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Human Bone Marrow-Derived CD133$^+$ Cells Delivered to a Collagen Patch on Cryoinjured Rat Heart Promote Angiogenesis and Arteriogenesis

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Transplanting hematopoietic and peripheral blood-derived stem/progenitor cells can have beneficial effects in slowing the effects of heart failure. We investigated whether human bone marrow CD133$^+$-derived cells (BM-CD133$^+$ cells) might be used for cell therapy of heart injury in combination with tissue engineering. We examined these cells for: 1) their in vitro capacity to be converted into cardiomyocytes (CMs), and 2) their potential for in vivo differentiation when delivered to a tissue-engineered type I collagen patch placed on injured hearts (group II). To ensure a microvascular network ready for use by the transplanted cells, cardiac injury and patching were scheduled 2 weeks before cell injection. The cardiovascular potential of the BM-CD133$^+$ cells was compared with that of a direct injection (group I) of the same cells in heart tissue damaged according to the same schedule as for group II. While a small fraction ($2 \pm 0.5\%$) of BM-CD133$^+$cells cocultured with rat CMs switched in vitro to a CM-like cell phenotype, in vivo—and in both groups of nude rats transplanted with BM-CD133$^+$—there was no evidence of any CM differentiation (as detected by cardiac troponin I expression), but there were signs instead of new capillaries and small arterioles. While capillaries prevailed over arterioles in group II, the opposite occurred in group I. The transplanted cells further contributed to the formation of new microvessels induced by the patch (group II) but the number of vessels did not appear superior to the one developed after directly injecting the BM-CD133$^+$cells into the injured heart. Although chimeric human–rat microvessels were consistently found in the hearts of both groups I and II, they represented a minority (1.5–2.3%) compared with those of rat origin. Smooth muscle myosin isoform expression suggested that the arterioles achieved complete differentiation irrespective of the presence or absence of the collagen patch. These findings suggest that: 1) BM-CD133$^+$ cells display a limited propensity for in vitro conversion to CMs; 2) the preliminarily vascularized bioscaffold did not confer a selective homing and differentiation advantage for the phenotypic conversion of BM-CD133$^+$ cells into CMs; and 3) combined patching and cell transplantation is suitable for angiogenesis and arteriogenesis, but it does not produce better results, in terms of endothelial and smooth muscle cell differentiation, than the "traditional" method of cell injection into the myocardium.

Key words: Stem cells; Xenotransplantation; Collagen; Cardiac tissue engineering

INTRODUCTION

Cellular cardiomyoplasty has recently been proposed as an innovative strategy for repairing myocardial damage after experimental and spontaneous injury (9). In particular, an improved cardiac function has been reported after cell transplantation and/or extracardiac mobilization of fetal cardiomyocytes, skeletal myoblasts, smooth muscle (SM) cells, fibroblasts, endothelial progenitor cells (EPC), hematopoietic stem cells, bone marrow-derived mesenchymal stem cells (BM-MSC), fetal amniotic stem cells, umbilical cord stem/blood cells, and embryonic stem cells (30). Despite some contradictory results (13), there is a general consensus on the efficacy of these cells in generating new capillaries, salvaging dysfunctioning cardiomyocytes, and limiting postische-

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mic scarring (21,26). Although great efforts have been made to evaluate new protocols dealing with the number and type of cells, the route of injection and its timing after the injury, and engraftment and survival procedures (2,8,11,12,28), regeneration of lost CMs appears to be negligible in these situations.

A tissue engineering approach might help to improve the efficacy of stem/progenitor cell transplantation or mobilization from endogenous sources (7). Ideally, a biomaterial for use in cardiac tissue engineering should be able to allocate the stem/progenitor cell population of choice and establish appropriate interactions if not identical, at least similar to the native tissue. So far, three-dimensional (3D) cell constructs developed ex vivo lack the vascular network existing in normal tissues. New blood vessel formation through angiogenesis, vasculogenesis, and arteriogenesis (17) seems to be the most realistic endpoint achievable in experimental settings and clinical conditions (1,37). On these grounds, we reasoned that the lack of any significant phenotypic conversion of the transplanted stem cells in the injured heart might be attributable, at least in part, to the absence of an adequate vascularization and the presence of an altered/excessive extracellular matrix (ECM) (39). When rat tail type I collagen matrix engineered with human BM-MSC was applied as a "cardiac patch" in a model of myocardial infarction, it resulted in an improved cell engraftment and functional remodeling (36). Similarly, when a patch of type I collagen was applied to a cryoinjured rat heart, we found a marked vascular colonization by an ingrowth of capillaries and arterioles from the underlying damaged myocardial tissue (3). This bioscaffold is therefore useful for ascertaining whether multipotent stem/progenitor cells can inherently be converted into CMs.

Human BM-CD133$^+$ cells have been assessed for their cardiovascular potential in a vascularized collagen patch applied to cryoinjured hearts of nude rats. These cells can give rise to EPCs (40), and therefore to endothelial cells, and they can also be converted in vitro into cells of mesodermal and nonmesodermal lineages (33). In keeping with this performance, CD133$^+$ hematopoietic/angiogenic stem/progenitor cell populations from circulating blood can improve perfusion in patients with peripheral ischemia (39), as confirmed in vitro (22). Other related cells [e.g., mouse c-kit$^+$ and Sca-1$^+$ (43)] also have a key role in myocardial regeneration in models of heart injury. It is worth noting that BM-derived CD133$^+$ cells can also switch to nonmesodermal lineages when examined in vitro (30). Given these properties, we tested whether a tissue-engineered approach to using BM-CD133$^+$ cells in combination with a vascularized collagen bioscaffold can succeed in inducing heart repair in a nude rat model of acute cryoinjury.

MATERIALS AND METHODS

BM-CD133$^+$ Cell Isolation and Characterization

Cells were collected from leftover human BM samples obtained for transplantation purposes from consenting healthy volunteer donors (30–45 years old), according to the guidelines of the Ethical Committee of Padua Hospital, Padua, Italy. Cells were selected and stem cells were characterized as described elsewhere (33), and the antigen profile expressed by the cultured cells was as previously described (33).

Isolation of CMs From Wild-Type Rat

Wild-type rat CMs (rCMs) were obtained from 2–3-day-old Sprague-Dawley rats by enzymatic digestion according to Radisic et al. (34). Briefly, ventricles were quartered, incubated overnight at 4°C in a 0.06% (w/v) solution of trypsin (Gibco, Invitrogen, Italy) in Hank's balanced salt solution (HBSS, Gibco, Invitrogen, Italy), then washed in cardiac growth medium (CGM; Dulbecco's modified Eagle' medium, Gibco, containing 4.5 g/L glucose supplemented with 10% FBS, 1% HEPES, 1% L-glutamine, 1% penicillin and streptomycin, Gibco, Invitrogen, Italy), and serially digested (4 min, 37°C) in 0.1% solution (w/v) of type II collagenase (Worthington Biochemicals Corporation, USA) in HBSS. The cells were collected by centrifugation and then preplated for 1 h to enrich the cell suspension for rCMs, which were then seeded on 1% gelatin-coated petri dishes (Falcon, BD Biosciences, Italy). On the day after seeding, the cells were rinsed with culture medium to remove nonadhering cells. The culture medium was replaced daily.

Coculture of BM-CD133$^+$ With rCMs

To induce CM differentiation, 2–7 passages of BM-CD133$^+$ cells, cultured as described elsewhere (33) at a density of 2×10^3 cells/cm^2, were seeded on 1% gelatin-coated dishes (Falcon, BD) in the presence of neonatal rCMs and grown in CGM. Cocultures were established by admixing rCMs with BM-CD133$^+$ cells in the ratio of 4:1 and seeding the cell mixture on gelatin-coated glass coverslips (8×10^3 cells/cm^2 and 2×10^3 cells/cm^2, respectively). BM-CD133$^+$ were labeled with the green cell tracker chloro-methyl-fluorescein diacetate (CMFDA) according to the manufacturer's instructions (Molecular Probes, OR). After cell labeling, cell viability was monitored using the trypan blue exclusion test.

Detection of BM-CD133$^+$ Differentiated to CMs

The effect of coculturing BM-CD133$^+$ cells with rCMs was studied after 4, 6, and 9 days. Myogenic and CMs marker expression in cocultured cells was assessed by immunofluorescence. Cells were washed with PBS 1× solution (Gibco), fixed with 2% *p*-formaldehyde for

20 min at 4°C, permeabilized with 0.1% Triton X-100/PBS solution (Sigma) for 10 s at room temperature, and then incubated for 25 min at 37°C with primary antibody anti-cardiac troponin T (Chemicon, Italy) and anti-cardiac troponin I (Abcam, UK) diluted in a 1% PBS/BSA (Sigma) solution. Cells were incubated for 25 min at 37°C with secondary Alexa Fluor 594-conjugated (Molecular Probes, Invitrogen) antibody diluted in a 1% PBS/BSA and human serum solution, and finally counterstained with DAPI solution (Vector Laboratories, UK). Immunostaining was detected under the Axioplan Zeiss fluorescent microscope and the Leica TCS SP5 confocal microscope.

Differentiation of rCMs in cocultures was also analyzed by RT-PCR using human cardiac GATA4 (NM_002052.2, 158 bp, forward: 5′-TCCCTCTTCCCTCCTCAAAT-3′; reverse: 5′-TCAGCGTGTAAAGGCATCTG-3′; 58°C annealing temperature, 35 cycles) and human cardiac β-MHC (NM_000257.2, 152 bp, forward: 5′-CAAGTTTGGCCACACCAA-3′; reverse: 5′-GTTCCAGCAGCTTTTTGT-3′; 60°C annealing temperature, 35 cycles). All primer sequences were built using the http://fokker.wi.mit.edu/primer3/input.htm website and were purchased from Invitrogen, Italy. cDNA from human heart tissue (obtained from endomyocardial biopsies, a gift of Prof. Gerosa, Padua, Italy) and rCM cells were used as positive and negative controls of human cardiac primer specificity.

T-Lymphocyte Proliferation Assay

The assay was carried out as described elsewhere (25). Briefly, BM-CD133+ cells were irradiated with 3000 centi-Gray (cGy) and then added to PHA (PhycoHemAgglutinin)-stimulated lymphocytes at different concentrations (density 2×10^5, 2×10^4, 2×10^3 cells/well). Untreated T lymphocytes and hMSC (density 2×10^5 cells/well) and culture medium were used as negative controls. Each experiment was conducted in triplicate. After 3 days, 1 μCu of tritiated thymidine (Amersham Biosciences, UK) was added to the PHA-stimulated cultures; the cells were harvested using the Combi Cell Harvester (Skatron Instruments, Norway) and transferred onto fiberglass filters (Skatron Instruments, Lier, Norway) covered with 4 ml of scintillation medium (Ultima Gold, Perkin Elmer Life and Analytical Sciences, Boston, MA). Radioactivity was measured with a β-counter (Liquid Scintillation Analyzer, Tricarb, Canberra Packard) and results were expressed as counts per minute.

Induction of Acute Necrotizing Injury (ANI)

Experiments were performed with 10–11-week-old athymic nude rats (rNu; $n = 17$) (Harlan, Milan, Italy) weighing about 190–200 g, housed and maintained in a controlled environment. The investigation complied with the Guide for Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, USA, and the Italian Health Ministry Guidelines for Animal Research. The protocol was approved by the University of Padua Animal Care Committee. The animals were anesthetized by IM injection of Zoletil (9 mg/100 g body weight) with atropine (SC; 5 μg/100 g) and xylazine (IP; 0.4 mg/100 g), then intubated and ventilated mechanically in room air (Harvard, South Natick, MA). The heart was exposed through a left thoracotomy (third or fourth intercostal space) and a left ventricular ANI was induced (using the freeze–thaw procedure) by means of two sequential applications (lasting 30 s each with a 30-s interval between them) of a cryoprobe (a stainless steel cylinder, 5 mm in diameter, cooled in liquid nitrogen). The onset of ANI was confirmed by wall blanching followed by hyperemia.

Collagen Patch Application

Ultrafoam® (code # 1050050), made of lyophilized (freeze-dried) Avitene® flour and water, with which it shares a similar microfibrillar structure, was obtained from Davol Inc. (Cranston, RI). Within 10 min of inducing the injury, the Ultrafoam® patch was placed over the epicardial anterolateral region corresponding to the cryoinjured area (about 12 mm^2) identifiable from a pale appearance with respect to the surrounding myocardium. The patches were attached to the epicardium with a cranially positioned suture. The chest was then closed and the animals weaned from the respirator, extubated, and treated with antibiotics (20 mg/100 g; Baytril, Bayer, Milan). All animals underwent cryoinjury and the patch was applied in animals of groups II and III, followed by randomization for the assessment of the effects of patching versus cell transplantation.

Cell Transplantation

After 4–5 passages, BM-CD133+ cells were detached from the plastic culture dishes with a solution of trypsin 0.05%/EDTA 0.02% w/v (Biochrom AG) and centrifuged for 5 min at $310 \times g$. In vivo cell tracking was performed using the human-specific anti-mitochondria antibody (clone MTC02, Bio Optica, Milan, Italy) (14,27). To test the specificity of this antibody, cytospins prepared with different mixtures of rat MSC-GFP and hMSC cells (in rat to human cell ratios of 5:1, 1:5, and 2:5) were examined within 30 min to confirm that the original proportion of cells counted was also detected in the immunofluorescence tests (data not shown). Rat MSC-GFP were obtained by adhesion from fresh BM of GFP-expressing Sprague-Dawley rats (a gift of

Prof. S. Schiaffino, Padova), and hMSC were obtained as explained elsewhere (18,33). The operated rats were divided into three groups (see Fig. 1) and the cells were injected into the heart (group I, $n = 6$) or into the patch (group II, $n = 6$) 2 weeks after surgery via a second thoracotomy. This was the postinjury time needed for the optimal vascularization of the patch (3). The animals in group III were sacrificed 2 weeks after the first thoracotomy and formed the control group for assessing the effect of patching alone. BM-CD133$^+$ cells were selected for transplantation at passage 5, when the CD133 antigen was expressed in $5.63 \pm 1.5\%$ cells. Then 4×10^6 cells in 90 µl of DMEM high-glucose medium with HEPES (Gibco) and rNU serum (1:100 solution) were injected into the cryoinjured area of the heart or into the patch. One animal from groups I and II was sacrificed 24 h after the cell injection.

Histology and Cell Tracking of Transplanted BM-CD133$^+$ Cells

Frozen sections (8 µm thick) were cut from the animals' hearts and stained with hematoxylin and eosin, and Masson's trichrome (Sigma). Several cryosections were processed by immunofluorescence to identify car-

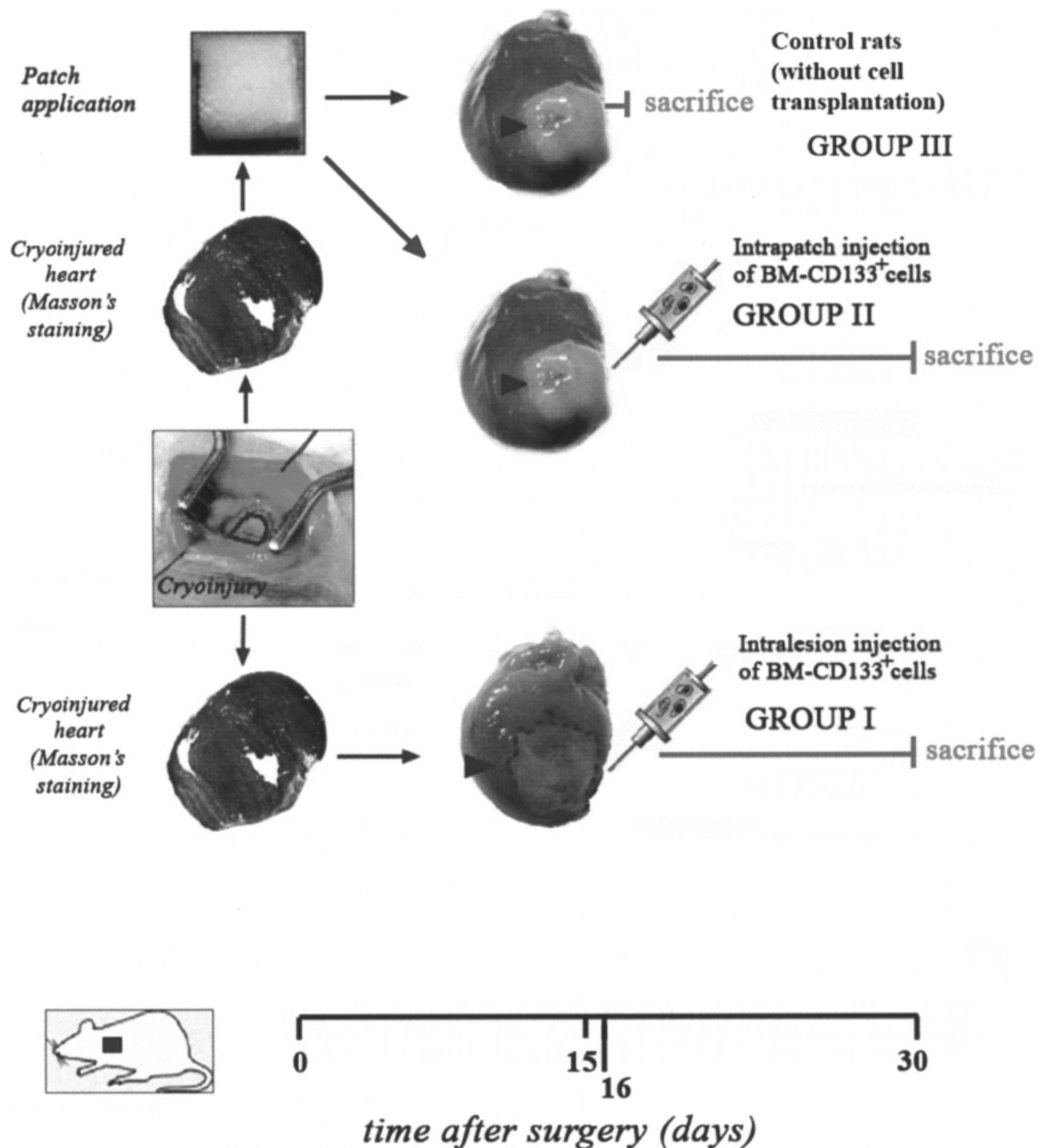

Figure 1. Procedures adopted for in vivo experiments.

diac, immune response, and inflammatory markers: e.g., anti-cardiac troponin I (Abcam, UK), anti-smooth muscle α-actin (SMA; clone 1a4; Sigma), FITC-conjugated SMA (Sigma), anti-von Willebrand factor (vWf; rabbit, DAKO), anti-CD79a (B lymphocyte, rabbit; Abcam), mouse monoclonal anti-rat SM myosin heavy chains -1 (SM-MyHC1; clone 1C10; Abcam) and -2 (SM-MyHC2; clone 1G12; Abcam); anti-rat macrophage (ED2; Chemicon); anti-rat NK (CD161, clone 10/78; FITC conjugated; Abcam); anti-rat pan-T (clone MRC OX-52, Chemicon); and anti-rat DC (OX-62, clone MRC 0X-62; Serotec).

Briefly, tissue slides were fixed in 4% *p*-formaldehyde for 5 min at room temperature, then incubated at 37°C for 25 min with the appropriate dilution of the primary antibody in PBS + 1% bovine serum albumin (BSA; Gibco). Cells were reincubated at 37°C for 25 min with the secondary antibody (goat anti-mouse IgG conjugated with Alexa Fluorescence 564, Molecular Probes; goat anti-rabbit IgG conjugated with Alexa Fluorescence 488, Molecular Probes) diluted in PBS + 1% BSA with human and rat serum. The slides were washed in PBS and mounted in fluorescence mounting medium (DakoCytomation) containing DAPI (Sigma).

The tissues were analyzed under the Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and the Leica TCS SP5 confocal microscope, and images were obtained using a Leica DC300F digital video camera.

Statistical Analysis

The density of capillaries and arterioles, and the expression of differentiation-specific antigens, normalized by mm^2, were evaluated blindly by two independent investigators. The Leica Qwin software was used for data collection and statistical analysis. Statistical significance was assessed with a paired Student's *t*-test. A probability of $p < 0.05$ was considered significant.

RESULTS

In Vitro

In vitro experiments were performed using BM-CD133+ cells at passage 4–5 (when 5% of cells expressed CD133 antigen). CM differentiation of BM-CD133+ cells in coculture with neonatal rCMs was assessed by immunofluorescence and PCR. A minority of cells labeled with CMFDA expressed a "CM-like" phenotype (i.e., 2 ± 0.5% of the whole stem cell population were positive for cardiac troponin I) (Fig. 2) and some cells expressing the "CM-like" phenotype contained double nuclei. Moreover, early transcription factor GATA4, which pertains to the myocardial cell lineage, and β-MyHC sarcomeric protein were detected by PCR after 4 (Fig. 2b) and 9 (not shown) days of coculture. The inherent immunomodulatory potential of BM-CD133+, which may be important for the survival of these cells once they have been transferred in vivo, was studied using the PHA test. In fact, even though rNu are athymic, a small percentage of lymphocytes still persist and might destroy the inoculum (16). Not surprisingly, the profile of the surface antigens expressed by the BM-CD133+ cells [for details, see (33)] resembled the one expressed by BM-derived mesenchymal stem cells (hMSC) and, like the latter, our BM-CD133+ cells have an immunomodulatory capacity, as shown by their ability to inhibit the proliferation of allogeneic PHA-activated human lymphocytes in a dose-dependent manner (Fig. 2d).

In Vivo

The potential for CM differentiation of the BM-CD133+ cells was assessed in vivo using a heart cryoinjury model in which the cells were delivered to the patch of type I collagen applied over the lesion (group I; Fig. 1). We assumed that: 1) Group II had to be compared with group III to distinguish the vascular response (capillaries and small arterioles) attributable to the patch (3) from the one due to the contribution of the BM-CD133+ cells; and 2) the efficiency of this cell transdifferentiation procedure had to be assessed in animals in which the same cells had been injected directly into the lesion.

There was no difference in the total number of surviving hMit-Ag+ BM-CD133+ cells after delivery to the patch or to the cryoinjured tissue: only about 25% of the original inoculum survived the first 24 h (data not shown; see also Fig. 3b, left). Whole heart sectioning 30 days after surgery revealed that the surviving cells were still attached to the bioscaffold, with no signs of any migration away from the patch (data not shown).

There was no transdifferentiation of injected cells positive for CD133, c-Kit, SSEA4, and OCT4 (31) into cardiac troponin I-expressing cells in group II (see also Fig. 4a, b); it was only in group I that rare cells (≤1%) were positive for this myocardial marker (Fig. 3b, right). On these grounds, we decided to focus on the vascular response, which appeared to be the only effect that might realistically be expected to be produced in our experimental setting.

Table 1 summarizes the morphometric analysis on vascularization induced by the combined effect of cardiac patching and cell transplantation (group II) by comparison with the effect of patching alone (group III) or cell transplantation into the lesion (group I), and the standard distribution of the vascular network in the intact myocardium. Injected cells of both human and rat origin were found in the patch or in the injured myocardium of the hearts in groups II and I, while all vessels were naturally of local derivation in group III. The com-

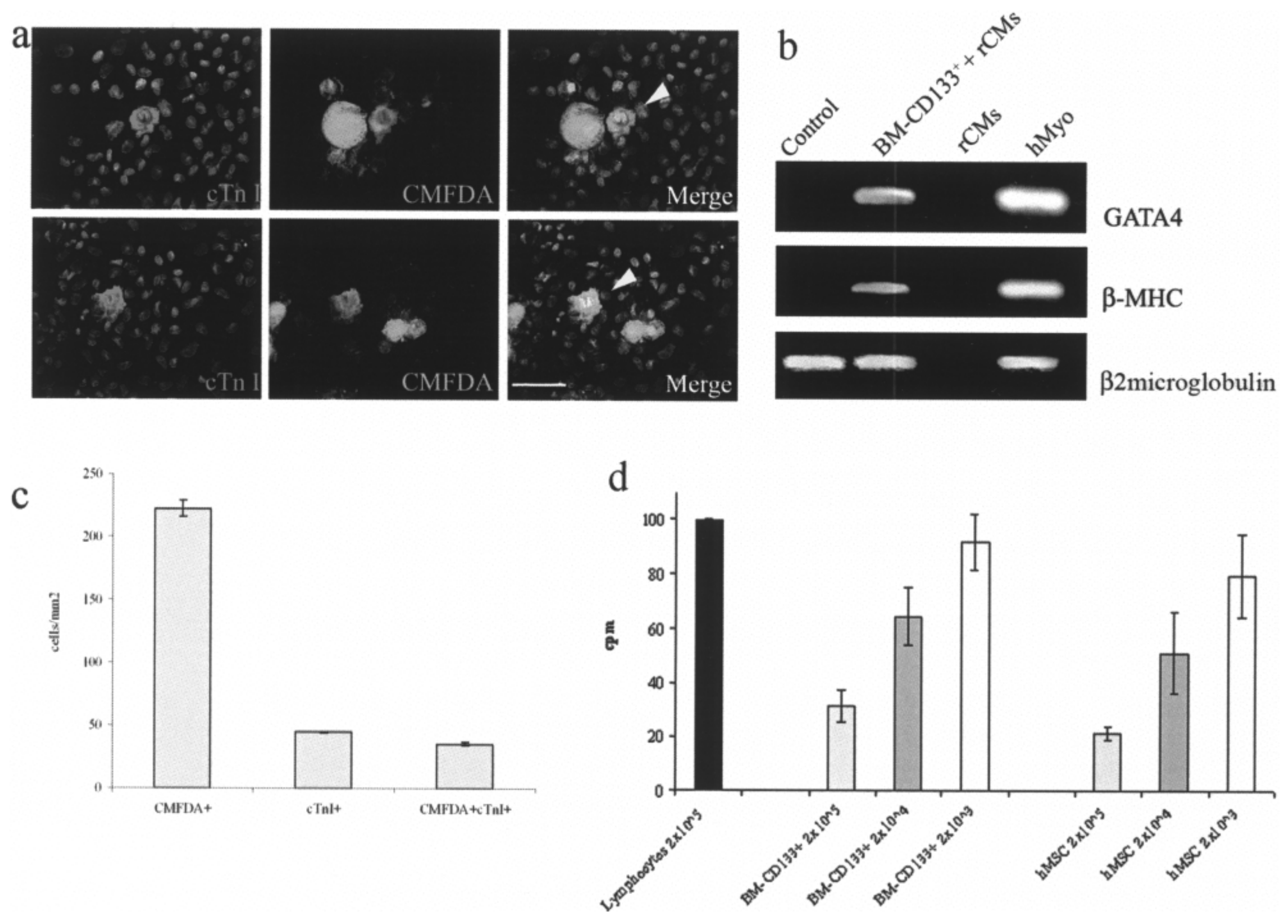

Figure 2. Analysis of CMs differentiation in vitro. (a) Immunofluorescence staining for cardiac troponin I (cTnI, red) expression in CMFDA-labeled (green) BM-CD133$^+$ cells cocultured with rCMs for 9 days. Nuclei were identified using DAPI. Yellow arrowheads indicate two BM-CD133$^+$ cells showing a "CMs-like" phenotype. Scale bar: 50 μm. (b) PCR profile of specific human cardiac genes expression (GATA4 and β-MHC) from BM-CD133$^+$ cells cocultured with rCMs for 4 days. Note that only BM-CD133$^+$ cells are positive for these two markers. hMyo: extract from human myocardium. (c) Graphic of number of cells/area TnI$^+$. $2 \pm 0.5\%$ of CD133$^+$ differentiate into cardiomyocytes. (d) PHA assay for BM-CD133$^+$ cells and hMSC. The dose-dependent immunomodulatory effect of the two stem cells preparations is comparable. Results are given as the mean cpm (count per minute) ± SD obtained from triplicate experiments.

bination of cardiac patching plus cell transplantation gave rise to much the same amount of vessels as in the intact heart, but there were fewer capillaries and, more importantly, far fewer arterioles (about three times less) than when the cells were injected directly into the injured heart. At the same time, adding BM-CD133$^+$ cells was clearly useful in improving the total number of microvessels obtainable with the application of the cardiac patch alone (group III).

As for the contribution of injected BM-CD133$^+$ cells to the formation of new capillaries and arterioles, most of the surviving hMit-Ag$^+$ BM-CD133$^+$ cells were positive for vWf or SMA (Figs. 3a, 4, and Fig. 5), but they were a minority with respect to those of rat origin. In group II, these human cells were $2.2 \pm 0.67\%$ positive for vWf and $1.5 \pm 0.56\%$ positive for SMA and, likewise, in group I they were $2.3 \pm 0.9\%$ positive for vWf and $1.5 \pm 0.6\%$ positive for SMA. Only a minority of hMit-Ag$^+$ cells became incorporated in the vessel walls (Fig. 4c), the vast majority being dispersed as single cells or small clusters in the interstitium.

On the whole, the most relevant consequence of BM-CD133$^+$ cell transplantation into the hearts of these two groups of animals was the appearance of numerous blood vessels of various size, but not of CMs (except for the hearts in group I, where they were expressed in a tiny amount). A part of the capillaries identified by vWf staining without SMA antigenicity were composed of human (hMit-Ag$^+$) and rat (hMit-Ag$^-$) cells, thus accounting for the chimeric vascular structures. The contribution of (hMit-Ag$^+$) cells varied from one microvessel to another (Fig. 4c). At 30 days, the chimeric vessels

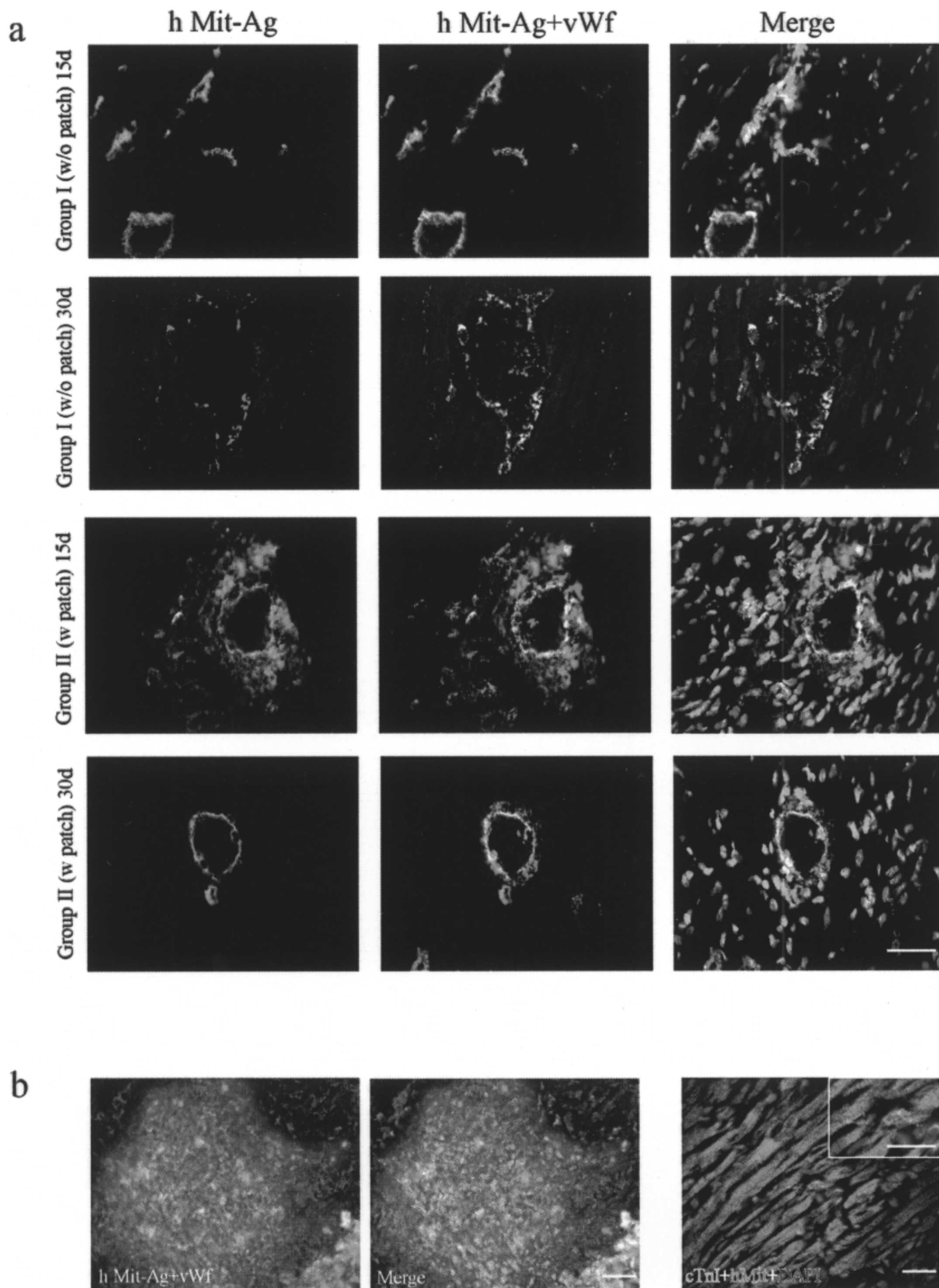

Figure 3. (a) Immunofluorescence staining for vWf antigen of newly formed vessels in the cryoinjury area and in the collagen patch following local BM-CD133^{+} cell delivery. Nuclei were identified using DAPI. (b) Left: Anti-human mitochondria staining after 24-h intrapatch cell injection. Right: Example of anti-human mitochondria TnI^{+} staining found only after 30 days in group I treated animals. Scale bar: 100 μm, inset: 50 μm.

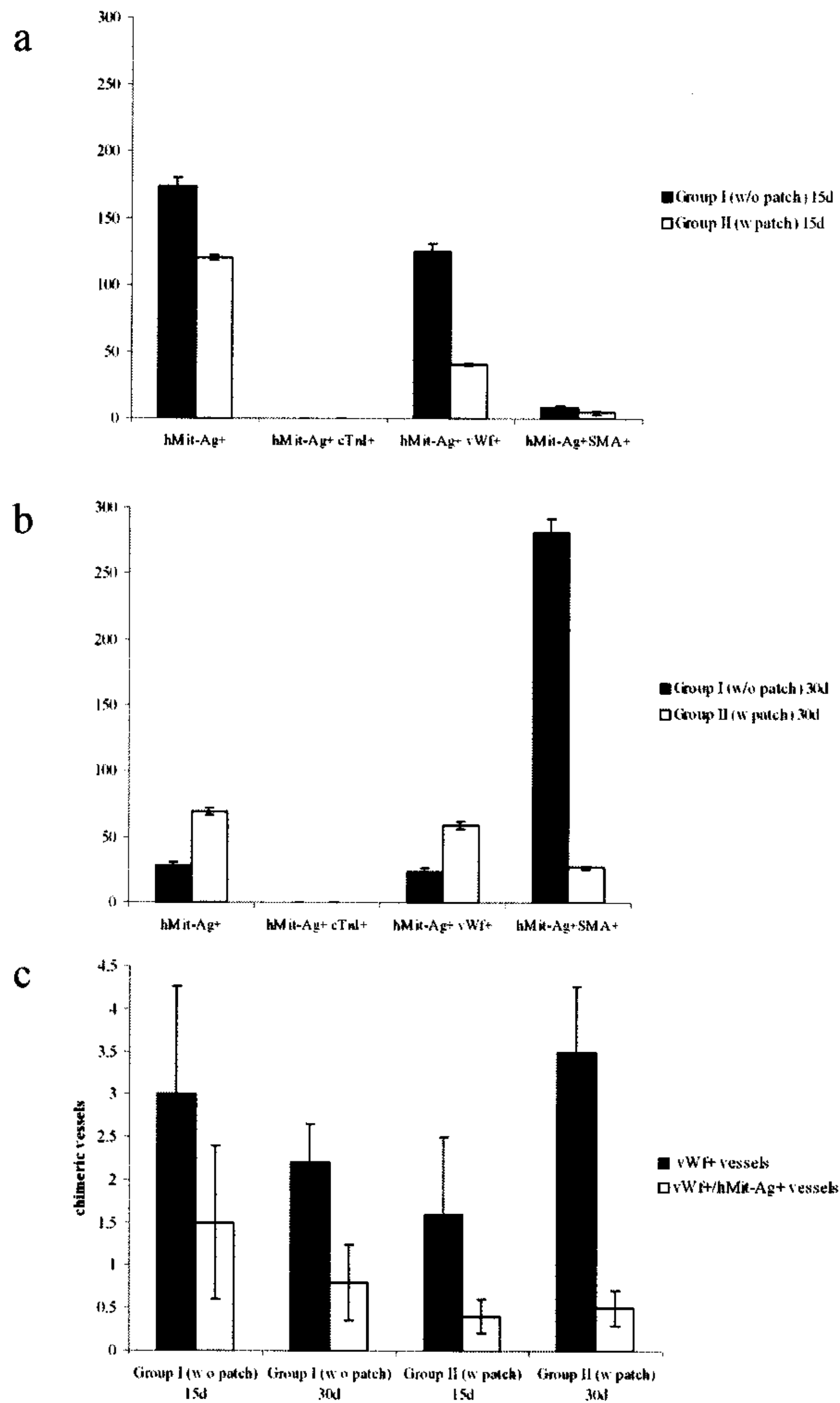

Figure 4. (a) Counting of the cell populations immunoreactive for hMit-Ag, hMit-Ag-cTnI, hMit-Ag-vWf, or hMit-Ag-SMA present in cryoinjury and in the collagen patch 15 days after BM-CD133$^+$ cell injection. (b) Counting of the cell populations immunoreactive for hMit-Ag, hMit-Ag-cTnI, hMit-Ag-vWf, or hMit-Ag-SMA present in cryoinjury and in the collagen patch 30 days after BM-CD133$^+$ cell injection. (c) Morphometric evaluation, in groups I and II, of chimeric capillaries (vWf$^+$ and hMit-Ag$^+$) in comparison with the whole population of chimeric and non-chimeric capillaries (vWf$^+$) formed in the cryoinjury area and in the collagen patch after BM-CD133$^+$ cell delivery. Twenty-five samples were used for each counting.

Table 1. Morphometric Analysis of Vessel Formation After Cell Transplantation

	Group I (30 Days)	Group II (30 Days)	Group III (15 Days)	Nude Rat (Intact Heart)
Total number of capillaries (vWf^+/SMA^-)	241.56 ± 16.61	288.77 ± 8.31	220.16 ± 8.47	288.77 ± 61.8
Total number of arterioles (SMA^+/vWf^-)	357.14 ± 14.38	112.29 ± 5.71	90.46 ± 5.66	117.64 ± 51.64

Morphometric analysis of newly formed vessels after cell transplantation into the cryoinjured area (group I) or into the collagen patch applied over the cryolesion (group II), compared with vessels forming after cryoinjury and patching alone (group III) and with those expressed in the hearts of intact nude rat. $p < 0.05$ comparing group I versus group II, and group II versus group III or nude rats. Twenty-five tissue samples from each animal were considered for statistical analysis.

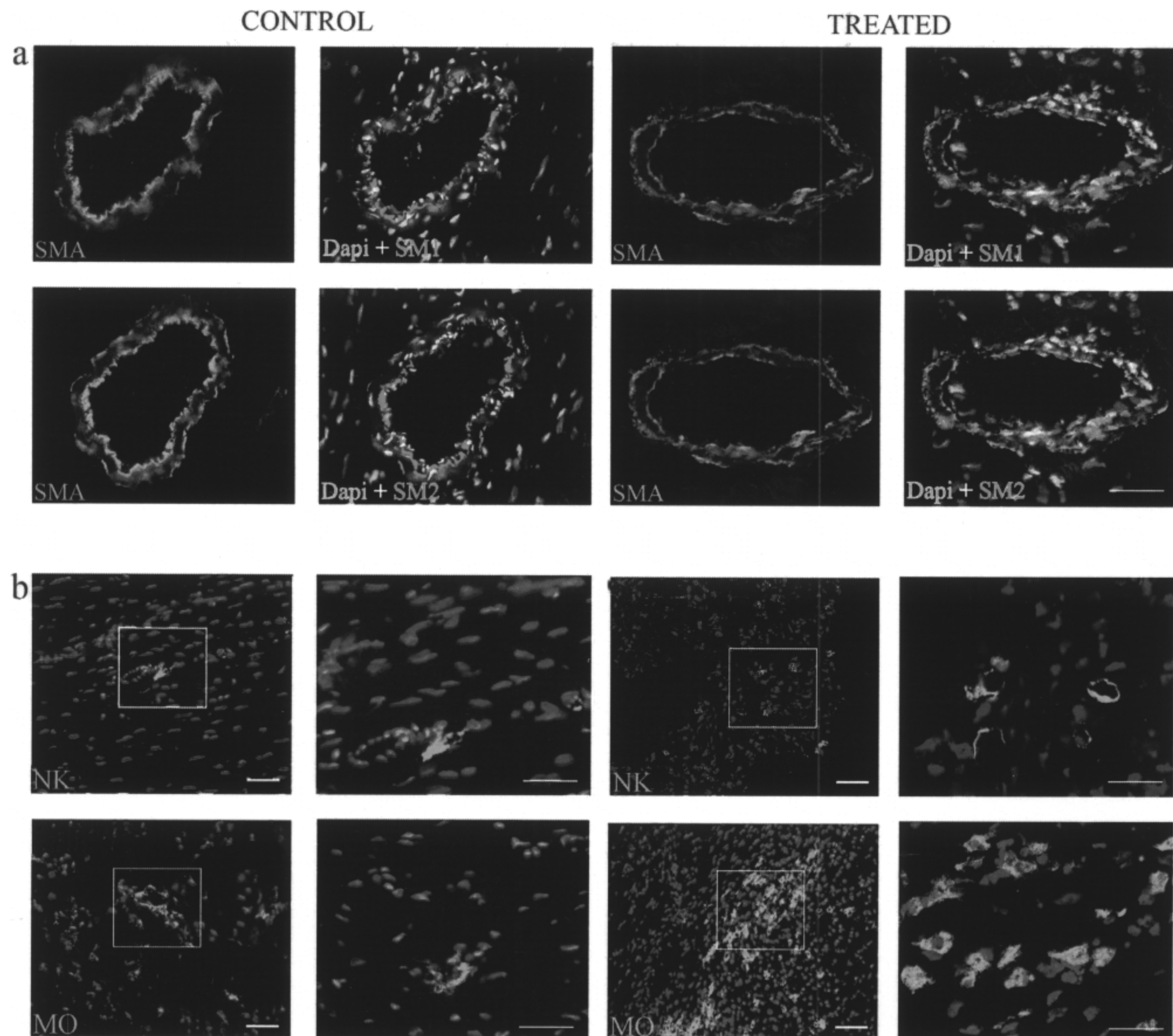

Figure 5. (a) Left: Evaluation of SMA, SM1, and SM2 expression in vessels of native rNu. Right: Example of differentiation pattern showed by SM cells in the newly formed vessels in the cryoinjury and in the patch after ANI and local delivery of BM-CD133+ cells (groups I and II). Note that arterioles formed in both native control animals and treated animals display same expression of SMA, SM1, and SM2 isoforms. Nuclei were identified using DAPI. Scale bar: 50 µm. (b) Left: Immunofluorescence staining of NK and macrophages antigens in native rNu. Right: Immunofluorescence staining of NK and macrophages antigens in group II. Nuclei were identified using DAPI. Scale bar: 100 µm and 50 µm.

containing human cells were fewer in group II than in group I (the proportion being 0.8:1; Fig. 4c).

We then investigated whether the small arterioles that formed in the collagen patch had a distinctive pattern of differentiation with respect to those forming within the lesion. We used two monoclonal antibodies directed against the SM1 and SM2 isoforms of the SM-type MyHC, because their expression in the peripheral muscle vessels changes during development (24). SM1 and SM2 can thus give an indication of the maturity reached by the new vessels in our system. The chimeric arterioles newly created after cell injection directly into the cryolesion (group I) or into the patch (group II) all contained cells positive for SMA, SM1, and SM2 isoforms (Fig. 6, right), as in the heart of intact rNu rats (Fig. 6a, left).

Finally, we wondered whether the relative paucity of the BM-CD133$^+$ cells surviving transplantation and the presence of the cells shown in Figure 3b might be attributable to the innate immune response (and NK cells and macrophages in particular) responsible for immune rejection in other xenotransplantation settings (4). A considerable presence of macrophages was seen in both groups of treated animals (Fig. 6b, at right, shows the pattern of the cells injected into the patch) by comparison with normal rNu, although the NK cells were not differently represented.

DISCUSSION

Our working hypothesis was that an absent or insufficient blood perfusion and an altered ECM might be responsible for a suboptimal engraftment and cardiovascular differentiation of transplanted stem/progenitor cells in cardiac repair. To test this hypothesis, we used human BM-CD133$^+$ cells that we had previously characterized in vitro as a multipotent stem cell population capable of being converted into different cell lineages (33). The inherently cardiomyogenic propensity of human BM-CD133$^+$ cells was first ascertained in a coculture with rCMs, and then the human–rat combination was repeated in vivo by transplanting these cells into a collagen bioscaffold implanted in a xenogeneic model of heart cryoinjury. In our hands, patching the cryoinjured rat heart proved a powerful stimulus for new microvessel formation, including small arterioles (3). We assumed that, in this model, the transplanted cells could take advantage of a newly formed vascular network and soluble factors (growth factors and cytokines) of local and blood-borne origin inherent in cardiac repair. To minimize the immunoinflammatory response that the human cells might elicit via a discordant cell transplantation, the BM-CD133$^+$ cells were transplanted into immunodeficient nude rats. The cryoinjury model made the surgical procedure simple and the heart lesion reproducible, thus affording better, more reliable morphometric assessments of the cardiovascular structures.

The model of heart cryoinjury does not fit completely with the human myocardial ischemia as occurs with all the current models applied to small or large animals involving the permanent or transient occlusion of a branch of the left coronary artery. We are aware that some differences between our method and the occlusion method exist such as: 1) the immunoinflammatory response is increased after cryoinjury, 2) the wound healing is accelerated after cryoinjury, 3) the electrophysiological response is uniquely expressed in the two models, and 4) the cryoinjured mice have only modest LV remodeling and a noticeable better functional outcome than ligation-induced infarcted mice (10,38). Nevertheless, the sequence of histological and cytological changes that take place after injury as a consequence of the application of a cryoprobe is, in comparison with other models and spontaneous event in humans, quite stereotyped. We think that for the morphometric measurements such as those reported in Table 1, the cryoinjury (ANI) has undoubtedly a major advantage: it gives a lesion of the same size throughout the three animal group used in our study whereas ligation typically leads to apical infarcts with large aneurysm formation causing a particular ventricular geometry (41).

The results obtained using this tissue engineering approach indicate that a small proportion of BM-CD133$^+$ cells can be converted in vitro into CMs when cocultured with rCMs, but they cannot be converted in vivo into CMs (29), despite a supposedly favorable microenvironment that favors their phenotypic conversion into endothelial and smooth muscle cells instead, which are partially involved in the formation of new capillaries and arterioles.

In agreement with the majority of studies performed in vitro using the cocultivation of stem/progenitor cells of BM or non-BM origin, we demonstrated that a very low percentage of BM-CD133$^+$ cells can transdifferentiate into a "CM-like" cell phenotype in vitro. Using fresh human BM, Kucia et al. (23) found that a population of CD34$^+$ CD133$^+$ enriched cells expressed the early cardiac markers GATA4 and Nkx2.5, whereas our expanded CD34$^-$ CD133$^+$ population only expressed said markers after coculture in a xenogeneic model. Our transplanted cell population clearly differs from the one used by Kucia et al., as regards both CD34 content and the percentage of cells expressing the CD133 antigen. We cannot say whether the failure to express CD133 marker has to do with the inability to implement a phenotypic myocardial cell switching. Certainly, the paracrine factors and cell–cell contacts available from neonatal rCM cocultures may to some extent retrieve the myocardial cell commitment in BM-CD133$^+$-derived

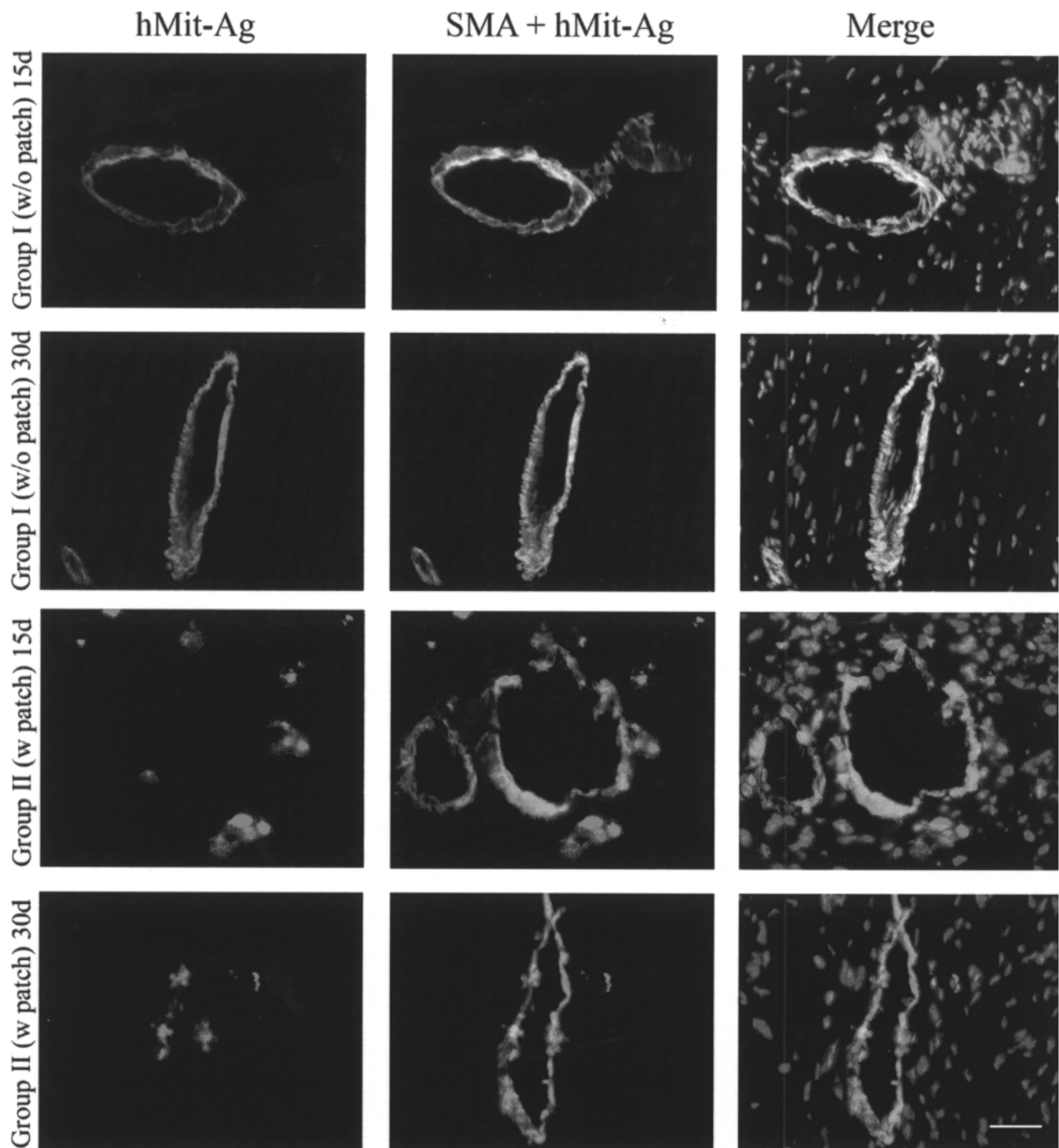

Figure 6. Immunofluorescence staining for SMA antigen of newly formed vessels (hMit-Ag−) in the cryoinjury area and in the collagen patch following local BM-CD133+ cell delivery. Nuclei were identified using DAPI. Scale bar: 50 μm.

cells (35). It is important to add that we cannot rule out the possibility of the in vitro expression of myocardial antigens being linked, at least in part, to the fusion of human cells with neonatal rCMs (15).

Despite the presence of a supporting vascular network that reproduced the cardiac microenvironment (see Table 1), the BM-CD133+ cells showed no capacity for in vivo transdifferentiation to CMs when assayed 15 days after their transplantation. Although it remains to be seen whether prolonged in vivo times can rescue the cardiomyogenic cell phenotype, the results obtained in group I [in which BM-CD133+ cells were transplanted directly into the injured myocardium and a small number of CMs-like cells were found (≤0.8%)] suggest that what matters in this context is the composition of the microenvironment. Another explanation for this in vitro versus in vivo mismatch lies in the inherent phenotypic stability of BM-CD133+ cells. In fact, Kucia et al. (23),

who used a fresh cell preparation of CD133+ cells in their experiments, came to a different conclusion. If the BM-CD133+ cell population is susceptible to a CM cell lineage commitment, a reasonable explanation for this discrepancy could lie in the reduction of this marker with cell passaging (30). Despite the CD133 antigen being present in 98% of freshly selected cells, the antigen was lost or internalized after expansion (passage 4–5), inasmuch as it was present in 5% of the cells used for transplantation and it was consistently maintained with passaging (33). We cannot rule out the possibility of the new cell phenotype attained in vitro being paradoxically less able to find its way in the new, oxygenated environment, which is different from its original niche in BM, where CD133+ cells have low oxygen levels (6).

The use of our tissue engineering approach, which combined cardiac patching and cell transplantation, proved superior to patching alone in terms of extending the vascular network obtained, but it was still inferior to direct cell injection, which particularly facilitated the development of small arterioles (Table 1). This suggests that trophic factors for SM cell lineage commitment are scarcely expressed in the patch (32). That such a tissue engineering approach can indeed be useful for evaluating the potential for differentiation of BM-CD133+ cells has been demonstrated by Simpson et al. (36) and Suuronen et al. (39): the former used hMSC embedded in a "collagen cardiopatch" applied on the epicardial surface of infarcted rats; the latter combined peripheral blood CD133+ cells with a collagen matrix injected into the ischemic hind limb of athymic rats. We used the same collagen patch as in Callegari et al. (3), who found that a type I "cardiopatch" was able to stimulate the formation of a network of capillaries and arterioles when applied to a cryoinjured rat heart, and this effect peaked 15 days after surgery. The fact that a strong angiogenic/arteriogenic response is achieved in the bioscaffold suggests that this engineered tissue meets the requirements for use in improving heart perfusion. This could be exploited in ischemic heart repair by combining a bypass with patching to enhance vascularization and improve patient outcome (42). It is worth mentioning here that the chimeric arteriolar vessels possess both isoforms of the SM-type myosin heavy chains, suggesting that they can achieve a maturity comparable to that of the vessels of untreated, normal animals.

In principle, the inability of BM-CD133+-derived cells to be converted into CMs in the vascularized collagen scaffold may be due to an inadequate or even hostile (immunoinflammatory) microenvironment. The acquired immune response, though negligible in rNu, is compensated by the inherent immunomodulatory effect of BM-CD133+-derived cells. Nude rats possess NK cells, macrophages, and complement and natural antibodies that can potentially participate in the rejection process. It may be that some of the transplanted BM-CD133+ cells were destroyed by an immune reaction brought about by the innate system (5) (see also Fig. 6b). It will be useful to transplant BM-CD133+ cells into an animal system devoid of either acquired or innate immune response to clarify this point.

If passaged BM-CD133+-derived cells have to be used (and this is a prerequisite for clinical applications when large numbers of cells are needed), then the option involving activation of the CMs probably has to be pursued. It is important to notice that the cardiomyogenic differentiation potential of various progenitor cells has been repeatedly demonstrated in vitro (20,31), when the same cells are tested in vivo, a functional improvement can be frequently observed without much evidence of either a presence of a relevant number or a complete differentiation. For instance, improvement in cardiac function after IV infusion of human MSC in immunodeficient mice after coronary ligation occurs in about five cells detected in the heart after 3 weeks (19). In vivo studies of cell therapy on different animal models of cardiac injury report a very small percentage of injected cells integrated into the vascular tissues of the recipients, so that our percentage of cells is consistent with such studies existing in the literature.

CM regeneration conceivably does not only rely on the presence of a type I collagen network and an efficient vascular support, but also requires: 1) locally available, powerful CM differentiation factors; 2) a specific environment that prevents or minimizes the loss of transplanted cells inherent in the chosen model; and 3) adequate multipotent stem/progenitor cells with an in vivo propensity for conversion into CM and endothelial and SM cells. For a functionally complete heart regeneration to take place, all three requirements must be met. To this end, for the purposes of future clinical applications, we have to consider several implementations: 1) a time window-restricted delivery of growth factors trapped in appropriate "cardiac scaffolds"; 2) human progenitor cells with an in vitro and in vivo enhanced potential for cardiovascular differentiation, such as induced pluripotent stem cells (iPS) (44); 3) the validation of transplanted human stem/progenitor cell survival in combined innate and acquired immunodeficient NOD/SCID mice; and 4) the functional evaluation of cardiac regeneration in models of acute/chronic lesions in small and large size animals. However, if a greater perfusion is the realistic endpoint of current clinical applications, then our tissue engineering procedure—which inherently improves the density of capillaries and arterioles—could be useful for ameliorating cardiac performance, particularly in the surgical treatment of heart failure (32).

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