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**Exogenous Endothelial Progenitor Cells Contribute to
Neovascularization in A Murine Model of Myocardial Injury**

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ABSTRACT

Circulating human hematopoietic stem cells, identified by their CD34 antigenicity, have been shown to contribute to peripheral *vasculogenesis* as evidenced by their incorporation into actively growing vessels of experimentally induced ischemic hind limbs. The purpose of the present study was to test whether CD34+ cells migrate, differentiate and assemble into new vessels at sites of myocardial injury. Hearts of 17 athymic mice were injured by multiple transmyocardial punctures with a 25g needle; 2 athymic mice underwent sham operations. Two days later, the main experimental group of 8 mice received an intravenous injection (via the tail vein) of approximately 2×10^5 CD34+ cells isolated from normal human donors which were labeled with the fluorescent dye DiI. The other mice were divided into 4 other groups which served as controls. These consisted of 2 sham operated mice followed by DiI labeled CD34+ cell injections, 2 mice with myocardial injuries without any cell injections, 5 mice with myocardial injury plus injection of DiI labeled peripheral mononuclear cells after extraction of CD34+ cells and 2 mice with myocardial injury followed by intravenous injection of DiI (no cells). Mice were sacrificed 14 days later and histologic analysis, including immunostaining and in situ hybridization, was performed. DiI fluorescence was identified exclusively in the injured regions of the hearts. Within these regions, capillaries and larger vessels were identified which were lined with elongated, flattened DiI+ cells; in many instances these vessels were completely composed of the DiI+ cells. Co-localization of DiI fluorescence and factor VIII, CD31 and HLA1 confirmed that these were the injected human cells which had been incorporated into, or perhaps formed, vascular structures. A large number of DiI+ cells localized to the interstitial areas of the injury also stained positive for factor VIII, CD31 and eNOS gene expression even though they did not incorporate into vessels nor take on the appearance of

endothelial cells. Lung, kidney, liver and spleen of all animals and hearts of sham operated animals were devoid of DiI+ fluorescence. Thus, exogenous endothelial progenitor cells migrate to injured myocardium. Complete vessels can be formed de novo in the heart from these cells (true *vasculogenesis*). The use of exogenous progenitor cells may provide be useful as one component of strategies to form new vessels in the hearts of patients with severe coronary artery disease.

INTRODUCTION

Bypass surgery and angioplasty are effective therapies for treating discrete coronary stenoses. However, there is a significant number of patients with diffuse arterial stenoses who cannot be adequately revascularized by these techniques. Accordingly, significant efforts are being devoted to investigating new forms of therapies to induce vascular growth in the heart such as the use of growth-factors (1-5). Results of recent studies have suggested that certain hematopoietic stem cells, identified by their surface CD34 antigenicity, are endothelial precursor cells (4,6-11). These cells have been shown to assemble into vascular-like tubes in cell culture (8). *In vivo*, they colonize surfaces of vascular prostheses and left ventricular assist devices, differentiate into endothelial cells and contribute to endothelialization of these artificial structures (6,12). In a murine model, they have been shown to migrate to ischemic hind limb tissue where they incorporate into vessels and differentiate into functional endothelial cells (8). Such data have collectively been interpreted as indicating that vasculogenesis, a process previously considered to be confined to the embryonic period, can be present in adults. The therapeutic implication of these findings is that endothelial progenitor cellular therapy may provide an approach to building new vessels *in situ*. Until recently (28), prior studies of the ability of endothelial progenitor cells to contribute to vascular growth have been confined to studies in the ischemic hind limb and to surfaces of prostheses. The purpose of this study was to test the hypothesis that endothelial progenitor cells could contribute to neovascularization in a murine model of myocardial injury. Rather than employing a model of myocardial ischemia in which, based on the prior study by Asahara *et al.* incorporation of progenitor cells into otherwise preexistent vessels would be expected, we employed a model of more severe tissue injury in order to test whether more complete vascular structures, such as were identified *in vitro*, could be identified (8). The results of this study show that vascular tubes composed of the progeny of these circulating CD34⁺ cells can assemble *in vivo*, thus providing

further support for the notion that vasculogenesis can occur in adults and further suggesting a potential therapeutic role of these cells.

METHODS

Preparation of CD34⁺ Cells. Peripheral blood from normal human donors was anticoagulated with citrate-phosphate dextrose (Sigma) and diluted 1:3 by volume with phosphate buffered saline (PBS). 30 cc of diluted blood was then loaded on top of 15 cc Histopaque-1077 (Sigma) in a 50 cc Falcon tube which was then centrifuged at 400g for 30 minutes. The mononuclear phase was collected and the remaining red blood cells were lysed with NH₄Cl solution (NH₄Cl 0.155M, NaHCO₃ 12mM, EDTA 10uM) at room temperature for 6 min. Cells were washed with PBS containing 2% fetal bovine serum (FBS) 3 times with centrifugation at low speed (250g, 5 minutes each) and the nucleated cells were collected and resuspended at a concentration of $2-8 \times 10^7$ cells/ml. This suspension was then incubated on ice for 30 minutes with human monoclonal antibody cocktail (MoAb, catalogue number ST-230H, StemCell Technologies Inc, Canada) optimized to capture cells other than CD34⁺ cells; as specified in the product instructions, 0.1 ml MoAb solution was used for each 1 ml of cell suspension. Cells were then incubated with magnetic colloid (StemCell Technologies, Inc, 0.06 ml colloid added for each 1.1 ml of cell suspension) on ice for another 30 min. The cells were then eluted through a gravity-fed magnetic column system (StemCell Technologies, Inc.) and MoAb free-colloid free CD34⁺ cells passed through and were collected. Eluted cells were then washed and resuspended in Dulbecco's Modified Eagle Medium (GibcoBRL, Life Technologies, Grand Island, NY) with 2% FBS for subsequent use.

Fluorescence-activated cell sorting (FACS) was performed to determine the CD34 purity of the eluted cells. The eluted cells were incubated with mouse anti-CD34 IgG followed by anti-mouse IgG conjugated with FITC at room temperature for 30 min each. After washing with PBS, the cells

were analyzed using standard techniques with a FACScan Flow Cytometer.

To further characterize the cells isolated in this manner, cells were placed on glass slides and fixed with 2% paraformaldehyde. To confirm CD34 positivity, the slides were incubated with mouse anti-CD34 antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA). After PBS wash, the slide was incubated with a goat anti-mouse IgG conjugated with FITC (Becton Dickinson) and was visualized under fluorescent microscope. To investigate the endothelial phenotype of these cells, separate slides were hybridized with RNA probe for eNOS (*in situ* hybridization, detailed below), were stained with antibody against Von Willebrand Factor (Factor VIII, DAKO) and were stained with antibodies against CD31 (Becton Dickinson).

For use in animal experiments, the eluted cells were labeled with the fluorescent dye DiI (StemCell Technologies Inc) at a concentration of 10 μ M at 37°C for 2 hours. After labeling, the cells were washed and resuspended in medium for injection into animals.

Animal Preparation. 6-week old male athymic nude mice (Harlan Sprague Dawley, Inc.) were anesthetized with peritoneal injection of ketamine (90 mg/kg) and xylazine (6 mg/kg), the trachea intubated and the lungs mechanically ventilated. A left thoracotomy was performed and the heart exposed. Myocardial injury was created by 10-20 transmural myocardial punctures with a 25 gauge needle. It was determined in preliminary studies that on a histological basis, the injury created by this method was indistinguishable from myocardial infarction but resulted in a more uniformly sized injury from mouse to mouse and a lower overall mortality. The chest was closed in layers and the animals allowed to recover from anesthesia. Two days after the operation, approximately 2×10^5 CD34+ cells labeled with fluorescent dye were administered intravenously to each animal via the tail vein. Mice were sacrificed 14 days later, organs harvested and histologic analysis was performed. Kidneys, liver, spleen, lung and brain were examined in addition to heart to determine whether there was any appearance of DiI or DiI labeled cells outside of the heart.

Nineteen mice, each assigned to one of five groups, were used for this study. The main experimental group (Group A) consisted of 8 mice receiving myocardial injury followed by injection with DiI-labeled CD34+ cells. The remaining four groups of animals constituted various control groups. Sham operations (thoracotomy without myocardial injury) followed by DiI labeled CD34+ cell injections were performed in 2 animals (Group B). Myocardial injury without any cell injections were performed in 2 animals (Group C). Myocardial injury followed by injection of DiI labeled peripheral mononuclear cells after extraction of CD34 positive cells were performed in 5 animals (Group D). Myocardial injury followed by intravenous injection of DiI was performed in 2 animals (Group E).

Histologic analysis. At the time of sacrifice, tissues were harvested and submersed in a Formal calcium solution containing 1% calcium chloride and 4% formaldehyde at 4°C for 18 hours. After H₂O rinse, the tissues were fixed in a 1% gum acacia and 30% sucrose solution at 4°C for another 18 hours. Tissues were then frozen at B20°C, cut into 6-8 um sections and mounted on slides (Superfrost Microscope Slides, Fisher Scientific).

Frozen tissue sections were visualized without any further preparation with fluorescence microscopy in order to detect the presence of DiI fluorescence. To identify the injured regions, hematoxylin-eosin (H&E) staining was performed using standard techniques. In brief, the sections were submersed in Harris hematoxylin solution (Poly Scientific) for 5 min, rinsed with 0.5% ethanol acid and then rinsed 3 times (0.5 min each) in ammonium hydroxide solution at pH 8.0 (Fisher). The section was then placed in eosin solution (Sigma) for 3-5 seconds followed immediately by gradient dehydrating in ethanol and xylene and was then permounted.

Standard immunohistochemical techniques were used to further characterize the tissue samples. Monoclonal anti-human Factor VIII, anti-human HLA1 (Accurate Chemical & Science, NY) and anti-human CD31 and CD34 antibodies were used for this purpose. eNOS *in situ*

hybridization was also performed. For immunostaining, the sections were digested with 50 μ g/ml of proteinase K (Sigma) in Tris solution containing 50 mM EDTA, 0.1 M Tris-HCl, pH 8.0, for 15 min at 37°C. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in PBS for 7 min. After PBS wash, the sections were blocked with 10% horse serum for 20 min followed by different antibody incubations. For Factor VIII staining, the sections were incubated with Factor VIII monoclonal antibody conjugated with horseradish peroxidase (DAKO, Denmark) for 1 hour at 37°C followed by peroxidase activity reaction with diaminobenzidine (DAB, Sigma). For HLA1 staining, the blocked sections were incubated with mouse anti-human HLA1 monoclonal antibody at 4°C over night. With intervening washes in PBS, sections were then incubated over night again at 4°C with goat anti-mouse IgG conjugated with fluorescence FITC. Free fluorescence was washed off in PBS the next day. For CD31 and CD34 immunostaining, the serum blocked sections were incubated with mouse anti-human CD31 and mouse anti-human CD34 monoclonal antibodies separately at room temperature for 1 hour. After washing with PBS, the sections were then incubated with a biotinylated horse anti-mouse IgG (Vector Laboratory Inc., CA.) and the avidin-biotin-immunoperoxidase complex (ABC, Vector) at room temperature for 30 min each. With intense PBS wash, the peroxidase activity was visualized with DAB substrate yielding a brown cytoplasmic reaction product.

In Situ Hybridization for eNOS mRNA. Freshly isolated CD34+ cells fixed on slides and the 6-8 μ m thick sections were digested with 15 μ g/ml proteinase K in Tris solution for 30 min at 37°C. After rinsing in 0.2% glycine to stop digestion, the sections were acetylated in 0.25% acetic anhydride containing 0.1 M triethanolamine for 10 min. The sections were arranged in moist chambers and prehybridized for 2 hours at 37°C in a solution containing 50% formamide, 2x SSC, 1x Denhardts, 10% dextran sulphate, 0.1% SDS, 4 mM EDTA and denatured salmon testis DNA. Hybridization was then performed overnight at 42°C with the addition of eNOS RNA probe labeled

by transcription of cDNA with digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN). An antisense probe for eNOS was used for each sample as a positive hybridization. Each section was covered with 15-30 μ l of solution with a probe concentration of 10 μ g/ml. The slides were washed in 2 to 0.1x SSC and digoxigenin buffer (0.1 M Tris-HCl; PH 7.5; 0.15 M NaCl). After blocking with 10% normal rat serum, the sections were incubated for 2 hours with alkaline phosphatase conjugated anti-digoxigenin polyclonal sera (Boehringer Mannheim), diluted 1:750 in digoxigenin buffer containing 10% normal rat serum. The bound antibody was detected by a standard immunoalkaline phosphatase reaction using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as substrate (Boehringer Mannheim). After development, sections were lightly counterstained with ethyl green and permounted. The mRNA signals were then observed under bright field microscopy.

Probe generation. RNA probes for eNOS *in situ* hybridization were synthesized by *in vitro* transcription of cDNA labeled with digoxigenin-UTP. Bovine eNOS cDNA of 340 base pairs 5' was inserted into Bluescript II KS +/- vector of T7 promotor side. The orientation of T3 polymerase transcription is antisense. Xba I and PVU II linearized antisense DNA template was purified from electrophoresed agarose gel and "run off" transcript was created using digoxigenin RNA labeling system (Boehringer Mannheim). Prior to hybridization, the probe was analyzed for size on a 1% agarose gel and labeling efficiency was detected by hybridizing the dot blot using T7 sense transcript as negative control.

The use of animals and the collection of human blood samples for this protocol were approved by Columbia University's Institutional Animal Use and Care Committee and Institutional Review Board, respectively.

RESULTS

Isolation and Characterization of CD34+ Cells in Vitro. Fluorescence-activated cell sorting analysis indicated that significantly less than 1% of circulating mononuclear cells (identified by CD45 positivity) were positive for CD34 (Fig. 1A). Between 0.3% and 1% of all peripheral mononuclear cells were freely eluted through the StemSep Magnetic Column. Approximately 64% of the mononuclear cells isolated in this manner were CD34 positive (Fig. 1B) indicating significant CD34 enrichment using this technique.

Immunostaining of the selected cells plated on slides with antibodies against CD34 showed cell surface expression of this antigen (Fig. 2A). Immunostaining with antibodies against human HLA1 showed more uniform cellular expression (Fig. 2B). Factor VIII antigenicity co-localized with CD34 positivity (Figs. 2C and 2D), but the cells were negative for CD31 (Fig. 2E), indicating a partial endothelial phenotype of these circulating cells. *In situ* hybridization for eNOS expression was also positive (Fig. 2F), further suggesting at least partial endothelial phenotype of these cells.

Needle Puncture Creates Chronic Myocardial Injury. Repeated myocardial puncture with a 25 gauge needle produced grossly recognizable myocardial injury which was evident 16 days after surgery. Histologically, the hearts showed characteristic changes of myocardial injury, including mononuclear inflammatory cell infiltration, myocyte necrosis, connective tissue proliferation and fibrosis in the injured area. Two examples of the histologic appearance of 16 day old injury are shown in Fig. 3. By varying the number of needle punctures, the degree of myocardial injury can be controlled to involve a relatively small, discreet region (Fig. 3A) or a large, transmural region (Fig. 3B). The histologic appearance of myocardium injured in this manner is similar to that of a myocardial infarction due to an ischemic insult.

CD34+ Cells Migrate to Sites of Myocardial Injury and Participate in Neovascularization. Eight mice with myocardial injury were injected with DiI labeled CD34+ cells. The low magnification microscopic image of Fig. 4A shows the overall appearance of a region of injured myocardium of a mouse into which the CD34+ DiI cells were injected; the presence of DiI labeling is more clearly seen in the higher power magnification view of Fig. 4B. The DiI fluorescence can be identified even in this low magnification image and is seen to be limited exclusively to the injured region, with no fluorescence in the adjacent areas of normal myocardium. As seen in these figures, DiI labeled cells sometimes appeared randomly in the interstitium (arrow *a*), sometimes appeared to be arranged in lines (arrow *b*), and sometimes appeared to be forming circular rings with a centrally cleared space (arrow *c*). In comparison, animals in which myocardial injury was induced and DiI was injected intravenously exhibited no fluorescence in either injured or non-injured myocardial regions.

Figure 5 shows images from adjacent tissue sections of a new vessel identified within a region of myocardial injury near the epicardial surface. A fresh frozen section was visualized under fluorescence microscopy with a DiI filter to reveal a vascular structure (panel A). This same section was then immunostained for human HLA-1 with a FITC-conjugated antibody (panel B). The positive staining confirms that the cells which compose this structure are of human origin; the DiI signal of this section was lost after HLA-1 staining procedure (image not shown). This same section next showed strong Factor VIII immuno-positivity (panel C) which confirms the endothelial phenotype of the cells lining this structure. Immunostaining of a different adjacent tissue sections showed this structure to be CD34 negative (panel D) but CD31 positive (panel E). Thus, the injected cells had differentiated further into endothelial cells, having lost CD34 and gaining CD31 antigenicity.

Other examples of vessels with DiI positivity and positive Factor VIII staining are shown in Fig. 6 (panels A-F). As in the previous example, these vessels were located exclusively in regions of myocardial injury. However, not all vessels in the injured area incorporated DiI positive cells

(Fig. 7A-C); in particular, large vessels with at least one layer of smooth muscle never showed incorporation of DiI labeled cells. Additionally, vessels in the normal region were never DiI positive (Fig. 7A-C) no matter what their size..

As noted above, a large number of DiI labeled cells appearing in the injured area do not appear to be incorporating into blood vessels. Nevertheless, these cells expressed Factor VIII, eNOS and CD31 (Fig 8).

Examination of other organs, including kidney, liver and lung of all animals were devoid of DiI+ cells or DiI fluorescence. This suggests that the migration and concentration of the cells to the heart was mediated by injury related chemotactic factors and/or local expression of adhesion molecules in the injured area. These cells did appear, however, in the bone marrow with relatively high frequency.

In animals receiving myocardial injury followed by injection of DiI labeled peripheral mononuclear cells after extraction of CD34 positive cells (Group D, n=5) interstitial DiI fluorescence was observed in the injured area similar to that identified with the CD34 positive cells. However, DiI+ vessels were never seen in these animals. Animals receiving sham operations (thoracotomy without myocardial injury) followed by DiI labeled CD34+ cell injections (Group B), animals receiving myocardial injury without cellular injection (Group C), and animals receiving myocardial injury followed by intravenous injection of DiI (Group E) showed no DiI fluorescence in the heart or any other organ examined.

DISCUSSION

Circulating cells with CD34 surface antigenicity have been considered to represent endothelial progenitor cells (11,13,14). The finding that these cells, when freshly isolated from peripheral blood of normal human subjects, are morphologically similar to other circulating mononuclear cells but express Factor VIII and eNOS suggests that these cells are already differentiating along the endothelial cell lineage. In a recent study, it was further demonstrated that even after releasing bone marrow cells into the blood stream using granulocyte-colony stimulating factor (G-CSF), only a small portion of circulating CD34⁺ cells have cell surface markers consistent with a more primitive progenitor state (i.e., CD117) (28). Nevertheless, the CD34 antigenicity and the lack of CD31 surface antigenicity after isolation indicates that the cells are not fully differentiated. The change in morphology of cells incorporating into new vessels, the appearance of CD31 surface antigenicity and loss of surface CD34 antigenicity are evidence of further differentiation once they incorporate into the tissue.

Prior studies of circulating CD34⁺ cells have demonstrated their ability to localize to non-endothelialized sites in contact with blood, such as synthetic vascular grafts (6) and the surface of left ventricular assist devices (12). These cells have been shown to appear in the endothelial lining of vessels in the ischemic hind limbs in mice (8). They have been shown to assemble into tubular, vascular-like structures *in vitro* (8). However, the present findings are the first to demonstrate that these cells can assemble into complete vascular structures *in vivo*.

The finding that the same tissue section serially examined for DiI fluorescence and then for human HLA-1 antigenicity provided independent confirmation of the human origin of the cells composing the new vessels. This excludes the possibility that the DiI label diffused out of the loaded cells and was taken up by preexistent endothelial cells.

Injected CD34⁺ cells were found to distribute exclusively in the area of myocardial injury; no

cells were ever identified in normal regions of myocardium or in any other organ other than bone marrow, including the lung. The latter finding is significant since the cells were injected intravenously and had to pass through the lung in order to reach the heart. These findings were true for both CD34⁺ cells and for the peripheral mononuclear (i.e., the non-CD34 enriched) cells. In both cases, cells incorporated abundantly into the interstitium and, only for the case of CD34⁺ enriched cell injections, formed the entire circumference of small endothelialized vessels. In mice receiving intravenous DiI, no fluorescence was found in any organ, indicating lack of nonspecific DiI uptake in any region of the body, including the injured hearts.

Although beyond the intended scope of the present study, it is pertinent to note that the mechanisms controlling the injury site-specific adhesion and migration of the progenitor cells into the myocardial interstitium with their subsequent incorporation and assembly into tubular vascular structures is not known. In fact, the mechanisms controlling migration and assembly of these cells into tubes are not even understood for the simpler *in vitro* situation (8). The mechanisms underlying adhesion and migration are clearly not specific for CD34⁺ cells, since the CD34⁻ cells exhibited the same propensities and injury-site specificity. However, only the CD34⁺ cells assembled into tubes. Cardiac injury of any kind is accompanied by an acute inflammatory reaction mediated initially by neutrophils. Directional rearrangement of the actin cytoskeleton within the neutrophil, along with modulation of integrin-mediated adhesion appear to be necessary for neutrophil migration and adhesion to inflamed blood vessels (15,16). Recent data indicate that stromal cell-derived factor-1 (SDF-1) is a potential chemo-attractant for human pro- and pre-B cells including CD34⁺ cells, which acts via the chemokine receptor CXCR4 (17-21). Furthermore, the chemotactic function of SDF-1 appears to be enhanced by IL-3 (20). Additional data demonstrating an increased rate of integrin-supported neutrophil migration induced by platelet/endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and p-selectin suggest that signals from other chemokines may also play a role in the mobilization and adhesion of human CD34⁺ to blood vessels (22,23).

CD34 is a cell surface glycoprotein that is selectively expressed within the hematopoietic system on stem cells and progenitor cells, and in primitive vascular endothelial cells (11,24,25). The loss of CD34 surface antigenicity on DiI labeled cells in hearts in this study is consistent with the loss of this antigen on endothelial cells postnatally (26). Disappearance of CD34 antigenicity after differentiation has suggested to some investigators that the CD34 molecule itself may participate in migration and differentiation, and possibly may itself be a cell adhesion molecule (13). CD31, another integral membrane glycoprotein, is also expressed on endothelial cells and appears to play a role in inter-endothelial cell adhesion, trans-endothelial migration and angiogenesis (27). In our study, CD31 antigenicity intensively emerged on DiI⁺ cells lining the inner layer of the new vessels in the injured area.

A vast majority of DiI labeled cells in the CD34⁺ group appeared in the interstitium of the injured area and did not appear to be forming blood vessels. It should be recalled that in the group of cells considered to be CD34⁺, however, approximately 40% of the cells were actually CD34⁻ as determined by FACS analysis. Thus, the appearance of DiI labeled cells in the injured region likely reflects the adhesion and migration of both CD34⁺ and CD34⁻ cells. However, it is certain that not all of the cells found in the interstitium were of CD34⁻ origin, since we observed strong Factor VIII expression in these cells, a feature not expected from cells in the CD34⁻ lineage.

The present animal model mimics instances of severe myocardial injury, and the results are meant as proof of the concept that new blood vessels can be formed from progenitor cells. From a therapeutic standpoint, the goal would be to create such vessels in the absence of overt injury in regions which are subject to periodic episodes of myocardial ischemia (typically occurring only during physical stress). In addition, it would be advantageous to be able to generate larger conduit vessels which typically include smooth muscle layers. In the present study, CD34⁺ cells never incorporated into such large vessels (even in the core of the injured area) nor did any vessels composed of CD34⁺ cells ever exhibit any smooth muscle layers. Deeper understanding of the

factors controlling a host of processes involved in the generation of conduit vessels will be required to achieve such goals. Information derived from studies of the embryology of vascular growth may provide important clues.

In summary, the present study provides evidence that circulating human endothelial progenitor cells migrate to injured murine myocardium and can form new blood vessels. In this experimental model, migration and differentiation of these cells occur in close temporal association with the process of cardiac wound healing and neovascular angiogenesis, suggesting regulation by chemotactic factors released by damaged tissues. Deeper understanding of the mechanisms which control adhesion, migration and assembly of these cells into mature vascular structures may lead to novel forms of therapy for patients with severe vascular disease. The model employed (needle injury of the heart), although not mimicking the setting in which such therapies would ideally be applied, provided a convenient and important setting for studying these phenomena *in vivo*.

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FIGURE LEGENDS

Figure 1. Fluorescence activated cell sorting (FACS) was used to determine the purity of CD34+ cells obtained with the antibody-based cell separation technique described in **METHODS**. Cells were double stained with CD45 and CD34 antibodies to determine lymphocyte and stem cell populations, respectively. **A.** Staining of all isolated cells prior to CD34 enrichment showed that approximately 0.4% of all peripheral blood lymphocytes (PBL) are CD34+. **B.** Approximately 64% of cells passing through the separation column are CD34+. In each panel, the shaded area shows the CD34 staining while the unshaded area shows the isotype control.

Figure 2. Histologic examinations of isolated cells. **A.** Immunostaining confirms a predominance of CD34 antigenicity of isolated cells. **B.** Immunostaining with antibodies against human HLA1 showed more uniform cellular expression of this antigen. Factor VIII antigenicity (**C**) co-localized with CD34 positivity (**D**) on the same slide. **E.** Isolated cells were CD31 negative. **F.** *In situ* hybridization for eNOS expression was also positive, indicating an endothelial phenotype of these cells. Calibration bar shows 20µm for panel A, 50 µm for panels B-D and 100 µm for E and F.

Figure 3. Histologic appearance of myocardium 16 days after needle injury. By varying the number of needle punctures, the degree of myocardial injury can be controlled to involve a relatively small, discrete region (**A**) or a large, transmural region (**B**). Calibration bar shows 0.5 mm for panel A and 0.125 mm for panel B.

Figure 4. DiI fluorescence appears exclusively in the region of injured myocardium. **A.** Low magnification image of a region of injured myocardium. **B.** Same region at higher magnification. DiI labeled cells typically appeared randomly in the interstitium (arrow *a*), but elongated DiI labeled cells were also seen to be arranged in straight lines (arrow *b*), and sometimes appeared to form circular rings with centrally cleared space like a vessel (arrow *c*). Calibration bar shows 200 μm for panel A and 50 μm in panel B.

Figure 5. Images of a new vessel identified within a region of myocardial injury. **A.** DiI fluorescence reveals a vascular structure. **B.** The very same section shown in A was next immunostained with FITC-conjugated human HLA-1 confirming the human origin of these cells. DiI fluorescence was lost through the immunostaining process. **C.** The very same section was next immunostained for Factor VIII which was positive. **D.** Immunostaining of an adjacent tissue sections showed this structure to be CD34 negative. **E.** However, the cells were CD31 positive. Calibration bar is 100 μm for all panels.

Figure 6. Other examples of vessels identified on H&E sections (panels A and D) which are DiI positive (panels B and E, respectively) and also stain positive for Factor VIII (panels C and F, respectively). As in Fig. 5, these vessels were located in regions of myocardial injury. Calibration bar shows 100 μm for panels A, B, C and E and 200 μm for panels D and F.

Figure 7. Large, mature vessels in normal region were never DiI positive (A-C) no matter what their size. Additionally, most vessels in the injured area did not incorporate DiI (D-F), especially large vessels with at least one layer of smooth muscle. Calibration bar shows 100 μm for panels A-C and 25 μm for panels D-E.

Figure 8. A large number of DiI labeled cells appeared in the interstitium of the injured area which did not appear to form vessels (A, C and E). Nevertheless, these cells expressed Factor VIII (B, corresponding to A), eNOS (D, corresponding to C) and CD31 (F, corresponding to E). Calibration bar is 200 μm for all panels.

FIGURE 1

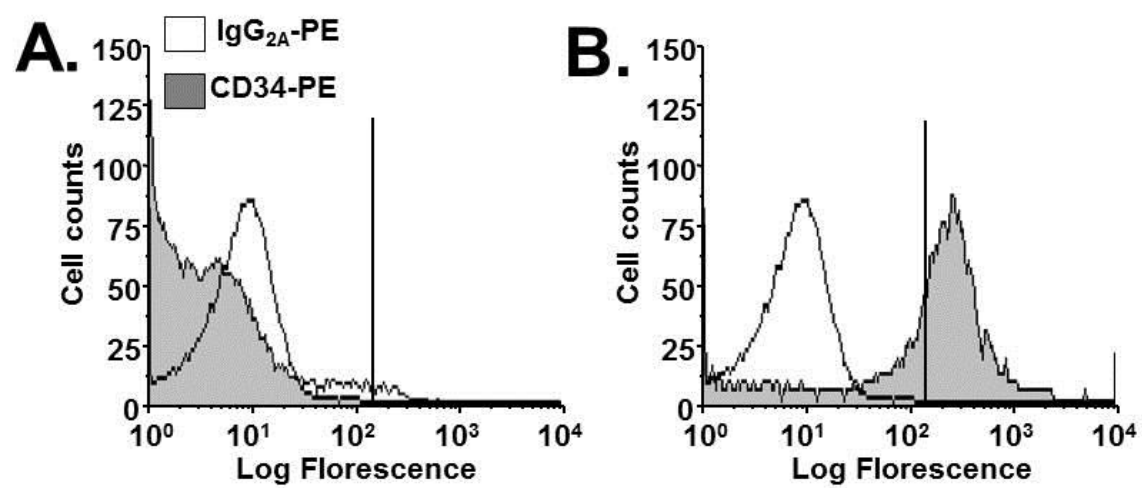


FIGURE 2

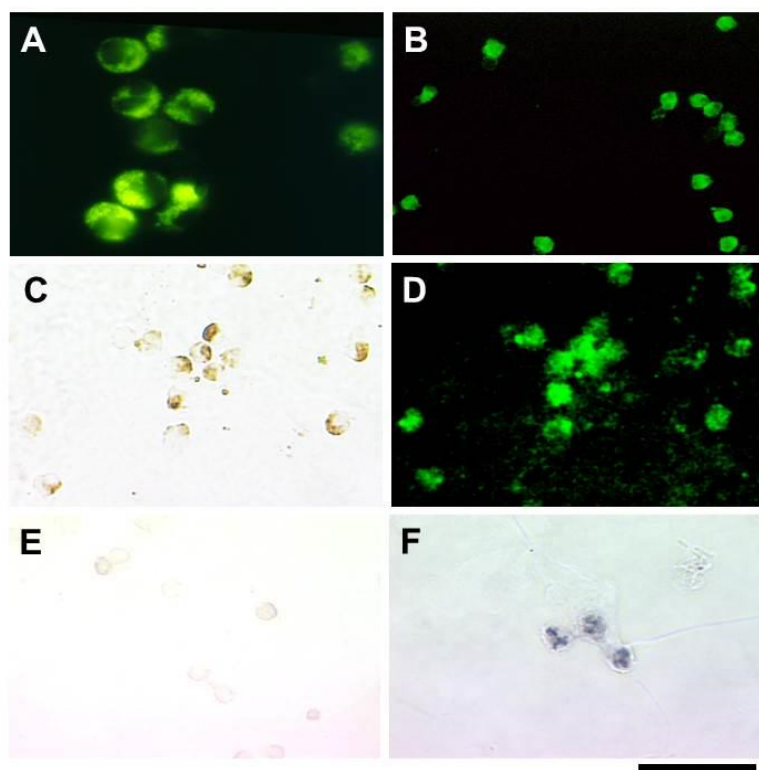


FIGURE 3

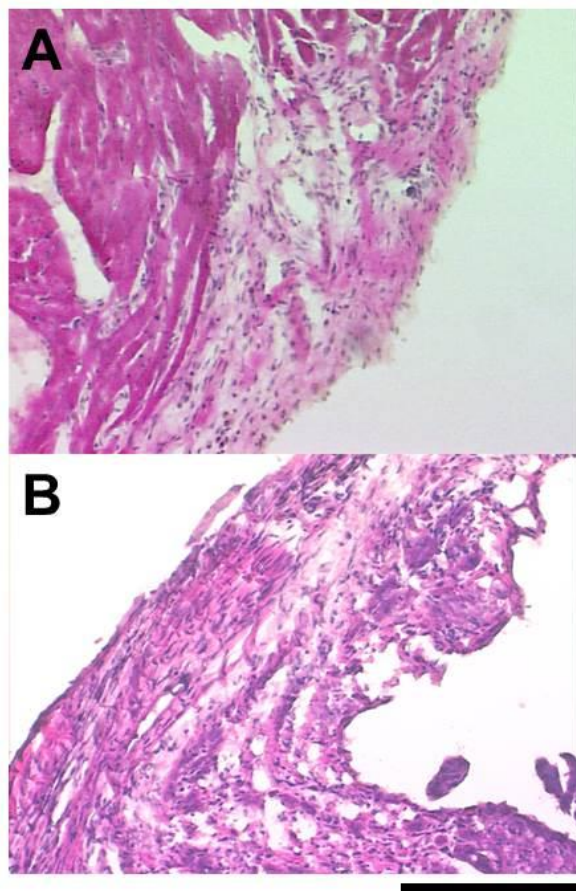


FIGURE 4

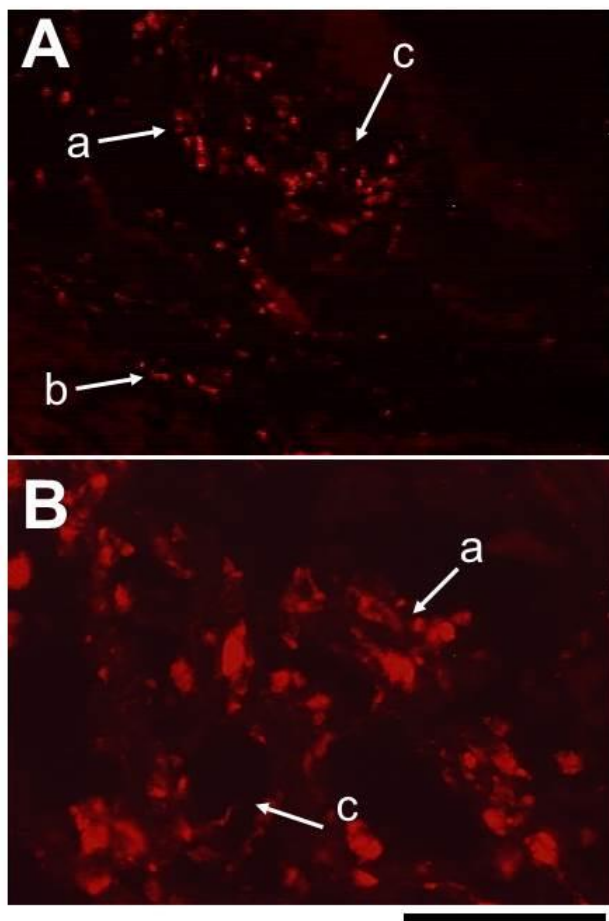


FIGURE 5

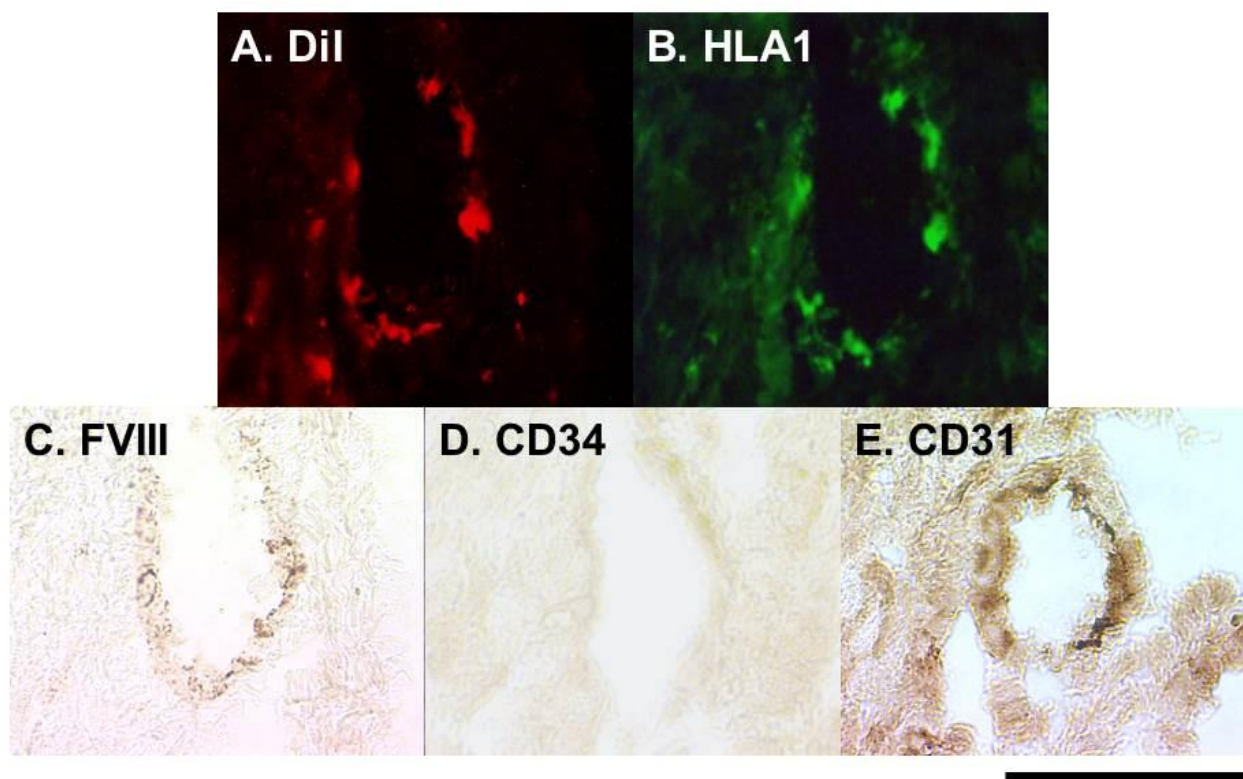


FIGURE 6

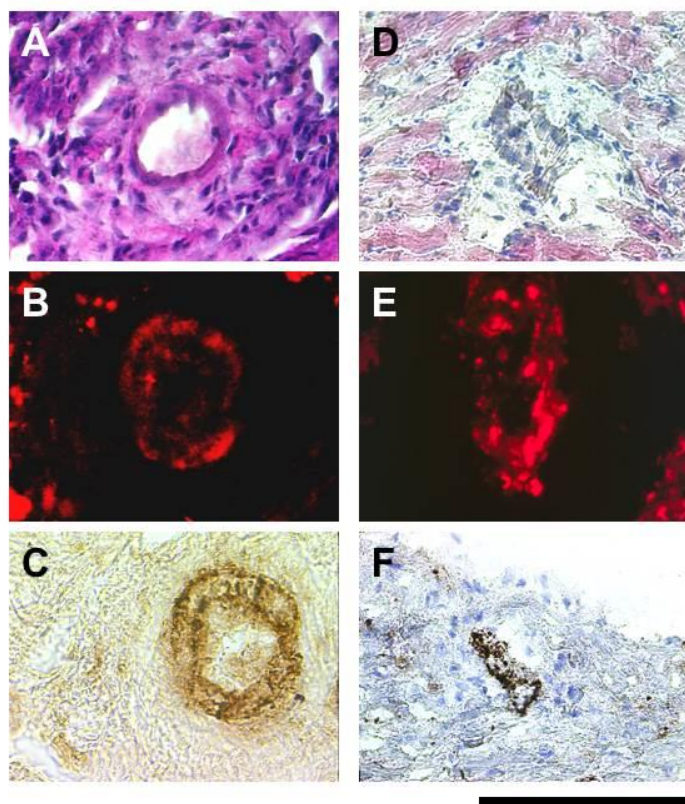


FIGURE 7

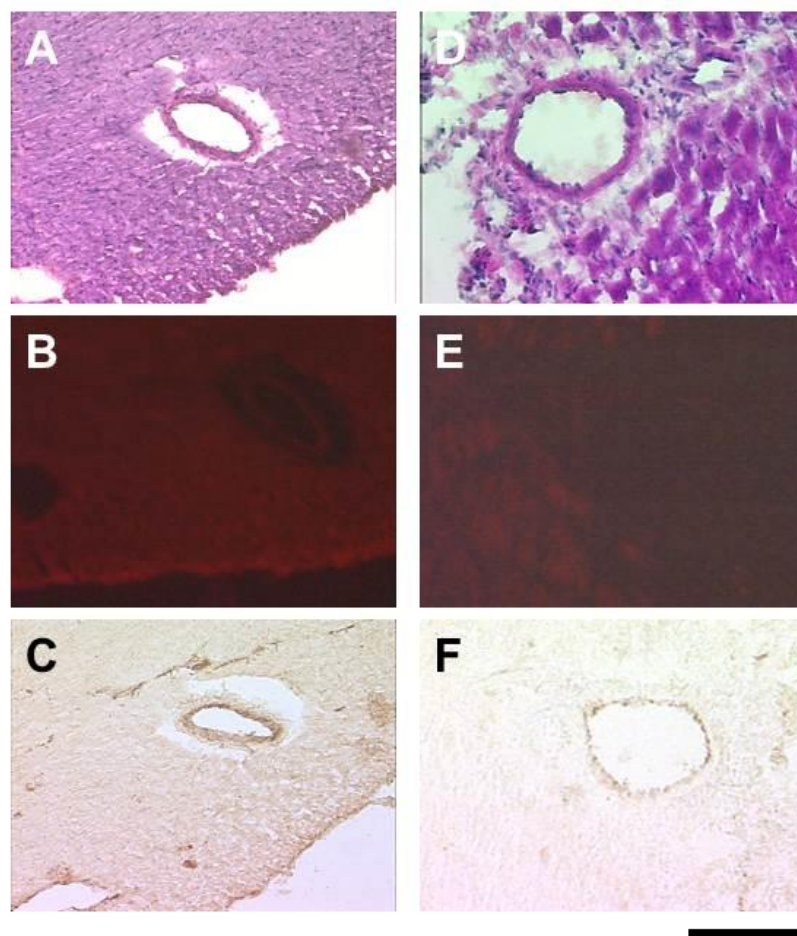


FIGURE 8

