Infarct healing is regulated by a well-orchestrated inflammatory response that ultimately leads to formation of a scar (1,2). Myocardial necrosis results in marked induction of inflammatory mediators and recruitment of leukocytes in the infarcted myocardium. Activated neutrophils and mononuclear cells secrete proteolytic enzymes and debride the infarcted area from dead cells and extracellular matrix fragments. The inflammatory phase is followed by the proliferative phase of healing, when fibroblasts accumulate in the infarct granulation tissue and deposit extracellular matrix proteins (3). During the proliferative phase the wound is highly cellular and metabolically active and requires a rich vascular supply to provide the proliferating cells with oxygen and nutrients. Thus, formation of infarct neovessels is a critical part of the healing process. As the wound matures, resolution of the inflammatory infiltrate occurs and the highly vascular granulation tissue is replaced by collagen-rich scar. The infarct vasculature undergoes dynamic changes: infarct angiogenesis is suppressed, and many infarct neovessels acquire a muscular coat (4,5) while uncoated vessels regress. As a result, the mature scar contains few capillaries and a large number of coated arterioles (5). Resolution of the inflammatory response and maturation of the neovasculature may be important steps in infarct healing, necessary to prevent uncontrolled angiogenesis and expansion of granulation tissue formation.

Platelet-derived growth factor (PDGF) signaling regulates events critical to fibrous tissue deposition and angiogenesis through interactions with its 2 PDGF receptors (PDGFR) α and β. Activation of both PDGFR-α and PDGFR-β pathways stimulates fibroblast migration, proliferation, and activation (6–8). In addition, PDGF-BB/PDGFR-β interactions play a crucial role for investment of developing vessels with pericytes, a key process in vascular maturation (9,10). Endothelial cells form vascular tubes and direct the recruitment of mural cell precursors by secreting PDGF-BB (11), whereas PDGFR-β is expressed by pericytes and smooth muscle cells. Although the critical significance of PDGFR-β-mediated pericyte coating in embryonic vascular development has been established (12,13), the importance of vascular maturation and acquisition of a
muscular coat in healing wounds has not been investigated. In the retina, investment of the vessels with mural cells marks the end of the plasticity window and leads to vascular stabilization (14) and decreased angiogenic potential. We hypothesized that activation of the PDGFR-β pathway in the infarcted myocardium is crucial for maturation of infarct neovessels and acquisition of a muscular coat. Thus, recruitment of mural cells by infarct neovessels may be important in mediating suppression of the angiogenic process and resolution of the inflammatory infiltrate during wound maturation.

Our study examined the effects of disrupted PDGFR-β and PDGFR-α signaling, through systemic injection of neutralizing antibodies (15), on infarct angiogenesis, wound maturation, and scar formation in reperfused murine infarcts. The PDGFR-β inhibition resulted in impaired pericyte coating, formation of a chaotic and tortuous infarct vasculature, increased capillary density, prolonged extravasation of blood cells, and impaired collagen deposition in the infarct. In contrast, PDGFR-α inhibition had no effect on vascular maturation but significantly attenuated collagen deposition in the infarcted myocardium. Acquisition of a muscular coat through PDGF-BB/PDGFR-β interactions is critical for stabilization of the infarct neovessels and resolution of the postinfarction inflammatory response.

METHODS

Murine ischemia/reperfusion protocols. All animal protocols were approved by the Baylor College of Medicine Institutional Review Board. Wild-type C57/BL/6 mice, 8 to 12 weeks of age (purchased from Charles River, Wilmington, Massachusetts), were used for myocardial infarction experiments. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (10 μg/g). A closed-chest mouse model of reperfused myocardial infarction was used as previously described (16,17) to avoid the confounding effects of surgical trauma and inflammation. The left anterior descending coronary artery was occluded for 1 h and then reperfused for 6 h to 7 days. At the end of the experiment, the chest was opened and the heart was immediately excised, fixed in zinc formalin, and embedded in paraffin for histological studies, or snap frozen and stored at −80°C for RNA isolation. Sham animals were prepared identically without undergoing coronary occlusion/reperfusion. Animals used for histology underwent 72-h and 7-day reperfusion protocols (5 animals per group). Mice used for RNA extraction underwent 6 h, 24 h, 48 h, 72 h, and 7 days of reperfusion (3 animals per group).

Immunohistochemistry and quantitative histology. Sections were cut at 3 μm and stained immunohistochemically with the following antibodies: anti-α smooth muscle actin (α-SMA) (Sigma, St. Louis, Missouri), rat anti–mouse macrophage antibody clone F4/80 (Research Diagnostics Inc., Flanders New Jersey), rat anti-neutrophil antibody (Serotec, Raleigh, North Carolina), anti-phosphorylated PDGFR-β antibody (Upstate Biotechnology, Charlotteville, Virginia), and rat anti-mouse CD31 antibody (Pharmingen, San Diego, California). Staining was performed using a peroxidase-based technique with the Vectastain ELITE rat or goat kit (Vector Labs, Burlingame, California). Negative control procedures were performed with omission of the primary antibodies. The Mouse-on-Mouse kit (Vector) was used for α-SMA immunohistochemistry. For CD31 staining, sections were pretreated with trypsin and staining was performed using the Tyramide Signal Amplification kit (Perkin Elmer, Boston, Massachusetts). Collagen staining was performed using picrosirius red. Quantitative assessment of macrophage and neutrophil density was performed by counting the number of F4/80 positive cells and immunoreactive neutrophils respectively in the infarcted area (18). Macrophage and neutrophil density was expressed in cells/mm². Infarct microvascular density was assessed by counting the number of CD31-stained vascular profiles in infarcted murine hearts. To assess the acquisition of a muscular coat by infarct vessels, the density of coated vessels in the infarct was measured by using a section stained for α-SMA. The density of uncoated vessels was assessed by subtracting the density of coated vessels from the total vascular density in the infarct. The percentage of the infarcted area stained for collagen with picrosirius red (collagen percent staining) was quantitatively assessed in infarcts after 7 days of reperfusion using Image Pro software (Image Pro Plus, version 4.5, Media Cybernetics Inc., Silver Spring, Maryland) as previously described (19).

RNA extraction and quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from frozen hearts using a commercially available acid-phenol reagent (TRIZol, Invitrogen, Carlsbad, California). Total RNA was treated with DNase to remove any genomic contamination as described by the manufacturer (DNase-free, Ambion, Austin, Texas). First-strand cDNA was synthesized using SuperScript II reverse transcriptase and random hexamer primers as described in the manufacturer's protocol (Invitrogen). Relative standard curve method was used to measure the expression levels of PDGF-BB, -AA, PDGFR-α, and PDGFR-β. TaqMan primers and probes for PDGF-BB, PDGF-AA, PDGFR-α, and PDGFR-β were obtained from Applied Biosystems (Foster City, California). Real-time PCR was performed and analyzed with

### Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APA</td>
<td>anti–PDGFR-α antibody</td>
</tr>
<tr>
<td>APB</td>
<td>anti–PDGFR-β antibody</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
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<td>SMA</td>
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1:10 diluted cDNA according to the manufacturer’s instructions on an ABI Prism 7000 Sequence Detection System. Target gene expression was normalized to an internal control, ribosomal protein S3 (RPS3). The RPS3 was measured using SYBR Green chemistry and the relative standard curve method. At the end of the PCR cycle, target gene expression was normalized to an internal control, ribosomal protein S3 (RPS3). The RPS3 was measured using SYBR Green chemistry and the relative standard curve method. At the end of the PCR cycle,
dissociation curve analysis was performed to ascertain the amplification of a single PCR product. Sequence of the murine primers is as follows: RPS3 forward (5'-ATCAGAGAGTTGACCGCAGTTG-3'), RPS3 reverse (5'-AATGAACCGAAGCACACCATAG-3').

**PDGFR-α and PDGFR-β inhibition in mouse infarcts.** To examine the effects of PDGFR-β inhibition on myocardial infarct healing, mice undergoing coronary occlusion/reperfusion received daily intraperitoneal injections (200 μg/day, the first dose was administered after 24 h of reperfusion) with the neutralizing rat anti-mouse PDGFR-β antibody (APB) 5, the rat anti-mouse PDGFR-α antibody (APA) 5 (both from eBioscience, San Diego, California), or rat immunoglobulin G (IgG) (Sigma) (APA5, n = 7; APB5, n = 7; rat IgG, n = 14). Selection of the dose was based on previously published studies using these antibodies in vivo to inhibit PDGFR signaling in mouse models (15,20,21). After 7 days of reperfusion the animals were killed, and the hearts were fixed in zinc formalin (Z-fix; Anatech, Battle Creek, Michigan) and embedded in paraffin for histological studies.

**Statistical analysis.** Statistical analysis was performed using 1-way ANOVA followed by t test corrected for multiple comparisons (Student-Newman-Keuls). Data were expressed as mean values ± SEM. Statistical significance was set at 0.05.

**Figure 3.** Assessment of mRNA expression of platelet-derived growth factor receptor (PDGFR)-α (A), platelet-derived growth factor (PDGF)-A chain (B), PDGFR-β (C), and PDGFR-B chain (D) in mouse infarcts using quantitative real-time polymerase chain reaction. (A) The PDGFR-α mRNA expression in the heart peaked after 6 h of reperfusion (**p < 0.01 vs. sham). (B) The PDGF-A mRNA expression also increased after 6 h of reperfusion; however, the difference with sham hearts did not reach statistical significance (p = 0.08). (C and D) The PDGFR-β (C) and PDGFR-B (D) mRNA levels show a similar time course, peaking after 7 days of reperfusion (p < 0.05 vs. sham, *p < 0.01 vs. sham).

**Figure 4.** Activation of the platelet-derived growth factor receptor (PDGFR)-β signaling pathway in healing murine myocardial infarcts was detected using immunohistochemical staining with an antibody to phosphorylated PDGFR-β (pPDGFR-β). (A) Noninfarcted areas showed minimal staining for pPDGFR-β. (B) After 7 days of reperfusion, perivascular and mononuclear-like cells (arrowheads) with intense immunoreactivity to pPDGFR-β were found in the infarcted myocardium. (C) Negative control with omission of the primary antibody shows no staining. Counterstained with eosin (magnification 200×).
RESULTS

Vascular maturation in healing mouse infarcts. CD31 immunohistochemistry identified the vascular network of the mouse heart, specifically labeling endothelial cells (Figs. 1A and 1B), whereas α-SMA immunoreactivity was localized in smooth muscle cells (Figs. 2A and 2B). After 72 h of reperfusion, dead cardiomyocytes in the infarcted area were replaced with granulation tissue that contained an extensive capillary network (Fig. 1C). At the same time point, α-SMA immunohistochemistry labeled a large number of myofibroblasts and occasional vessels coated with smooth muscle cells (Fig. 2C). As the wound matured, more infarct vessels acquired a muscular coat, and after 7 days of reperfusion (Fig. 1D) the scar contained a significant number of mature, coated neovessels (Fig. 2D). Acquisition of a muscular coat confers stability and indicates maturation of the vasculature.

Expression of PDGF-A, PDGFR-α, PDGF-B, and PDGFR-β in healing infarcts. The PDGFR-α mRNA expression was markedly induced in the infarcted myocardium after 6 h of reperfusion (p < 0.01, corrected for multiple comparisons) and decreased after 24 h of reperfusion (Fig. 3A). Although PDGF-A mRNA expression also peaked after 6 h of reperfusion, the difference between PDGF-A mRNA expression in infarcted and sham animals was not statistically significant (Fig. 3B). In contrast, PDGFR-β and PDGF-B mRNA showed late up-regulation, peaking after 7 days of reperfusion (Figs. 3C and 3D).

Phosphorylated PDGFR-β localization in the infarcted heart. Many perivascular cells in the healing infarct showed expression of phosphorylated PDGFR-β, suggesting activation of the PDGF-B/PDGFR-β signaling pathway (Fig. 4B). In contrast, minimal phosphorylated PDGFR-β immunoreactivity was noted in the noninfarcted heart (Fig. 4A).

PDGFR-β and PDGFR-α neutralization inhibit collagen deposition in the healing infarct. After 7 days of reperfusion, mouse infarcts showed replacement of the dead cardiomyocytes with collagen-based scar. Healing infarcts in antibody-treated animals showed decreased matrix deposition in the infarcted myocardium (Fig. 5). The PDGFR-α neutralization resulted in significantly diminished collagen content in the infarcted area (anti-PDGFR-α: 12.45 ± 0.48, n = 7; vs. rat IgG: 20.34 ± 1.94, n = 14; p < 0.05, corrected for multiple comparisons). The PDGFR-β inhibition also attenuated collagen deposition in the infarcted area (anti-PDGFR-β: 13.46 ± 1.88, n = 7; vs. rat IgG: 20.34 ± 1.94, n = 14, p < 0.05, corrected for multiple comparisons).

PDGFR-β but not PDGFR-α neutralization results in impaired maturation of the infarct vasculature. The PDGFR-β inhibition impaired vascular maturation and increased microvascular density in healing infarcts (anti-PDGFR-β: 471.6 ± 73.8 vessels/mm² vs. IgG: 267.7 ±

Figure 5. (A) Platelet-derived growth factor receptor (PDGFR)-α and PDGFR-β inhibition decreased collagen deposition in the infarcted myocardium. (A) After 7 days of reperfusion, Sirius red staining identified a well-organized collagen network in the infarcted heart from animals treated with control rat immunoglobulin (IgG). (B) Mice treated with anti–PDGFR-α antibody (APAS) had attenuated collagen deposition in the infarcted myocardium. (C) Mice injected with anti–PDGFR-β antibody (APB5) showed decreased and disorganized collagen deposition. Note the presence of dilated vessels (arrows). (D) Quantitative assessment of collagen deposition in the infarcted heart (*p < 0.05 vs. IgG control group). Magnification 400×.
13.98 vessels/mm$^2$, $p < 0.01$, corrected for multiple comparisons). Anti–PDGFR-$\beta$ treatment resulted in formation of a disorganized, “chaotic” vasculature comprised of irregular uncoated vessels (Figs. 6C to 6F). The PDGFR-$\beta$ antibody-treated mice had a significantly lower density of coated vessels (anti–PDGFR-$\beta$: $70.02 \pm 6.75$ vessels/mm$^2$ vs. IgG: $117.5 \pm 15.7$ vessels/mm$^2$, $p < 0.05$, corrected for multiple comparisons) and a higher number of uncoated vessels compared with IgG-injected animals (anti–PDGFR-$\beta$: $401.61 \pm 68.94$ vessels/mm$^2$ vs. IgG: $150.16 \pm 21.83$ vessels/mm$^2$, $p < 0.01$, corrected for multiple comparisons). In contrast, PDGFR-$\alpha$ inhibition did not affect microvessel formation in the healing infarct (anti–PDGFR-$\alpha$: $216.77 \pm 15.2$ vessels/mm$^2$, $p = \text{NS}$, corrected for multiple comparisons) (Fig. 6).

**Effects of PDGFR-$\alpha$ and PDGFR-$\beta$ inhibition on leukocyte infiltration in the healing infarct.** We have previously shown that reperfused mouse infarcts show an intense but transient inflammatory reaction leading to formation of a mature scar after 7 to 14 days of reperfusion (17). After 7 days of reperfusion, murine myocardial scars contained large amounts of matrix (Fig. 4A) and relatively low numbers of leukocytes. The PDGFR-$\beta$ inhibition resulted in prolonged extravasation of blood cells in the healing infarct. After 7 days of reperfusion, anti–PDGFR-$\beta$–treated mice, but not IgG-injected control mice, showed hemorrhagic areas containing extravasated red blood cells (Figs. 7A and 7B). In addition, PDGFR-$\beta$ neutralization resulted in increased macrophage density in the infarcted area compared with IgG-treated animals (Fig. 7D). The PDGFR-$\alpha$ inhibition also increased macrophage density.
but did not induce hemorrhagic infiltrates in the scar. In contrast, neutrophil density after 7 days of reperfusion was low in both antibody-treated and control animals (Fig. 7E).

**DISCUSSION**

**PDGF signaling modulates fibrosis and angiogenesis.** Platelet-derived growth factors are a family of disulfide-linked dimeric proteins encoded by four genes, PDGF-A, -B, -C, and -D, that signal by binding to homodimers or heterodimers of the 2 PDGF receptor proteins, PDGFR-α and PDGFR-β. The PDGF isoforms mediate fibrogenic actions and play an important role in regulation of angiogenesis. PDGFR-α and PDGFR-β activation stimulates fibroblast migration and gene expression (6); ligands of these receptors are known inducers of cardiac fibrosis. Cardiac overexpression of the PDGFR-α ligand PDGF-C induced fibrosis, collagen deposition, vascular defects, and myocardial hypertrophy (22). In addition, transgenic overexpression of the active core domain of PDGF-D, a PDGFR-β ligand, resulted in cardiac fibrosis followed by dilated cardiomyopathy and subsequent cardiac failure. On the other hand, extensive evidence supports the crucial role of PDGFR-β–mediated interactions in recruitment of mural cells by newly formed vessels (23–25). Genetic disruption of PDGF-B or PDGFR-β in mice results in virtually identical phenotypes, leading to the development of microvascular leakage, lethal hemorrhage, and edema in late embryogenesis (26,27); these defects are caused by a severe deficit in pericyte investment (12). Thus, PDGFR-β signaling plays an important role in stabilizing the vasculature through acquisition of a mural coat. **PDGFR-β signaling critically regulates maturation of the infarct vasculature.** Our study explored for the first time the functional role of PDGF signaling in healing myocardial infarcts. We showed that both PDGF receptors exert important and distinct actions in regulation of the cellular events associated with formation of the scar. Although both receptors mediate fibrogenic effects (Fig. 5), PDGFR-β signaling also plays a unique and crucial role in vascular maturation by regulating coating of infarct microvessels with mural cells (Fig. 6). The PDGFR-β inhibition through injection of the neutralizing antibody APB5 (15) critically impaired vascular maturation in healing infarcts. The APB5 injection resulted in defective investment of infarct microvessels with mural cells, increased capillary density, and formation of tortuous and dilated vascular structures (Fig. 6). The role of PDGFR-β signaling in healing infarcts likely involves a paracrine interaction between endothelial and mural cells: endothelial cells secrete PDGF-B, whereas pericytes and smooth muscle cells express PDGFR-β (28). The importance of the endothelial cell as a source of PDGF-B is illustrated by the severe pericyte deficiency noted in mice with endothelium-specific ablation of PDGF-B (29,30). In contrast, selective PDGF-B disruption in hematopoietic cells has no obvious effects on the vasculature (31).

The dilated and tortuous vasculature noted in APB5-treated infarcts suggests an important role for PDGFR-β signaling in regulating vessel size and shape. Hellstrom et al.
(13) showed that PDGFB- and PDGFR-β-deficient embryos show increased and variable capillary diameter with high- and low-diameter segments. In addition, pericyte coating inhibits endothelial proliferation and suppresses angiogenesis (32). Our findings suggest that PDGFR-β-mediated mural cell recruitment may be a crucial step in maturation of the infarct vasculature and in suppression of the angiogenic process in the mature scar.

**PDGFR-α signaling does not regulate vascular maturation but promotes collagen deposition.** In contrast, PDGFR-α inhibition did not affect vascular maturation but significantly decreased collagen deposition in the infarcted myocardium (Fig. 5). This finding is consistent with the established role of PDGFR-α ligands (such as PDGF-AA and PDGF-C) in the development of myocardial fibrosis. PDGF-AA potently stimulates cardiac fibroblast proliferation inducing cell division to the same extent and with the same kinetics as PDGF-BB (33); PDGF-AA may also mediate, at least in part, the fibrogenic actions of angiotensin II in the heart (34). In addition, PDGF-C induces fibroblast proliferation (35) and promotes cardiac fibrosis when overexpressed in transgenic mice (22).

**PDGFR-β signaling stabilizes the infarct vasculature promoting maturation of the scar and resolution of inflammation.** Resolution of inflammation is critical for effective cardiac repair after myocardial infarction. Chemokine and cytokine expression is markedly but transiently induced in the infarcted myocardium (17); suppression of inflammatory gene synthesis is followed by resolution of the inflammatory infiltrate. We have recently identified thrombospondin-1 mediated transforming growth factor-β activation as a crucial mechanism for suppression of the inflammatory response (36) in healing myocardial infarcts. Thrombospondin-1 −/− infarcts showed increased and prolonged inflammation and expansion of the inflammatory process into the noninfarcted myocardium; this resulted in adverse postinfarction remodeling. Thus, timely suppression of the inflammatory response is critical for optimal repair. Our current study identifies another important mechanism with a key role for resolution of inflammation. Disruption of PDGFR-β signaling results in prolonged red blood cell and monocyte/macrophage extravasation in the infarcted myocardium (Fig. 7), suggesting that PDGFR-β-mediated vascular maturation is critical for resolution of the inflammatory process. The pro-inflammatory effects of PDGFR-β inhibition lead to formation of a more cellular wound, with lower collagen content. In addition, inhibition of the direct stimulatory effects of PDGFR-β signaling on fibroblast migration, proliferation, and activation (37,38) may further suppress fibrous tissue deposition in the healing infarct. Impaired maturation of the infarct and decreased collagen content may decrease tensile strength, adversely affecting postinfarction remodeling.

**Study limitations.** Antibody neutralization is a valuable tool for temporary inhibition of the biological effects of PDGF. However, this strategy has significant limitations. The exact dose of neutralizing antibody needed to inhibit a particular biological response cannot be predicted. In addition, the delivery of the antibody to the reperfused myocardium may vary among experiments. We did not quantitatively evaluate the effectiveness of the anti-PDGF antibodies in inhibition of PDGF signaling in the infarcts. Although these antibodies have been extensively used for in vivo neutralization of PDGF-mediated effects (15,20,21) in murine models, the dose we used may have resulted in partial inhibition of PDGF signaling. In addition, our study did not quantitatively assess PDGF-AA and -B chain protein levels in the infarcted heart and did not identify the cell types expressing PDGFR-β in the infarct.

**Conclusions.** The PDGF-mediated pathways play crucial but distinct roles in regulating postinfarction repair. Both PDGFR-β and PDGFR-α signaling pathways mediate fibrogenic responses, probably through direct effects on fibroblasts. However, activation of the PDGF-B/PDGFR-β pathway is also involved in recruitment of mural cells by infarct neovessels, critically regulating acquisition of a mural coat by the vasculature. Coating and maturation of the vasculature is an important step in suppression of the angiogenic process and promotes resolution of inflammation by preventing extravasation of blood cells into the mature scar. Disruption of PDGFR-β signaling results in formation of a defective infarct vasculature, impaired maturation of the scar, prolonged inflammation, and decreased collagen content in the wound. Defective scar formation may adversely affect function of the infarcted heart by enhancing left ventricular remodeling.

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