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Hypoxia Increases Placenta Growth Factor Expression in Human Myocardium and Cultured Neonatal Rat Cardiomyocytes

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Abstract

Background—Placenta growth factor (PIGF) plays an important role in pathological angiogenesis and is thought to be an independent biomarker in patients with coronary artery disease. However, little is known regarding the regulation of PIGF expression in heart tissue.

Methods—We determined expression changes in PIGF and its receptor, VEGFR1, in normal and abnormal biopsies from human cardiac allografts and in cardiomyocytes cultured under hypoxia or cyclical stretch conditions.

Results—Human donor myocardium and biopsies from allografts without fibrin deposits expressed PIGF and VEGFR1 mRNA. Biopsies (n = 7) with myocardial fibrin, elevated serum cardiac troponin I titers (p < 0.03) and cellular infiltrates (p < 0.05), expressed 1.6-fold more PIGF mRNA than biopsies from allografts without fibrin (n=11; p < 0.05). PIGF protein was localized in cardiomyocytes, extracellular matrix and some microvessels in areas with fibrin deposition. VEGFR1 mRNA expression was not different between groups. Cultured neonatal rat cardiomyocytes constitutively expressed PIGF/VEGFR1 under normoxia. PIGF expression was increased 3.88 ± 0.62 fold after 12 hours (n = 6; p ≤ 0.05) and 3.64 ± 0.41 fold after 24 hours of hypoxia (n = 6; p ≤ 0.05). Shorter periods of hypoxia, conditioned media from hypoxic cells and cyclical stretch did not significantly alter PIGF or VEGFR1 expression.

Conclusion—Cardiomyocyte PIGF expression is upregulated by hypoxia in vitro and its expression increases significantly in allografts with myocardial damage. Collectively, these results provide important temporal and spatial evidence that endogenous PIGF could facilitate cardiac healing following myocardial hypoxia/ischemia.

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Keywords

angiogenesis; hypoxia; growth factors; gene expression; cardiomyocyte

Introduction

A competent collateral circulation limits mortality/morbidity following myocardial infarction [1] and members of the vascular endothelial growth factor (VEGF) family play an important role in the growth and maintenance of blood vessels[2]. One VEGF family member that has received relatively little attention is placenta growth factor (PlGF)[3]. Although others have described the usefulness of plasma PlGF as an independent biomarker of favorable[4] or unfavorable[5-7] outcomes in patients with acute coronary syndromes, little is known regarding its regulation in heart tissue.

The association between angiogenesis and increased PlGF expression during proliferative retinopathy[8], cutaneous wound healing[9,10], bone fracture repair[11] and tumor growth [9,12] suggests PlGF can regulate vascularity during pathological conditions. PlGF binds VEGFR1 and neuropilin-1 (NP-1) receptors[13] and amplifies VEGF-driven myocardial angiogenesis during ischemia in mice[14]. Therefore, endogenous PlGF could play an important role in vascular growth/maintenance during myocardial ischemia.

Hypoxia is a potent stimulus for myocardial angiogenesis. However, hypoxia decreases PlGF expression in some cells [15,16] but increases PlGF expression in others[17,18]. No studies have directly assessed PlGF gene expression in ischemic human myocardium. Furthermore, cardiomyocyte stretch in vitro [19,20] and in vivo [21,22] increases VEGF expression and induces coronary angiogenesis independently of hypoxia[23]. The effect of cyclical stretch on PlGF expression in cardiomyocytes is not also known. Clearly, there is a fundamental lack of information regarding cardiomyocyte PlGF expression. Our results are the first to show that 1) human myocardium expresses PlGF mRNA and protein, 2) its expression increases with cellular and biochemical indices of myocyte damage, and 3) that hypoxia, but not mechanical stretch, upregulates PlGF expression in isolated cardiomyocytes. Collectively, these results provide important temporal and spatial evidence that endogenous PlGF could facilitate cardiac healing following myocardial hypoxia/ischemia.

Methods

PlGF expression in human myocardium

Right endomyocardial biopsy samples were obtained from donor hearts (n = 2) and cardiac allograft recipients (n = 16), embedded in medium, and snap frozen in liquid nitrogen. All biopsies after transplantation were for routine rejection surveillance and blood samples were obtained prior to biopsy to assess serum cardiac troponin-I titers as described[24,25]. Additional paraffin embedded tissue was submitted for light microscopy assessment of allograft rejection according to the International Society for Heart and Lung Transplantation revised grades[26]. Days between transplantation and biopsy were determined for each sample.

Immunohistochemical assessment of the presence of myocardial fibrin, vascular antithrombin and cellular infiltrates were performed on frozen sections as described[24,25,27,28]. For immunoperoxidase-based detection of PlGF in biopsies with (n = 7) and without (n = 7) fibrin deposition, 5µm sections obtained from paraffin blocks were deparaffinized, hydrated, rinsed and antigen-retrieved using DAKO Target Retrieval solution (pH 6.0). Endogenous biotin was blocked using an avidin/biotin blocking system (DAKO, Carpinteria, CA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Rabbit anti-human PlGF

antibody (ab9542, Abcam, Cambridge, MA) was applied for 60 minutes at room temperature, washed, and developed using DAKO's EnVision+ Dual Link HRP kit. PIGF localization was evaluated by 2 investigators unaware of the biopsy fibrin characteristics.

Total RNA was isolated from frozen blocks, 500 ng was reverse transcribed as described[29], and cDNA was used for analysis of PIGF, VEGFR1, VEGFR2, and NP-1 mRNA expression by RT-PCR. We designed primers to amplify 318 bp of the 5' region of human PIGF contained in all known splice variants (Table 1). Validity of the amplicon was confirmed by cloning into pCRII-TOPO vector and bi-directional sequencing of the insert. We also utilized primers to amplify 239 bp of human VEGFR1/flt-1 [15,30], 204 bp of human VEGFR2/KDR [15,31], and 210 bp of human NP-1 [32] (Table 1). Appropriate PCR conditions were determined [29] to ensure linear amplification of cDNA target over a range of PCR cycles. Primers and competimers for 18S ribosomal RNA (Ambion Inc, Austin, TX) were used to determine relative differences in gene expression [29]. Tissue samples were grouped according to the immunohistochemical presence or absence of fibrin[29] and relative changes in mRNA expression and protein, cellular infiltrates, plasma Tn-I, and ISHLT rejection grades were compared between groups.

Cardiomyocyte cultures

Ventricular cardiomyocytes were obtained from neonatal rats[20]. The Institutional Animal Care and Use Committee approved all animal procedures in accordance with guidelines. Culture media (DMEM, 10% FCS, 1mM sodium pyruvate, 100 IU penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B) was changed every 24 hours. Approximately 90% of the cells began to beat spontaneously[20] and cultures were used within 2-3 days of plating. Representative cell culture purity was assessed using monoclonal antibody to sarcomeric myosin (MF-20, University of Iowa Hybridoma Center) and rhodamine labeled goat anti-mouse IgG (1:100) with DAPI (1 µg/ml; Molecular Probes, Eugene, OR) counterstain. Cultures with >85% MF-20 positive or beating cells were used in the studies.

In vitro cyclical stretch experiments

We used a stretch protocol known to significantly increase VEGF expression in isolated cardiomyocytes[20]. Cardiomyocytes were plated on collagen I Bioflex plates and subjected to a 10% average surface elongation at 30 cycles/min for 1, 3, or 6 hours using a computerized Flexercell Strain Unit (Flexcell International, Hillsborough, NC). Cultures not subjected to cyclical stretch served as controls. At the end of each time period, total RNA was extracted [20] and stored at -80°C until used.

In vitro hypoxia experiments

Parallel hypoxic (< 2% O₂) and control (21% O₂) cardiomyocyte cultures were incubated for 1, 3, 6, 12, or 24 hours. In addition, conditioned media from cardiomyocytes incubated under hypoxic or normoxic conditions for 12 hours was collected, centrifuged, and added to fresh cardiomyocytes cultured under normoxia for 12 additional hours. Total RNA was isolated at the end of each time point and stored at -80°C.

Relative RT-PCR for rat PIGF and VEGFR-1

Total RNA was reverse transcribed as we have described[29]. Two microliters of cDNA was combined with 50 pmol of primer in the presence of PCR beads (Amersham Pharmacia Biotech) and subjected to PCR amplification for 1 minute each at 95°C, 61°C, and 72°C. We designed primers that amplified 224bp of rat PIGF based on its published sequence (Table 1). The single PCR product was purified and sequence analysis confirmed 99% homology to the published sequence of rat PIGF. Primers were also used to amplify 600bp of rat VEGFR-1

[33](Table 1). Multiplex PCR was performed using primers and competimers for 18S ribosomal RNA (Ambion Inc, Austin, TX) as we have described[29]. PCR products were separated by gel electrophoresis and PIGF/18S and VEGFR1/18S ratios were compared between culture conditions. No amplicon signals were produced from RNA samples not subjected to reverse transcription or from samples without cDNA template added.

Significant increases in PIGF expression by end-point PCR were confirmed by real-time PCR. Gel electrophoresis and melting point analysis confirmed single products of the correct size. Primers for ribosomal protein L32 were used as an endogenous standard[34] and relative changes in PIGF expression were calculated using[35]:

$$\text{Ratio} = \frac{E_{\text{PIGF}}^{(C_T \text{ Normoxia} - C_T \text{ Hypoxia})}}{E_{\text{RPL}}^{(C_T \text{ Normoxia} - C_T \text{ Hypoxia})}}$$

where E = efficiency of the PCR reaction for a primer set, C_T = threshold cycle for each product under each culture condition.

PIGF/VEGFR1 immunohistochemistry

Immunohistochemistry was used to localize PIGF and VEGFR1 protein in mixed cultures of cardiomyocytes and non-cardiomyocytes. Cells were fixed with 10% buffered formalin for 30 minutes at 4°C. For PIGF, cells were incubated overnight at 4°C with a 10 µg/ml mixture (1:1) of affinity-purified goat polyclonal antibodies against the C-terminus and an internal sequence of mouse PIGF (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 0.15% Triton X-100. For VEGFR1 cells were reacted with 4 µg/ml goat polyclonal antibody against VEGFR1 (Santa Cruz Biotechnology). Cells were washed and incubated with 10 µg/ml fluorescein isothiocyanate (FITC) conjugated donkey F(ab')₂ anti-goat IgG and counterstained with 1 µg/ml DAPI. To confirm cardiomyocyte localization, some cultures were sequentially reacted with monoclonal antibody (MF-20) against sarcomeric myosin primary antibody and 10 µg/ml of rhodamine (RITC) conjugated goat F(ab')₂ anti-mouse IgG. Specificity of PIGF/VEGFR1 immunoreactivity was confirmed by incubating cells with primary antibody pre-absorbed overnight with 15-fold excess immunizing peptide.

Statistics

All data are presented as mean ± SEM and all assays were performed in duplicate. Kruskal-Wallis analysis for multiple groups (cell culture data) or the Mann-Whitney U-test for two groups (biopsy data) was used to determine significant differences in immunohistochemical scores as well as fold changes in gene expression. Statistical differences in cardiac troponin I values were assessed by Wilcoxon's rank sum test[25]. Differences in the proportion of biopsies with ISHLT rejection grade ≥R1 or with positive plasma Tn-I titers between groups were assessed by Fisher's exact test. All differences were considered statistically significant if $P < 0.05$.

Results

PIGF mRNA and protein are elevated in damaged human myocardium

As we have shown[27], fibrin deposition in cardiac allografts occurs early after transplantation and biopsies with fibrin were characterized by a significantly shorter period between transplantation and biopsy compared to biopsies without fibrin (Table 2). Biopsies with fibrin were associated with increased macrophage and neutrophil infiltrates; however, there was no significant difference in T-cell infiltrates or proportion of biopsies with ISHLT rejection grade

$\geq 1R$ between groups (Table 2). Serum cardiac troponin-I titers were significantly greater in patients with fibrin in their biopsies (Table 2). Thus, biopsies with fibrin demonstrated immunohistochemical and biochemical indices of myocyte damage in the absence of cellular rejection.

Biopsies from donor hearts and transplanted hearts without myocardial fibrin expressed less PIGF mRNA compared to biopsies from transplanted hearts with fibrin (Figure 1). Fibrin deposition was associated with 1.6-fold increase in PIGF expression (no fibrin = 0.298 ± 0.05 relative units, positive fibrin = 0.472 ± 0.045 relative units; $p < 0.05$). By immunohistochemistry, biopsies with myocardial fibrin also demonstrated PIGF cytoplasmic immunoreactivity in cardiomyocytes and more intense myocyte plasma membrane and interstitial immunoreactivity. Microvessels in/near areas of fibrin deposition also demonstrated PIGF immunoreactivity (Figure 2A). In contrast, areas within these biopsies that were distant from sites of fibrin deposition did not demonstrate PIGF immunoreactivity. Similarly, biopsies without fibrin demonstrated little/no PIGF immunoreactivity (Figure 2B).

There was no significant association between PIGF mRNA expression and degree of macrophage, T-cell, or neutrophil infiltrates or ISHLT rejection grade in the biopsies. However, PIGF expression decreased with increased time since transplantation ($R = -0.792$; $p < 0.001$) and its expression was 1.6-fold greater (0.428 ± 0.06 relative units) in biopsies with reduced vascular antithrombin immunoreactivity compared to biopsies with unique capillary antithrombin (0.270 ± 0.04 relative units; $p < 0.05$). We have shown development of capillary antithrombin binding following fibrin deposition is associated with improved allograft outcome[28]. There was no significant change in VEGFR1, VEGFR2 or NP-1 expression between groups of biopsies (Figure 3). Collectively, these results demonstrate spatial and temporal relationships between increased PIGF expression in biopsies with immunohistochemical and biochemical evidence of myocardial damage. The factor(s) regulating cardiomyocyte PIGF expression in vivo are not known but two potential mechanisms include hypoxic stress or physical stretch.

Cardiomyocyte stretch does not significantly induce PIGF mRNA expression

We assessed expression of PIGF and VEGFR1 in cardiomyocytes that had undergone cyclical stretch for 1, 3, or 6 hours. Although we have shown that this degree/duration of stretch increases VEGF expression in cardiomyocytes[20], it did not significantly alter PIGF or VEGFR1 mRNA expression (Figure 4).

Hypoxia directly induces PIGF mRNA expression

Cardiomyocytes cultured in normoxia showed stable PIGF mRNA expression over 24 hours. Although 1, 3, or 6 hours of hypoxia did not significantly alter PIGF mRNA expression compared to time-matched controls; PIGF expression was significantly increased following 12 and 24 hours of hypoxia (Figure 5). By real-time RT-PCR, twelve hours of hypoxia produced a 3.88 ± 0.62 fold increase ($n = 6$; $p < 0.05$) while 24 hours produced a 3.64 ± 0.41 fold increase ($n = 6$; $p < 0.05$) in PIGF expression. Conditioned media from 12 hour hypoxic cultures did not alter PIGF expression (1.21 ± 0.40 of control; $n = 5$) compared to conditioned media from normoxic cultures. Hypoxia did not significantly alter VEGFR1 expression in cardiomyocytes (Figure 6).

PIGF/VEGFR1 localization in mixed cultures

Cultured cells demonstrated diffuse, cytoplasmic PIGF immunoreactivity that was consistent in both cardiomyocytes and noncardiomyocytes (Figure 7A-C). No immunoreactivity was detected when primary antibody, preabsorbed with excess PIGF antigen, was used (Figure 7D).

Double immunofluorescence experiments also confirmed that cardiomyocytes demonstrated prominent VEGFR1 immunoreactivity (not shown).

Discussion

PIGF plays a more prominent role in pathological rather than developmental cardiac angiogenesis[9,36]. However, factors modulating endogenous PIGF gene expression in myocardial tissue are not known. Our results are the first to demonstrate 1) the presence and upregulation of PIGF expression in human myocardium with myocardial damage, 2) a time course for hypoxic upregulation of PIGF expression in cardiomyocytes, 3) a lack of effect of cyclical stretch on PIGF expression in cardiomyocytes, and 4) the presence of PIGF and receptor protein in isolated cardiomyocytes. Collectively, these results provide important temporal and spatial evidence of increased endogenous PIGF expression following myocardial ischemia.

There is a paucity of data on the expression and function of PIGF in human myocardium. Others have shown that plasma PIGF titers are significantly increased in patients with coronary occlusion and developing collaterals[37] and patients with greater plasma PIGF titers showed greater improvement in left ventricular ejection fraction[4]. However, elevated plasma PIGF titers in patients with acute coronary syndromes have also been associated with greater morbidity/mortality[5-7] suggesting that PIGF is a biomarker of adverse outcomes. Unfortunately, the cellular source of PIGF was not determined in these studies. Our findings show that myocardium with fibrin deposition, cellular infiltrates and elevated troponin I titers exhibited 1.6-fold increase in PIGF mRNA expression while longer-term allografts and those with favorable vascular antithrombin binding had reduced PIGF expression. Furthermore, PIGF protein localization was confined to cardiomyocytes, interstitium and blood vessels in and around areas of fibrin deposition. Additional studies are needed to determine whether plasma PIGF protein titers are useful as a noninvasive biomarker in cardiac transplant patients, similar to acute coronary artery syndrome patients[4-6].

These findings in human allografts, which are similar to those we have shown with VEGF expression[25,29], do not provide a mechanism for the increased expression. Accordingly, we determined the independent effects of cyclical stretch and hypoxia on cardiomyocyte PIGF expression in vitro. We chose to concentrate our studies on isolated cardiomyocytes since they are the major source of VEGF in mouse[38] and human hearts[29] and non-myocyte cells cannot compensate for deficiencies in growth factor production[38].

Cardiomyocyte stretch in vitro [20] and in vivo [21,22] produces a significant increase in VEGF expression that contributes to increased myocardial capillarity[21,22]. However, PIGF mRNA expression does not change under similar stretch conditions. These results are consistent with findings that PIGF plays a more relevant role in pathological angiogenesis rather than physiological vascular growth associated with organ growth[9,36].

Our results show that chronic hypoxia significantly increased cardiomyocyte PIGF expression while shorter periods did not alter its expression. Others have shown increased PIGF expression in hypoxic cardiomyocytes[18] and immortalized fibroblasts[17] after 24 hours but its temporal effect on PIGF expression was not known. Furthermore, these results are strikingly different from those in human trophoblast[15], microvascular endothelial cells[16], and human hepatoma cells (hepG2)[39] where hypoxia reduces or does not alter PIGF expression. The mechanism for the delay in hypoxia-induced PIGF expression in cardiomyocytes is not known; however, these results correspond to clinical findings where a single brief episode of provoked cardiac ischemia in humans did not significantly increase plasma PIGF titers[40]. Clearly, the

effect of hypoxia on PlGF expression is cell type specific and temporally regulated and additional studies are needed to understand PlGF gene regulation in cardiomyocytes.

Conditioned media from hypoxic cardiomyocytes did not induce PlGF expression suggesting that hypoxia directly upregulates PlGF expression. The degree of hypoxia used in the present study (<2% O₂) produces ~10-fold increase in HIF-1 binding activity[41] and HIF-1_{alpha}, in the absence of hypoxia, has been shown to upregulate PlGF expression in cardiomyocytes [18]. PlGF promoter reporter studies in mouse fibroblasts have shown that hypoxic induction of PlGF is regulated, in part, by metal response element-binding transcription factor 1[17]. Nuclear factor – kappa B elements may also function to increase the hypoxic responses of PlGF in some cell types[42]. Thus, it remains to be determined whether hypoxic induction of PlGF expression in rat cardiomyocytes is wholly regulated by HIF-1_{alpha} activity. Our data also confirm that isolated cardiomyocytes express VEGFR1 mRNA and protein as others have shown in situ[43]. Although the physiological effect of PlGF-VEGFR1 interactions in cardiomyocytes is not known, it has been shown to protect other cell types from stress-induced apoptosis[9,44].

In summary, our results show that cardiomyocytes express PlGF as well as its receptor and that PlGF expression is increased by hypoxia in vitro and by ischemia in vivo. Coupled with functional data from relevant animal models[9,45], we hypothesize that increased cardiomyocyte PlGF expression may function to amplify the angiogenic response and/or provide a novel protection mechanism against cell death following myocardial hypoxia.

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Figure 1.

Expression of PIGF mRNA in biopsies of human myocardium. Top panel: Total RNA from biopsies of donor myocardium (D) and biopsies from allografts without (-) and with (+) myocardial fibrin was reverse transcribed and subjected to PCR amplification for human PIGF. Products: PIGF = 318bp; 18S (classic) = 488bp. 100bp = 100bp ladder. Bottom panel: Biopsies with fibrin deposition (n = 7) expressed 1.6-fold more PIGF mRNA than biopsies without fibrin deposition (n=11; *p < 0.05).

Figure 2.

Localization of PlGF immunoreactivity in biopsies of human myocardium with (A) and without (B) fibrin deposition. A) Areas of myocardium with fibrin deposition also demonstrated PlGF immunoreactivity in the interstitium, cytoplasm of cardiomyocytes and the endothelium of some microvessels (arrows). There was little/no PlGF reactivity in areas within these biopsies that were distant from sites of fibrin deposition. B) Little/no PlGF immunoreactivity was noted in biopsies devoid of fibrin deposition. Hematoxylin counterstain; magnification = 400x in both panels.

Figure 3.

Expression of PIGF and VEGF receptor mRNA in biopsies of human myocardium. Top panel: Total RNA was reverse transcribed and subjected to PCR amplification for VEGF/PIGF family of receptors (VEGFR-1, VEGFR-2, NP-1). Products: VEGFR-1 = 239bp; VEGFR-2 = 201bp; NP-1 = 210bp; 18S (classic) = 488bp. (-) = no fibrin; (+) = positive fibrin; 100bp = 100bp ladder. Bottom panel: Biopsies with fibrin (n = 7) showed no statistically significant changes in VEGFR1, VEGFR2, or NP-1 mRNA expression compared to biopsies without myocardial fibrin deposition (n = 11).

Figure 4.

Effect of cyclical stretch (10% elongation) on PIGF and VEGFR1 mRNA expression in isolated neonatal rat cardiomyocytes. Total RNA from cardiomyocytes that were not stretched (0 hour) or stretched for 1, 3, or 6 hours was reverse transcribed and subjected to PCR amplification.

Top panel: cyclical stretch (n=5 at each time point) did not significantly alter PIGF mRNA expression compared to control cells. Products: PIGF = 225bp; 18S (classic) = 488bp. 100bp = 100 bp ladder. Bottom panel: cyclical stretch (n=5 at each time point) did not significantly alter VEGFR1 mRNA expression compared to control cells. Products: VEGFR1 = 600bp; 18S (classic II) = 324bp. 100bp = 100bp ladder.

Figure 5.

Effect of hypoxia on PlGF mRNA expression in isolated neonatal rat cardiomyocytes. Total RNA from cardiomyocytes cultured for 1, 3, 6, 12, or 24 hours in $<2\% \text{O}_2$ or control ($21\% \text{O}_2$) conditions was reverse transcribed and subjected to PCR amplification. Compared to time-matched normoxic cultures, hypoxia did not significantly alter PlGF mRNA expression at 1, 3 or 6 hours. Twelve and 24 hours of hypoxia significantly increased PlGF mRNA expression by 108% and 118%, respectively. $*p < 0.03$ from normoxic controls ($n = 6-7$ cultures at each time point). N = normoxia, H = hypoxia. Products: PlGF = 225bp; 18S (classic) = 488bp. 100bp = 100bp ladder.

Figure 6.

Effect of hypoxia on VEGFR1 mRNA expression in isolated neonatal rat cardiomyocytes. Total RNA from cardiomyocytes cultured for 1, 3, 6, 12, or 24 hours in $<2\% \text{O}_2$ or control ($21\% \text{O}_2$) conditions was reverse transcribed and subjected to PCR amplification. Compared to time-matched normoxic cultures, hypoxia did not significantly alter VEGFR1 mRNA expression throughout the culture periods ($n = 6-7$ cultures at each time point). N = normoxia, H = hypoxia. Products: VEGFR1 = 600bp; 18S (classic II) = 324bp. 100bp = 100bp ladder.

Figure 7.

Immunolocalization of PIGF protein in cardiomyocytes and non-cardiomyocytes. A) Micrograph showing PIGF immunoreactivity (green) and nuclear DAPI reactivity (blue). Note the diffuse, cytoplasmic reactivity for PIGF in all cells within the field. B) Photomicrograph showing monoclonal antibody to sarcomeric myosin (red) and nuclear DAPI reactivity (blue). Note that the majority of cells in the field are cardiomyocytes (closed arrowhead), but noncardiomyocytes, presumably cardiac fibroblasts, are also present (open arrowhead). C) Merged panels A+B showing PIGF (green), sarcomeric myosin (red) and nuclear DAPI (blue). Note that both cardiomyocytes and non-cardiomyocytes demonstrate relatively similar PIGF immunoreactivity. D) Negative control photomicrograph using anti-PIGF antibodies pre-incubated with excess immunizing peptide followed by FITC-conjugated secondary IgG and DAPI (1ug/ml). Note the lack of PIGF immunoreactivity (green) in the cells. Magnification = 400x.

Table 1**Human and Rat Primers**Human Primers:

PIGF(S): AAG ATG CCG GTC ATG AGG C

PIGF(AS): CTG CAT GGT GAC ATT GGC

VEGFR1(S): GAC GTC TAG AGT TTG ACA CGA AGC

VEGFR1(AS): GCA TGC AAC ACT GAG TAA CAT GAC

VEGFR2(S): TAA GCT TAC CAA AGG GGC ACG ATT CCG T

VEGFR2(AS): TGG ATC CCT GTA ACA GAT GAG ATG CTC C

NP-1(S): AGG ACA GAG ACT GCA AGT ATG AC

NP-1(AS): AAC ATT CAG GAC CTC TCT TGA

Rat primers:

PIGF(S): CAG CCA ACA TCA CTA TGC AG

PIGF(AS): TCC TCT GAG TGG CTG GTT A

VEGFR-1(S): TCA TGC AAG CGG GCC AGA CTC TCT TTC

VEGFR-1(AS): ATC TCA CGG GGC TCG GCG GGC TTA TTT

PIGF = placenta growth factor; VEGFR1 = vascular endothelial growth factor receptor 1; VEGFR2 = vascular endothelial growth factor receptor 2.

Table 2

Characteristics of biopsies without (–) and with (+) fibrin deposition

	(–) Fibrin	(+) Fibrin	p-value
Number	11	7	
Fibrin score[27]	1.32 ± 0.12	2.57 ± 0.07	< 0.01
Time since transplant (days):*			
Mean ± SEM	3105 ± 584	1138 ± 537	< 0.03
Range	36 – 4985	11 – 3851	
Macrophage score	1.55 ± 0.18	2.29 ± 0.15	< 0.03
T-cell score	1.50 ± 0.19	1.86 ± 0.21	NS
Neutrophil score	1.36 ± 0.15	1.93 ± 0.17	= 0.05
ISHLT Grade (# ≥ 1R)[26]	2/11 (18%)	2/7 (29%)	NS
Plasma Tn-I:*			
Mean ± SEM (ng/ml)	0.043 ± 0.04	1.31 ± 0.46	< 0.002
# Tn-I positive samples	1/9 (11%)	7/7 (100%)	< 0.002

* Excludes donor tissue; NS = not statistically significant.