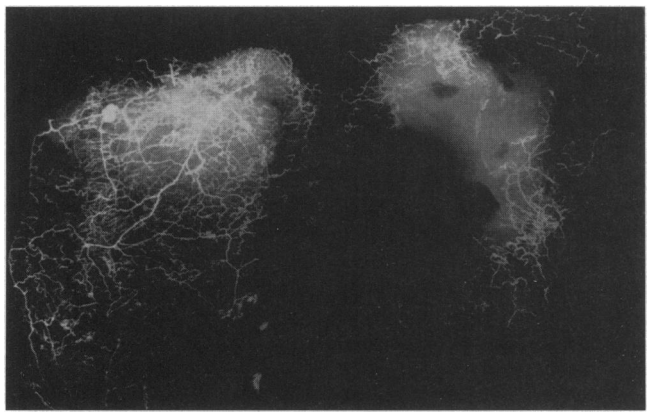

FIG. 4. Microangiogram of bilateral skin flaps in which the nutritive vessels were ligated 5 days postoperatively. The control (*Right*) shows minimal neovascularization restricted primarily to the flap margins. In contrast, the flap treated with PDGF-BB (*Left*) shows extensive neovascularization, which fills the epigastric artery through numerous new vessels at the wound edge.

DISCUSSION

Normal wound healing contains an angiogenic component in which the microvasculature returns after the leading edge of

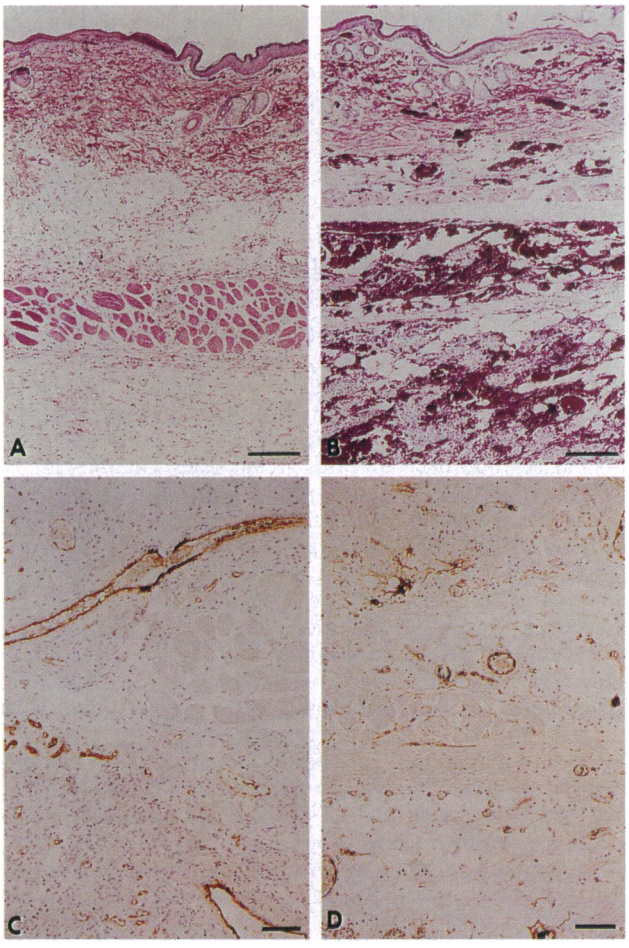

FIG. 5. Photomicrograph of sections from PDGF-BB-treated (*A* and *C*) and control (*B* and *D*) flaps at day 5 stained with hematoxylin/eosin (*A* and *B*) and with antibodies to von Willebrand factor (*C* and *D*). The treated flaps show increased granulation tissue, complete epidermal coverage (*A*), and the presence of numerous patent vessels in the periphery of the flaps (*C*). The control flaps had loss of dermal and epidermal tissue (*B*) and extensive vascular occlusion and stasis (*B* and *D*). (*A* and *B*, bar = 200 μm; *C* and *D*, bar = 100 μm.)

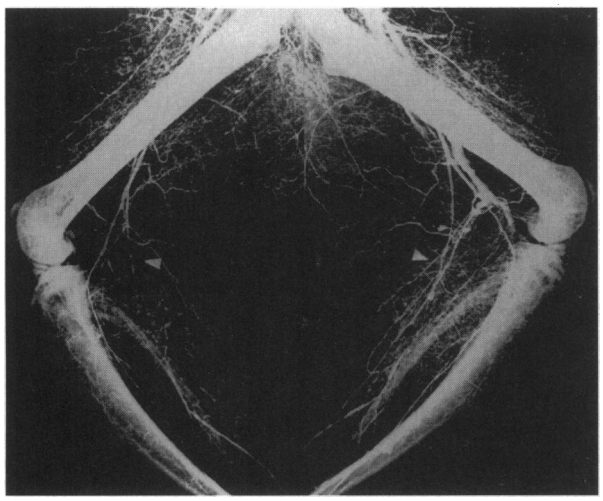

FIG. 6. Microangiogram of the divided rat femoral vessels 3 weeks postoperatively. The vessel gap injected with PDGF-BB (*Right*) shows a regenerated vascular conduit that restored patency in the desired vessel. In contrast, the mutant variant of PDGF-BB (*Left*) failed to achieve patency.

tissue regeneration (41, 42). This normal physiologic response was responsible for tissue survival seen in the mutant PDGF-treated flaps when the nutritive vessels were ligated several days later. Previous studies have shown that PDGF-BB can accelerate wound healing in dermal incisions (43) and rabbit and human ulcers (29, 44, 45), via the induction of a richly vascularized extracellular matrix (29, 46). By adding PDGF-BB-impregnated collagen disks where the neovascular response in the grafted flap normally occurs, microvascular growth and anastomoses with vessels in the periflap skin were accelerated, which permitted vessel ligation at an earlier time with good tissue survival.

The present results support the capacity of PDGF-BB to induce a local angiogenic response that is capable of protecting tissue from ischemic injury. Further, the rapid generation of large-caliber vessels from a mesenchymal tissue bed indicates that PDGF-BB not only acts at the microvascular level but also can initiate the *de novo* coordinated generation and anastomoses of an artery composed of multiple-cell layers.

Endothelial cells make both the A and B chains of PDGF (21–24). Although endothelial cells were initially found to be unresponsive to PDGF, more recent studies have shown that many microvascular endothelial cells express the PDGF-α and PDGF-β receptors and can proliferate in response to exogenous PDGF (17, 18, 27, 31, 32, 34). Of interest, Battegay and coworkers (34) recently showed that aortic endothelial cells that displayed an "angiogenic" phenotype in culture have PDGF-β receptors and respond to PDGF-BB to form cords and tubes. Thus, initial stimulation of microvascular endothelial cells, and potentially macrovessel endothelial cells, coupled with a positive autocrine PDGF loop may play a role in the angiogenic response. PDGF autocrine loops also may be operant for smooth muscle cells and fibroblasts (25, 26, 47) as well as pericytes, the smooth muscle-like cells within the microvasculature (48, 49). Furthermore, since PDGF is synthesized by and stimulates proliferation (12–14) and migration (reviewed in ref. 14) of smooth muscle cells and fibroblasts, these cell types, alone or working in concert with the transiently increased macrophages and platelets, may exert a paracrine effect on the other cells in both small and large vessels, contributing to vessel wall assembly (50). By addition of exogenous PDGF, the angiogenic potential appears to be more rapidly induced and augmented.

Hypoxic gradients locally produced within the tissue are considered to play a role in the neovascular response generated in both models. PDGF-BB is upregulated under hypoxic conditions (4) and can stimulate dermal repair in an ischemic environment (30). Notably, growth factors such as vascular endothelial growth factor/vascular permeability factor, which stimulate angiogenesis under ischemic conditions (5, 51), are induced by PDGF (52). Thus, the initial chemotactic and proliferative stimuli of exogenously administered PDGF-BB likely triggered a cascade of synergistic autocrine and paracrine activities for PDGF and other relevant endogenous cytokines and growth factors (53). The long duration of the enhanced vascular responses induced by a single injection of PDGF-BB also may be due, at least in part, to binding and localization of this isoform to extracellular matrix (54), via a sequence present in exon 6 of the B chain. This sequence confers binding of PDGF-BB to heparan sulfate proteoglycans (54).

Other investigators have shown that basic and acidic fibroblast growth factor and vascular endothelial growth factor can accelerate development of collateral circulation in ischemic limbs and myocardium (55–60). A striking finding in this report is the *de novo* generation of a multiple-cell-layer artery by injection of PDGF-BB into the surrounding tissue of a large vascular gap. Spontaneous anastomoses have been observed to take place between vessels across short distances, but this occurs rarely in large arteries and occurred in none of the control limbs. Although PDGF is active on microvessels, this study provides direct evidence for its ability to augment large vessel regrowth, elongation, and anastomoses. This effect probably works not by recruitment of stem cells but by stimulating proliferation and migration of vascular wall cells from existing capillaries within the tissue and/or the occluded artery itself. It is most interesting that only one vessel of large caliber was formed, suggesting that a single anastomosis precludes further large vessel formation. Temporal and spatial analyses of gene activation in the neovessel, such as al(I) collagen, may yield further insights on this process (61).

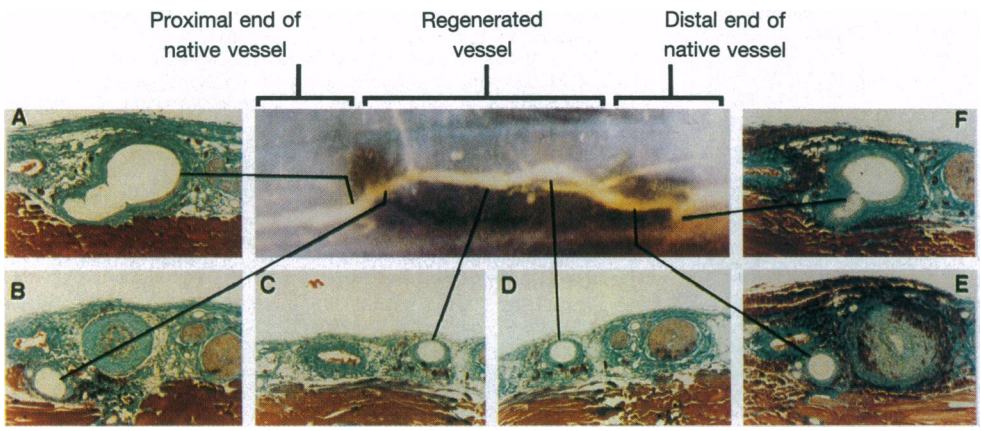

FIG. 7. A histologic cross-sectional reconstruction of a newly generated artery 3 weeks after injection of PDGF-BB into the vessel gap. The sequence of histologic figures covers ≈6 mm overlying the 3- to 4-mm gap site. The line indicates the regenerated vessel in each section. (Hematoxylin/eosin; ×120.)

There are certain parallels between the response induced by PDGF-BB in the present studies and the role of PDGF in intimal hyperplasia (19, 25, 26). Antibodies to PDGF can inhibit the formation of neointima in injured arteries in rats (19), and an inhibitor of PDGF, trapadil, may limit restenosis in humans (62). Since neointima formation and restenosis after angioplasty are thought to be primarily a consequence of smooth muscle cell proliferation and migration, this provides further evidence for the role of PDGF in generating large vessels.

Further work will be necessary to establish the mechanism by which exogenous application of PDGF-BB exerts these potent effects *in vivo*. In particular, in the flap model the granulation tissue response is robust and complex. However, the early onset and degree of protection conferred by PDGF-BB on the skin flap after ligation was dramatic, and this correlated with the high degree of vascularity observed in the flaps. Further development of reagents will facilitate the elucidation of the mechanisms of regeneration in these systems. Although extensive dose–response studies were not performed, the limited dose range in this and in *de novo* granulation tissue induction studies (37) suggests the dose range is appropriate in these model systems. Taken together, these studies, in conjunction with data from other *in vivo* and *in vitro* systems, suggest that complex growth factor networks can be pharmacologically manipulated and enhanced to trigger regenerative processes.

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