Endothelial progenitor cells in vascular regenerative medicine
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COMBINED IMPLANTATION OF CD34\(^+\) CELLS AND CD14\(^+\) CELLS INCREASES NEOVASCULARIZATION THROUGH AMPLIFIED PARACRINE SIGNALING

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ABSTRACT

Therapeutic neovascularization by autologous endothelial progenitor cells represents a novel treatment for ischemic injuries; however incorporation of CD34+ cells is debated. Recent reports indicate that CD14+ cells can also differentiate into endothelial cells following ischemic injury. We examined whether combined implantation of CD34+ cells and CD14+ cells would augment therapeutic neovascularization in a nude mouse model. Human CD34+ and CD14+ cells were isolated from peripheral blood and implanted subcutaneously into nude mice using Matrigel as carrier. There was no differential gene expression for IL-8, MCP-1, BFGF, HGF and VEGFα, but stimulating CD14+ cells with CD34+ conditioned medium (CM) amplified protein secretion of MCP-1 and BFGF. In vitro, mouse-derived monocytes migrated towards CD34+ and CD14+ CM, which was abrogated by neutralizing antibodies to IL-8 or MCP-1. Also, proliferation of mouse-derived endothelial cells increased by CD34+ and CD14+ CM in a HGF-dependent manner. Combined implantation of human CD34+ and CD14+ cells resulted in superior cell recruitment and neovascularization, compared to either cell type alone and was abrogated by neutralizing antibodies to IL-8 or MCP-1. Neutralizing antibodies to HGF reduced neovascularization without affecting cell recruitment. Combined implantation of human CD34+ and CD14+ cells induced superior neovascularization compared to either cell type alone. Neovascularization did not result from human cell incorporation but relied on amplified paracrine pro-angiogenic signaling.

INTRODUCTION

Cell therapy is considered to be a new treatment for ischemic diseases such as coronary artery disease and myocardial infarction. Endothelial progenitor cells (EPC) contribute to neovascularization [46;296], and therefore have therapeutic potential in regeneration of the ischemic heart. We have previously demonstrated that CD34+ cells are superior to CD133+ and KDR+ cells with regard to the induction of neovascularization and inflammatory cell recruitment [100], and these CD34+ cells are currently evaluated in clinical trials [297]. However, the mechanism by which CD34+ cells contribute to neovascularization is still unclear. Differentiation into endothelial cells and incorporation of CD34+ cells into the neovascularule, albeit in low numbers, has been described [199;274]. We, and others, have demonstrated a paracrine function of CD34+ cells, inducing neovascularization by the formation of an angiogenic microenvironment [100;199;274]. Additionally, human CD34+ cells secrete IL-8 and MCP-1 in vivo, which recruits monocytes [100;268]. In an angiogenic microenvironment, these monocytes contribute to neovascularization by the further secretion of angiogenic factors [64;202] or may differentiate into endothelial cells [101;113] and incorporate into the neovascularule [73;282].

Previously, we have demonstrated that co-cultivation of human CD34+ and CD14+ cells results in an increased differentiation of CD14+ cells into functional endothelial cells in vitro [113]. The enhanced differentiation was attributed to the secretion of HGF by CD34+ cells, since neutralizing antibodies to HGF inhibited the endothelial differentiation of the CD14+ cells for up to 60%. Furthermore, addition of HGF to CD14+ cells replaced the paracrine effect of co-cultured CD34+ cells [113]. Hence, we hypothesized that combined subcutaneous implantation of CD34+ and CD14+ cells would result in neovascularization with higher efficacy than either cell type alone. To this end, we investigated the CD34+ cell-induced neovascularization in our established model of Matrigel implants [100;268]. Furthermore, we dissected the contribution of human CD14+ cells to neovascularization through the secretion of paracrine factors or by incorporation into the neovascularule in vivo.

ANIMALS AND METHODS

ANIMALS

Male, athymic nude mice (Harlan, the Netherlands), 8-10 weeks of age, were held under specific pathogen-free conditions in individual ventilated cages and received sterile food and water ad libitum. Animal experimentation was approved by the local Ethical Committee on Animal Experiments (DEC4186) and was conducted according to governmental and institutional guidelines on animal experimentation.
CHAPTER 8

ISOLATION OF HUMAN CD34+ CELLS AND HUMAN CD14+ CELLS

Human CD34+ cells and CD14+ cells were isolated fromuffy coats of 0.5 L peripheral blood. (Sanquin, The Netherlands) as described previously [113]. Flow cytometric analysis using PE-conjugated mouse antibodies to human CD34 (BD Biosciences, CA) or CD14 (IQ Products, The Netherlands) revealed average purities of 99.17 ± 0.21% for isolated CD34+ cells (n=9) and 97.56 ± 1.07% for isolated CD14+ cells (n=9).

SUBCUTANEOUS INJECTION OF CELL-LOADED MATRIGEL PLUGS

Mice were anesthetized with a mixture of isoflurane (Forene/Abbott BV, The Netherlands) and oxygen. Isolated human CD34+ cell and CD14+ cells were mixed to ratios from 0:0, 1:0, 0:1, 1:10, 1:100 and 1:1000 (CD34 to CD14, respectively) and were labeled fluorescently using CM-Dil (Molecular Probes/Invitrogen, CA) according to manufacturer’s protocol. Aliquots of 10 000 cells were added to 200 μL Matrigel (BD Biosciences, CA) and injected subcutaneously into the flanks of the animals. In some cases, neutralizing goat antibodies to human HGF, IL-8, MCP-1 or Mock (3 μg/mL; all R&D System, MA) were added to the cell-loaded Matrigels. To investigate the influence of paracrine factors on neoangiostatization, some Matrigels were supplemented with recombinant human VEGFa (10 μg/mL), BFGF (10 μg/mL), HGF (20 μg/mL), IL-8 (10 ng/mL) and MCP-1 (10 ng/mL; all Peprotech, CA) without the addition of cells. At 14 days after implantation, the mice were anesthetized and terminated by cervical dislocation after explantation of the Matrigels. Matrigels were fixed in either 2% glutaraldehyde (in 0.1M sodium phosphate buffer) or Zinc fixative (0.1M Tris buffer, pH 7.4 with 0.5g CaCl2•2H2O and 5g ZnCl2 per liter; MERCK, Germany) for further tissue processing.

(IMMUNOHISTOCHEMICAL ANALYSES

For histochimical analyses, glutaraldehyde-fixed explants were dehydrated, resin (Technovit 7100, Heraeus Kulzer, Germany) embedded, sectioned at 2 μm and stained using toluidine blue according to standard procedures. Toluidine blue-stained sections in 0.1M sodium phosphate buffer) or Zinc fixative (0.1M Tris buffer, pH 7.4 with 0.5g CaCl2•2H2O and 5g ZnCl2 per liter; MERCK, Germany) were randomized and evaluated for cellular and capillary density. Photomicrographs of the whole tissue section were taken using a Leica DC300F camera mounted on a Leica DMLB microscope (Leica Microsystems, Germany). The cellular density was calculated by counting all nucleated cells and dividing their number by the total area of Matrigel. The vascular structures that were scored contained capillaries (tubular structures formed by a single-spindle-shaped cell) and small vessels (tubular structures consisting of 2-4 endothelial cells) as described previously [100,268]. The capillary density was calculated as the total number of vascular structures per unit area of Matrigel. For immunohistochimical analysis, 4 μm sections were prepared from zinc-fixed, paraffin-embedded explants, which were dewaxed before staining. Murine endothelium was detected with rat antibodies to mouse CD31 (BD Pharmingen, CA), murine monocytes/macrophages were detected with rat antibodies to mouse MoMa-2 (Serotec, UK). Additionally, sections were incubated with biotinylated rabbit antibodies to rat IgG and horse radish peroxidase-conjugated streptavidin-ABC-complex (all DakoCytomation, Denmark). 3-Amino-9-ethylcarbazole (Sigma-Aldrich, MO) was used as a substrate.

GENE TRANSCRIPT AND PROTEIN PRODUCTION ANALYSES

Human CD34+ cells and CD14+ cells were cultured on fibronectin-coated (1 μg/cm²; Harbor Bio-Products, MA) in RPMI1640, supplemented with 20% fetal bovine serum (both BioWhittaker, Belgium), 5 U/mL heparin (LeoPharma, Denmark), 2 mM L-glutamine (GIBCO/Invitrogen, CA) and 1% Penicillin/Streptomycin (Sigma-Aldrich, MO) for 48 hours. CD14+ cells were cultured in CD34+ cell conditioned medium (CD34+ CM) in triplicate, in order to determine CD34+ cell-induced changes in CD14+ cell gene expression. Thereafter, total RNA was isolated using a RNeasy Microprep Kit (Qiagen, CA) following manufacturer’s protocol. RNA integrity was determined by gel electrophoresis and RNA purity and concentration by spectrophotometry (Nanodrop Technologies, NC). Aliquots of 100 ng total RNA were reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas UAB, Lithuania) and 1 μL of cDNA was used for amplification in 384-well microtiter plates in a TaqMan ABI7900HT cycler (Applied Biosystems, CA) in a final reaction volume of 10 μL containing 5 μL TaqMan universal PCR Master Mix (Applied Biosystems, CA) and 0.5 μL primer/probe mix. Applied Biosystems’ assay on demand’ primer/probe sets were used to detect amplimers of β-2-Microglobulin (β2M; Hs99999907_m1), MCP-1 (Hs00234140_m1), IL-8 (Hs00174103_m1), BFGF (basic FGF; Hs00960934_m1), HGF (Hs00300159_m1), and VEGFa (Hs00990055_m1). Cycle threshold (Ct) values for individual reactions were determined using ABI Prism SDS 2.2 data processing software (Applied Biosystems, CA). To determine differences in gene transcripts between CD34+ cells and CD14+ or between CD14+ cells and stimulated CD14+ cells, Ct-values were normalized against β2M using the ΔCt-method (ΔCtgene = Ct(gene) – Ct(β2M)). To correct for interassay variance, ΔCt values were normalized against expression levels of an external calibrator (ΔΔCtgene = ΔCt(gene) – ΔCt(calibrator)). Fold variance in transcript levels between CD34+ cells and CD14+ cells, or between CD14+ cells and stimulated CD14+ cells, Ct-values were calculated as 2^(-ΔΔCt). For the analysis of protein production by human CD34+ cells, human CD14+ cells and CD34+ cell conditioned medium-stimulated CD14+ cells, cells were cultured as described above in RPMI1640 supplemented with 2% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-glutamine for 48 hours. Concentrations of growth factors and cytokines were determined using multiplex protein analysis by PerBio Sciences (Carmlington, UK).

MIGRATION ASSAYS

Human CD34+ cells and CD14+ cells were cultured as described above in RPMI1640 supplemented with 2% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-glutamine. After 48 hours, the ‘conditioned’ culture medium was removed and
filtered through a 0.2 μm filter to remove any cell debris. To generate CD34+/CD14+ cell conditioned medium, conditioned medium from CD34+ cell cultures was added to CD14+ cells and cultured for an additional 48 hours.

Migration assays were performed in triplicate for pooled CD34+ cell conditioned medium, CD14+ cell conditioned medium and CD34+/CD14+ cell conditioned medium using a 48-well micro-chemotaxis chamber (Neuroprobe, MD) according to manufacturer’s instructions. RAW264.7 responder cells (a murine monocyte/macrophage cell line) were used to study migration towards the conditioned media. Spontaneous migration of RAW264.7 responder cells was used as negative control and migration towards 10 μM histamine (Sigma-Aldrich, MO) was used as positive control. The migratory capacity was tested using conditioned media which was supplemented with neutralizing antibodies to human IL-8, MCP-1 and HGF or Mock IgGs (5 μg/mL; all R&D Systems, MN). The RAW264.7 responder cells were allowed to migrate at 37°C, 5% CO2 in 100% humidity for 4 hours. Thereafter, the chemotaxis chamber was disassembled and the filter (8 μm pore size) was fixed and stained using Diff-Quik (Medion Diagnostics, Switzerland). The filter was mounted on a glass slide and photomicrographs were taken on a 100x lens magnification. The migration towards 10 μM histamine was set at 100% and the migration towards conditioned media was calculated accordingly.

ENDOTHELIAL CELL PROLIFERATION ASSAYS

To study the effect of conditioned culture media on the proliferation of endothelial cells, H5V cells (a murine endothelial cell line) were cultured on gelatin-coated (10 μg/cm2; Sigma-Aldrich, MO) wells plates. H5V endothelial cells were a kind gift of Dr. Annunciata Vecchi (Istituto Clinico Humanitas, Rozzano, Italy). Before analysis, H5V cells were serum-starved by reducing the fetal bovine serum from 10% to 2% for 48 hours. Thereafter, culture medium was replaced by either CD34+ cell conditioned medium, CD14+ cell conditioned medium or CD34+/CD14+ cell conditioned medium and the H5V cells were cultured for an additional 48 hours. RPMI1640 containing 10% or 2% fetal bovine serum were used as positive and negative controls respectively.

To analyze cell proliferation, H5V cells were fixed using ice-cold methanol:acetone (1:1) and post-fixed using 2% paraformaldehyde (in 0.1M sodium phosphate buffer), rehydrated and stained using rabbit polyclonal antibodies to Ki67 (Monosan, UK), followed by fluorescein-conjugated donkey antibody fragments to rabbit IgGs (Jackson ImmunoResearch, UK). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, MO) and image analysis was performed on a Leica DMRXA fluorescence microscope (Leica Microsystems, Germany).

STATISTICAL ANALYSIS

All data are expressed as mean ± standard error of mean. The Mann-Whitney U-test was used to detect statistical differences between cell-loaded Matrigels and bare Matrigel controls. For multiple comparisons testing, one-way ANOVA followed by Bonferroni post hoc analyses were performed. Probabilities (p) of less than 0.05 were considered to be statistically significant.

RESULTS

CD34+ CELL INDUCED NEOVASCULARIZATION

CD34+ cell-loaded Matrigels and bare Matrigel controls were subcutaneously implanted in nude mice. After 14 days, Matrigels were explanted and processed for
(immuno-)histochemical analysis. Similar to our previous findings [100;268], we found vascularization of the cell-loaded Matrigel implants (Figure 1A), while vascularization was hardly observed in bare Matrigel controls (Figures 1B & D; p < 0.01). Some neovessels were functionally coupled to the host vasculature, indicated by the presence of erythrocytes in the lumen (Figure 1A, insert). Neovessels were of murine origin (Figure 1C) and did not contain human cells (data not shown).

Neovascularization was accompanied by recruitment of monocytes/macrophages, which aligned the capillary-like structures (Figures 1E & F). Furthermore, the amount of neovascularization correlated positively with the influx of monocytes/macrophages (r = 0.59, p = 0.007; Figure 1G).

**CD34+/CD14- CELL INDUCED NEOVASCULARIZATION**

We investigated the neovascularization efficacy of CD34+ and CD14+ cells either implanted alone or in mixed ratios varying from 1:10-1:1000 (CD34+/CD14+). All cell-loaded Matrigel implants showed higher capillary formation than bare Matrigel controls (p < 0.05). CD34+ cells induced the formation of, erythrocyte-filled, functional small-sized vessels in the Matrigel implants (Figure 2A). CD14+ cell-loaded Matrigels also induced neovascularization, although at a lower density compared to CD34+ cells (56.5 ± 7.4 versus 71.0 ± 8.7 capillaries/mm², respectively). The capillary-like structures in the CD14+ cell-loaded Matrigel implants were void of erythrocytes (Figure 2B). Vessel formation in Matrigels loaded with both CD34+ and CD14+ cells (1:10 ratio) was superior to either cell type alone (p < 0.05; Figures 2C & F). This effect was dose-dependent and was no longer observed when CD34+ cells were mixed with CD14+ cells in a ratio of 1:100 or more (Figure 2E). Erythrocytes in the lumen of these blood vessels indicated functional coupling to the host vasculature. Contrary to vessel formation in CD34+ cell-loaded Matrigels, human cells were identified in capillary-like structures (Figure 2D), arrow), albeit at low numbers.

**PRODUCTION OF AN ANGIogenic NICHE BY CD34+ CELLS AND CD14- CELLS**

We investigated the production of two chemoattractants of inflammatory cells, interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), and three angiogenic growth factors, fibroblast growth factor-2 (bFGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor-a (VEGFa), by human CD34+ and CD14− cells both on gene transcript level and on secreted protein level. CD34+ cells had a higher gene transcript level for MCP-1 (~9-fold) than human CD14− cells. In contrast, no difference in gene expression was found between the CD34+ cells and the CD14− cells for IL-8 (Figure 3A). The transcripts for BFGF (~8-fold), HGF (~7-fold) and VEGF (~4-fold) were expressed more in the CD34+ cells compared to the naive CD14− cells (Figure 3A). Surprisingly, when CD14− cells were incubated with conditioned medium from CD34+ cell cultures (CD34+ CM), expression of MCP-1 (~8-fold), bFGF (~7-fold), HGF (~6-fold) and VEGFA (~8-fold) increased (Figure 3B). No alteration in gene expression of IL-8 observed after stimulation of naive CD14− cells with CD34+ CM (Figure 3B).

Culture supernatants from CD34+ cells, naive CD14− cells and from CD34+ CM-stimulated CD14− cells (CD34+/CD14− CM) were analyzed for the presence of chemoattractants IL-8 and MCP-1, and the growth factors BFGF, HGF and VEGFA. The concentration of IL-8 was higher in the culture supernatant from CD34+ cells than in the supernatant from naive CD14− cells (3.14 ± 0.29 versus 2.02 ± 0.05 ng/mL, respectively; p < 0.05), but was similar to CD34+ /CD14+ CM (3.14 ± 0.29 versus 2.61 ± 0.21 ng/mL, respectively; Figure 3C). Similar amounts of MCP-1 were found in the culture supernatants of CD34+ cells and CD14− cells (3.94 ± 0.34 versus 3.03 ± 0.04 ng/mL, respectively). MCP-1 concentration in the CD34+/CD14+ CM was higher compared to both the CD34+ cell supernatant (~2-fold; p < 0.001) and CD14− cell supernatant (~2.5-fold; p < 0.001; Figure 3D). The concentration of bFGF was higher in the CD34+ cell supernatants than in the CD14− cell supernatants (2.11 ± 0.17 versus 0.85 ± 0.04 ng/mL, respectively; p < 0.001) and was even higher in the CD34+/CD14+ CM (3.30 ± 0.21 ng/mL; p < 0.01 versus CD14− cells, respectively; Figure 3E). The concentration of HGF was also higher in the CD34+ cell culture supernatants than in the CD14− cell supernatants (4.17 ± 0.27 versus 1.8 ± 0.29 ng/mL, respectively; p < 0.001), and tended to be higher than in the CD34+/CD14+ CM (4.17 ± 0.27 versus 3.16 ± 0.18 ng/mL, respectively; p < 0.10), indicating consumption of this growth factor (Figure 3F). The protein concentration of VEGFa was also higher in CD34+ cell supernatants than in the CD14− cell supernatants (1.26 ± 0.14 versus 0.43 ± 0.06 ng/mL, respectively; p < 0.01), but did not differ from the VEGFa concentration in the CD34+/CD14+ CM (1.26 ± 0.14 versus 0.93 ± 0.02 ng/mL, respectively; Figure 3G).
IN VITRO EFFECTS OF CD34+ CELL AND CD14+ CELL-DERIVED PARACRINE FACTORS

The conditioned media from CD34+ cells (CD34+ CM) and CD14+ cells (CD14+ CM) had a recruiting effect on the RAW264.7 monocytes (p < 0.001 versus spontaneous; Figures 4A & B). RAW264.7 migration towards CD34+ CM was higher than the CD14+ CM (p < 0.05). The CD34+ /CD14+ CM had the highest recruiting effect on RAW264.7 cells (p < 0.05 versus CD34+ CM and p < 0.001 versus CD14+ CM). Migration towards the CM-gradients decreased to baseline when either neutralizing antibodies against IL-8 or MCP-1 were added to the CM. Neutralizing antibodies to HGF did not affect RAW264.7 migration (Figures 4A-C).

We further analyzed if the conditioned media could affect proliferation of HSV cells, a mouse endothelial cell-line. All conditioned media increased proliferation of HSV cells (Figures 4D-F) but with different efficiencies. CD34+ CM increased HSV proliferation by 30% compared to 2% FBS controls, while CD14+ CM increased HSV proliferation by only 14%. CD34+/CD14+ CM induced the highest proliferation rates (p < 0.001 versus CD34+ CM and p < 0.001 versus CD14+ CM). HSV proliferation was increased by 47% compared to controls. Neutralizing antibodies to IL-8 or MCP-1 did not affect proliferation but neutralizing antibodies to HGF decreased HSV proliferation in all media tested (Figure 4F).
NEOVASCULARIZATION THROUGH PARACRINE SIGNALING OF CD34+/CD14+ CELLS

We investigated the effect of IL-8, MCP-1 and HGF on CD34(1:10)/CD14 cell-induced neovascularization. Implantation of CD34(1:10)/CD14 cell-loaded Matrigels into nude mice led to recruitment of host-derived cells (617.7 ± 45.3 cells/mm² Matrigel) and the formation of capillary-like structures (100.2 ± 15.0 capillaries/mm² Matrigel). The addition of Mock IgGs (Figure 5A) to the cell-loaded Matrigel had no effect on cell recruitment and neovascularization, but the addition of neutralizing antibodies to IL-8 (Figure 5B) decreased cell recruitment and capillary formation by ~35% (p < 0.05) and ~57% (p < 0.001), respectively. Similarly, neutralizing antibodies to MCP-1 (Figure 5C) showed a reduction in cell recruitment of ~42% (p < 0.01) and capillary formation of ~68% (p < 0.001) compared to IgG controls. Neutralizing antibodies to HGF (Figure 5D) had no effect on cell recruitment to the cell-loaded Matrigel implants (Figure 5E), but highly reduced capillary formation (~72% reduction; p < 0.001; Figure 5F) compared to Mock IgG controls.

NEOVASCULARIZATION THROUGH PARACRINE FACTORS

To analyze the dependence of neovascularization of Matrigels on paracrine signaling by human cells, Matrigels were loaded either with CD34(1:10)/CD14 cells (Figure 6B) or with a pentet of chemokines and growth factors (IL-8, MCP-1, bFGF, HGF and VEGFa), solely (Figure 6C) or with the addition of neutralizing antibodies to IL-8, MCP-1 and HGF (Figure 6D). The addition of the cytokine/growth factor pentet to the Matrigels induced similar cell recruitment and capillary formation as the cell-loaded Matrigels (Figures 6E-F). Addition of neutralizing antibodies to the cytokine/growth factor-loaded Matrigels reduced cell recruitment and capillary formation to the level of bare Matrigel controls (Figures 6E-F).

DISCUSSION

Current cell therapy strategies that use peripheral blood-derived CD34+ progenitor cells are hampered by low cell numbers and the low cellular incorporation into the neovasculature. Hence, the use of CD34+ progenitor cells to treat ischemic diseases is under debate. We dissected the function CD34+ cells in our subcutaneous model for therapeutic neovascularization [100;268] and show that (1) CD34+ cells contribute to neovascularization through paracrine signaling, which involves recruitment of (CD14+) monocytes and secretion of angiogenic growth factors, (2) combined implantation of CD34+ and CD14+ cells increases neovascularization through amplified paracrine signaling, and (3) that neovascularization of Matrigel implants depends primarily on paracrine signaling through IL-8, MCP-1 and HGF by both cell types.

CD34+ cells have been regarded as the archetype endothelial progenitor cell [46], however incorporation of CD34+ cells into the neovasculature is rarely observed and
only in low numbers [274]. However, clinical trials suggest a positive contribution of CD34+ cells in the prevention of tissue damage and restoration of the vasculature after myocardial infarction [297;298], suggesting a paracrine function for CD34+ cells in tissue revascularization. We dissected the paracrine role of CD34+ cells in our subcutaneous model for neovascularization and found that CD34+ cell-induced neovascularization is accompanied by influx of (CD14+) monocytes that spatially associate with the neovascularure. Previously, we demonstrated the importance of monocytes in neovascularization by showing that monocytes can act as endothelial precursors and differentiate into mature endothelial cells [101;113]. We furthermore showed that CD14+ cell-depletion decreases neovascularization following myocardial infarction in mice [270]. These findings are corroborated by recent reports that show induction of neovascularization following the injection CD14+ cells into mouse hearts after myocardial infarction or into ischemic hind limbs [64;299].

We questioned if the addition of CD14+ cells to the Matrigel implants would increase neovascularization and implanted Matrigel implants loaded with CD34+ and CD14+ cells subcutaneously into nude mice. Combined implantation of CD34+ cells and CD14+ cells increased neovascularization, in a dose-dependent manner, by ~41% compared to CD34+ cell-induced neovascularization. Furthermore, CD14+ cells could be detected in close proximity to the neovessels, albeit in low numbers. We contemplated that increased neovascularization, without the incorporation of CD14+ cells, could only be caused by differential amplification of paracrine signaling. Hence, we investigated the cytokine/growth factor secretion of both CD34+ cells and CD14+ cells in vitro.

CD34+ cells and CD14+ cells both contribute to a pro-angiogenic microenvironment by the production and secretion of several chemokines and growth factors. CD34+ cells produced IL-8, MCP-1, BFGF, HGF and VEGFa. Notably, we found an additive effect in the amount of MCP-1 and BFGF secretion and a small decrease HGF and VEGFa secretion when naive CD14+ cells were incubated with conditioned medium from CD34+ cells. This decrease may indicate consumption of CD34+ cell-derived HGF and VEGFa by CD14+ cells. Although we did not find differential secretion of chemokines and growth factors, Yoon et al. reported differential expression of matrix metalloproteinase (MMP)-2 and MMP-9 by CD14+ and CD34+ cells. In combination, these cells contributed to enhanced neovascularization [202], and corroborate our findings that interaction between CD34+ and CD14+ cells results in the formation of an enhanced angiogenic microenvironment, which is superior to the product of either cell type alone.

Neovascularization of Matrigel implants by host-derived cells may be caused by recruitment of mouse monocytes and their subsequent differentiation into endothelial cells [300], or by sprouting angiogenesis from the pre-existing vasculature [199]. We therefore examined the ability of paracrine signals to recruit mouse monocytes and to influence proliferation of mouse endothelial cells in vitro.

Migration of monocytes was increased by the conditioned media of CD34+ cell-stimulated CD34+ cells (CD34+/CD14+ CM) compared to either conditioned media alone. Notably, migration of mouse monocytes decreased after the addition of neutralizing antibodies to IL-8 and MCP-1, reflecting the importance of these factors in cell recruitment prior to neovascularization [290;293]. In vivo, neutralizing antibodies to IL-8 and MCP-1 decreased the recruitment of host-derived cells and consequently neovascularization of the Matrigel implants was reduced.

Proliferation of mouse endothelial cells was increased by paracrine factors in the CD34+/CD14+ CM and this increase was abrogated by the addition of HGF neutralizing antibodies. This indicates that HGF may contribute to neovascularization by increasing sprouting angiogenesis [301]. This was confirmed in vivo where HGF neutralizing antibodies reduced capillary formation without affecting cell recruitment. These data indicate a role for HGF in either sprouting angiogenesis, or endothelial cell differentiation and capillary formation by recruited monocytes. Supporting the latter, we have previously reported a major role for HGF in the endothelial differentiation of CD14+ cells [113]. Supporting the former, others reported HGF-dependence in capillary sprout formation [302].

We subsequently hypothesized that endothelial progenitor cell-induced neovascularization of Matrigel implants relied, for the major part, on the actions of IL-8, MCP-1, bFGF, HGF and VEGFa. Hence, we investigated neovascularization by incorporating this pentet of chemokines and growth factors into Matrigel and found similar cell recruitment and capillary density as in endothelial progenitor cell-induced neovascularization.

CONCLUSION

Taken together, combined implantation of CD34+ and CD14+ cells induces superior neovascularization compared to either cell type alone. Neovascularization is initiated through the production of angiogenic factors including IL-8 and MCP-1, for the active recruitment of monocytes, and growth factors bFGF, HGF and VEGFa, which induce sprouting angiogenesis and/or endothelial cell differentiation by recruited cells (Figure 7). These data provide new insights on neovascularization by cell transplantation and may contribute to cell therapeutic strategies for treatment of ischemic diseases.
REFERENCES


References


APPENDICES

REFERENCES


