Transfer of Endothelial Progenitor and Bone Marrow Cells Influences Atherosclerotic Plaque Size and Composition in Apolipoprotein E Knockout Mice

Jacob George, Arnon Afek, Anastasia Abashidze, Haim Shmilovich, Varda Deutsch, Juri Kopolovich, Hylton Miller, Gad Keren

Objectives—Recent clinical trials use cell therapy with bone marrow (BM) cells or endothelial progenitor cells (EPCs) for ischemic syndromes. We explored the effect of BM cell– or spleen cell–derived EPC transfer on plaque size and stability markers in the apolipoprotein E knockout (apoE KO) mouse model.

Methods and Results—ApoE KO mice aged 10 weeks served as recipients. Labeled BM cells and spleen cell–derived EPCs from age-matched apoE KO mice were injected intravenously to 2 groups of recipient mice each. Additional mice served as controls receiving saline. Both protocols were repeated 3 times at 2 weekly intervals. On killing, plaque size and character were studied, lipid profile analyzed, and serum and aortic cytokines assayed. Spleen cell–derived cells contained a significantly larger number of endothelial cell precursors. Labeled EPCs and BM cells were found abundantly in the spleens, yet also in the lesions of the recipient mice. Aortic sinus lesion size was significantly increased in mice receiving BM cells (n=10) in the EPC-treated group (n=10) compared with controls (n=10; a 54% and a 34% increase in aortic sinus plaque area, respectively). Mice receiving EPCs exhibited plaques with larger lipid cores and thinner fibrous caps and a higher number of infiltrating CD3 cells. RT-PCR analysis of aortas revealed reduced expression of mRNA for interleukin-10 (IL-10) in both cell transfer groups. Higher serum concentrations of IL-6 and monocyte chemoattractant protein-1 were found in sera from BM recipients, whereas lower IL-10 levels were found in mice transfused with spleen-derived EPCs.

Conclusions—Transfer of BM cells and EPCs may result in an increase in atherosclerotic lesion size, whereas EPC transfer could also potentially influence plaque stability. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words:

The pathogenesis of atherosclerosis involves a complex interaction of endothelial cells (ECs), macrophages, and lymphocytes, followed by smooth muscle cells and fibroblasts. Is it probable that factors that govern the initiation of atherosclerosis, which involves less complex cellular cross-talk, are not identical to determinants of plaque progression, in which additional matrix components and cell types are prevalent.

In both these processes, ECs have been proposed to play a major role forming the attachment surface on which monocytes role and adhere. ECs participate in the early fatty streak formation and in constituting the vasa vasorum network that acts to supply the inner growing neointima in more advanced lesions. These actions are regulated by expression of a set of adhesion molecules on the EC surface and by synthesis and secretion of regulatory humoral factors. Apparently, confounding data have been provided with regard to the effect of EC on plaque progression and phenotype. Atherosclerosis is a disorder with endothelial dysfunction, and it is thus conceivable that replenishment of ECs would result in attenuated EC activation with consequent inflammation. These findings are supported by a study by Rauscher et al showing that transfer of bone marrow (BM) cells from young apolipoprotein E knockout (apoE KO) mice reduces atherosclerotic plaque size. However, it appears that the angiogenesis inhibitor TNP-470 and angiostatin acting to inhibit plaque neovascularization were found to suppress atherosclerotic lesion development, whereas vascular endothelial growth factor promoted plaque growth. These findings support the hypothesis that vasa vasorum provides a delivery conduit through which inflammatory cells can gain access to the growing neointima and to further perpetuate lesion progression.
Endothelial progenitor cells (EPCs) have attracted major interest in recent years, particularly when found to be present as a subpopulation of peripheral mononuclear cells. They have been demonstrated to support postnatal angiogenesis and vasculogenesis in experimental models. Moreover, their number and functional properties are compromised in patients with atherosclerotic risk factors and restenosis, possibly as a compensatory mechanism. The convincing findings with regard to the therapeutic potential of EPC transfer were followed by recent small-scale trials in patients with myocardial infarction in which BM cell and EPC transfer was used for improving cardiac performance with promising initial findings.

In the current study, we investigated the effects of transfer of spleen cell–derived EPC and BM cells on the extent and nature of spontaneously arising atherosclerotic plaques of apoE KO mice.

### Materials and Methods

#### Experimental Design

Ten-week-old male apoE KO mice (Jackson Laboratories) were divided into 3 groups and served as recipients. Group A served as control receiving PBS. Group B received 3 twice-weekly intravenous injections of 10^6 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes)–labeled EPCs prepared as described below from spleens of healthy age-matched male apoE KO mice. Group C received 3 weekly injections of freshly prepared DiI-labeled BM cells (10^6 cells per mouse) from age-matched apoE KO mice euthanized before each transfer. Recipient mice from the 3 groups were euthanized at 18 weeks of age.

#### Preparation and Labeling of Cells for Transfer

Spleen-derived cells were loaded on ficoll, after which recovered mononuclear cells were seeded on 24-well plates (10^5 per well) coated with fibronectin (Sigma) in 0.5 mL endotoxin basal medium (CellSystems) supplemented with 1 µg/mL hydrocortisone, 3 µg/mL amphotericin B, and 10% FCS. After 5 days in culture, cells were washed with normal saline and resuspended. For transfection studies, 1×10^6 Dil-labeled cells were resuspended in 500 µL PBS for intravenous injection. BM cells were freshly prepared from tibias and femurs of donor age matched male apoE KO mice and suspended in PBS before labeling and injections. For long-term cultures, 1.5×10^6 spleen-derived EPCs or BM cells were cultured for 14 days in medium 199, with change of medium every third day.

#### Characterization of Cells Employed for Transfer

Fourteen days after culture in EC conditions, colonies with EC morphology were evident in both cultures (EPCs and BM cells). Immunofluorescence studies using rabbit polyclonal anti–Tie-2 (C-20), rat monoclonal anti–fak-1 (A-3) goat polyclonal anti-CD31 (platelet EC adhesion molecule-1; M-20), and rabbit monoclonal anti–von Willebrand factor (vWF; all from Santa Cruz Biotechnology) confirmed the EC-like identity of the colonies. EC lineage was further confirmed by indirect immunostaining with the use of Dil–acyethylated low-density lipoprotein (AcLDL) and containing with BS-1 lectin (Sigma).

#### Fluorescence-Activated Cell Sorter Analysis of Cells Used for Transfer

To determine the phenotype of cells used for transfer, the following antibodies were used: phycoerythrin rat anti-mouse Fk1-1 (PharMingen), fluorescein isothiocyanate rat anti–Sca-1 (Southern Biotechnology Associates, Inc.), and activated protein C rat anti–c-Kit (eBioscience), and read using a double-color FACSCalibur flow cytometer (Becton Dickinson).

### Determination of Circulating Inflammatory Cytokines by Flow Cytometry

Serum cytokines were measured serially by cytometric bead array (CBA) using a 4-color FACSCalibur flow cytometer (Becton Dickinson). In CBA, 6 bead populations with distinct fluorescence intensities had been coated with capturing antibodies specific for the respective cytokines. These bead populations could be resolved in the fluorescence channels of the flow cytometer. After beads were incubated with 50 µL of serum, cytokines were captured by their corresponding beads. The cytokine-captured beads were then mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. After incubation, washing, and acquisition of fluorescence data, the results were generated in graphical format using the BD CBA software. The concentrations of interferon-γ (IFN-γ), IL-2, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and IL-12p70 were measured using the inflammatory cytokine CBA kits (BD Pharmingen). The coefficients of variation for all cytokine assays were <10%.

### Determination of Serum-Antioxidized LDL Antibodies

Ninety-six–well plates were coated with either oxidized LDL (oLDL; at concentration of 10 µg/mL in PBS) or native LDL overnight at 4°C. The next steps were performed as described previously.

### RT-PCR of Aortas

Aortas from all groups, obtained on killing, were removed and cleaned of the surrounding tissues after perfusion with RNase-free PBS. Total RNA was obtained using the RNeasy Kit (Qiagen Ltd.) and primed with oligo(dT) according to protocol provided with the One-step RT-PCR kit (Roche Molecular Diagnostics). The PCR (40 cycles) was performed using the ReddyMix PCR Master Mix (Agene). The following primers were used: IFN-γ (sense) CTCTTCTGACAAAGCAAGGCAGAAA; IFN-γ (antisense) CCCCCAGA TACAACCCCAGCAATCA; IL-4 (sense) GAGCCATATCCACGGATGACCAA; IL-4 (antisense) CATTTGACCTGAGACCTAGGTA; IL-10 (sense) CTTGGAACCATCAGGCTA; IL-10 (antisense) ACTTCAATCAGGATGACCAA; TGF-β (sense) ACACGGAGAATTACACGGGAGAC; TGF-β (antisense) CTTACGACCTTGAGACCTAGGTA; CXCL12 (sense) GAGACCTTGAGACCTAGGTA; CXCL12 (antisense) GAGACCTTGAGACCTAGGTA; matrix metalloproteinase-2 (MMP-2; sense) GAGACCTTGAGACCTAGGTA; MMP-2 (antisense) CTTACGACCTTGAGACCTAGGTA; MMP-3 (sense) GAGACCTTGAGACCTAGGTA; MMP-3 (antisense) CTTACGACCTTGAGACCTAGGTA; TIMP-1 (sense) GAGACCTTGAGACCTAGGTA; TIMP-1 (antisense) CTTACGACCTTGAGACCTAGGTA; TIMP-2 (sense) GAGACCTTGAGACCTAGGTA; TIMP-2 (antisense) CTTACGACCTTGAGACCTAGGTA; TGF-β (sense) GAGACCTTGAGACCTAGGTA; TGF-β (antisense) CTTACGACCTTGAGACCTAGGTA; and IL-12p70 (sense) GAGACCTTGAGACCTAGGTA; IL-12p70 (antisense) CTTACGACCTTGAGACCTAGGTA.

### Assessment of Atherosclerosis

Quantification of atherosclerotic lesions was done by calculating the lesion size in the aortic sinuses as described previously.

### Assessment of Plaque Stability Markers by Histochemistry and Immunohistochemistry

Staining with Masson’s trichrome was used to determine fibrous cap to lipid core ratio determined by quantitative morphometry. CD3 lymphocytes and macrophage staining were performed as described previously.

### Statistical Analysis

Antibody and cytokine levels as well as plaque size and features were compared between the 3 groups using a 1-way ANOVA.
Results

Both cell populations used for transfer (total BM cells and spleen cell–derived EPCs) were phenotyped before their intravenous delivery. Both contained abundant cells double positive for DiI-AcLDL and BS-1 (70% to 80% in the BM cell group and nearly 90% in the EPC group; data not shown). By FACS analysis, a significantly higher number of EPCs expressed the Sca-1 marker (a mean of 5.04%) compared with BM cells (a mean of 0.85%). A similar trend was evident with regard to the expression of Flk-1; namely, spleen cell–derived EPCs were more likely to express this marker compared with BM cells (0.3% versus 0.1%; Figure 1A). The coexpression of Sca-1 and Flk-1 was 2× more abundant in spleen cell–derived EPCs compared with BM cells. Additionally, EPCs expressed higher numbers of cells double positive for Flk-1/c-Kit (1.18%) compared with BM cells (0.59%). Representative FACS sheets from these experiments are shown in Figure 1A.

We further pursued comparatively the ability of BM cells and spleen cell–derived EPCs to differentiate into ECs by monitoring their ability to form colony-forming units (cfu) after 14 days of culture in EC medium on fibronectin-coated surfaces. Indeed, cfu were formed by both cell populations and stained positive for several mature EC markers, namely: Tie-2, vascular endothelial growth factor receptor 2, CD31, and vWF (Figure 1B).

Transfer of either BM cells or EPCs did not alter the general health of the recipient apoE KO mice, nor were there differences evident in their weight on killing (data not shown). No significant differences between the 3 experimental groups were evident with respect to serum IFN-γ, TNF-α, MCP-1, or IL-12 (data not shown). However, we found that a significantly increased level of IL-6 was present in the BM cell–treated mice (144±6 pg/mL) compared with EPC-treated mice (128±2 pg/mL; P<0.05) or controls (132±2 pg/mL; P<0.05). A similar trend was evident with regard to MCP-1. However, IL-10 serum levels were also found in the spleen (28±7 versus 31±6 labeled cells per high-power field, respectively), in the lungs (9±3 versus 11±5 labeled cells per high-power field, respectively), and in the liver (5±3 versus 5±2 labeled cells per high-power field, respectively). The injected cells were found predominantly within the lipid core of the plaques and not in the endothelial or subendothelial regions.

We then evaluated a panel of serum cytokines thought to be involved in the pathogenesis of atherosclerotic plaques in apoE KO mice. No significant differences between the 3 experimental groups were evident with respect to serum IL-6, IL-10, IL-12, or MMPs, was statistically significant. Results are expressed as mean±SEM.

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Transfer of either BM cells or EPCs did not alter the general health of the recipient apoE KO mice, nor were there differences evident in their weight on killing (data not shown). No differences were evident between the 3 experimental groups with regard to total cholesterol levels (data not shown).

Next, we explored whether fluorescently labeled cells used for transfer were homing to the atherosclerotic plaques, spleens, lungs, and liver. We observed that intravenously injected cells were present in all plaques, BM cells more abundantly populating the lesions compared with EPCs, although not statistically significant (2.9±0.3 versus 2.4±0.4 labeled cells per plaque). Similar numbers of labeled cells were also found in the spleen (28±7 versus 31±6 labeled cells per high-power field, respectively), in the lungs (9±3 versus 11±5 labeled cells per high-power field, respectively), and in the liver (5±3 versus 5±2 labeled cells per high-power field, respectively). The injected cells were found predominantly within the lipid core of the plaques and not in the endothelial or subendothelial regions.

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content 39±3.1%) compared with BM cell–treated mice (51±3%; \(P<0.05\)) or controls (54±4%; \(P<0.05\); Figure 3).

We then explored the presence of antibodies to oxLDL in the 3 recipient groups and found that IgG levels significantly increased in EPC-treated mice (mean OD 0.44±0.1) compared with controls (OD 0.21±0.09; \(P<0.05\)) and BM cell–treated animals (0.16±0.01; \(P<0.05\); Figure 4A).

We then further studied plaques immunohistochemically and found that plaques from EPC-infused animals were more abundantly infiltrated by CD3 cells compared with lesions from BM cell–treated and control animals (Figure 4). No differences were observed in the number of macrophages infiltrating the plaques from the 3 groups (Figure 4).

**Discussion**

The purpose of the study was to explore the potential of BM cells and spleen cell–derived EPCs to influence atherosclerotic plaque size and phenotype in the apoE KO mouse model. There has been a growing number of studies involving intracoronary transfer of BM cells and EPCs in patients with ischemic heart disease and preliminary results to support a beneficial role in improving cardiac performance.\(^{18-20}\) However, BM cells and EPCs are principally mononuclear in origin-harboring proinflammatory properties, and we have thus reasoned that they may influence plaque size and stability markers.

Analysis of BM cells and spleen cell–derived EPCs demonstrated their in vitro ability to form colonies that stained positive for several mature EC markers such as Flk-1, Tie-2, vWF, and CD31, supporting their endothelial progenitor properties. When studied by FACS, we found that spleen cell–derived EPCs were more abundantly expressing several markers that are attributed to endothelial progenitors such as Sca-1, Flk-1, and both. This is consistent with the ex vivo culture and positive selection of spleen cell–derived mononuclear cells on fibronectin and EC medium for 5 days, similar to the conditions used by Werner et al.\(^{12}\) We used a 3-injection transfer protocol based on the realization that an intravenous administration of \(10^9\) cells per mouse is unlikely to simulate intracoronary administration because most of the cells are likely to be trapped within the spleen before reaching the plaque.\(^{12}\) Most of the clinical trials in patients with acute myocardial infarction and ischemic heart disease used a single dose of \(10^7–10^9\) cells per injection.\(^{21}\) However, the nature of the administered cells is different in each of the studies as well as the mode of delivery, preventing an accurate comparative analysis. In mice, it is extremely diffi-
cult to achieve selective intracoronary injection as has been done in humans, and therefore, the systemic route was used. This represents a potential confounder because it is expected that systemic cell delivery would achieve a less efficient mode of access to the target tissues. Nevertheless, based on the weight ratio between mice and humans, it is unlikely that delivery of a total of 3 million cells represent a number that is exaggerated and thus proatherogenic.

Next, to ensure that transferred cells were homing to the lesions, we studied the plaques and found the DiI-labeled cells were present at a density of 2 to 3 cells per plaque in the BM cell– and EPC-infused groups. In this respect, it is noteworthy that this number may represent an underestimation because quenching of the DiI cannot be completely excluded.

Interestingly, transfer of BM cells and EPCs significantly increased atherosclerotic lesion size in the apoE KO mice. These results are apparently contrasting those obtained by Silvestre et al.,23 which showed increased atherosclerosis but no change in plaque composition when BM cell–endothelial progenitors were transferred from young to old apoE KO mice. However, unlike both of these studies, we used a protocol with a larger number of cells, partially accounting for the possible proatherogenic effect. To exclude a possible confounding effect of age of the donor mice, animals from which cells were harvested were always age matched with the recipients. We also used a group of spleen cell–derived EPCs that were containing a larger number of endothelial progenitors compared with the BM cell group evident by a higher number of Ac-DiI–LDL/BS-1–positive cells and a larger population of Sca-1–enriched, Flk-1-positive, and Sca-1/Flk-1 double-positive cells. It is noteworthy that despite a higher “angiogenic efficiency” evident by a significantly larger number of endothelial progenitors in spleen cell–derived EPCs compared with BM cells, both were similarly proatherogenic in vivo. This finding may appear surprising in view of the antatherogenic effects attributed to endothelial progenitors and the protective role of EC in atherogenesis. We have thus reasoned that proinflammatory properties harbored by the transferred cells may contribute to enhanced atherogenesis. Indeed, we found that serum levels of IL-6 and MCP-1 were significantly increased in BM cell–treated recipients, whereas serum IL-10 concentrations were reduced in the EPC-infused mice. Analysis of atherosclerotic aortas disclosed a significantly reduced expression of mRNA for IL-10 in the EPC– and BM cell–treated mice compared with the controls, suggesting that a proinflammatory state and perhaps partial skewing of T-helper 2 to T-helper 1 may occur in the context cell transfer. These 3 cytokines were shown in several studies to influence atherosclerotic lesion size and could have thus partially mediated the proatherogenic effects.

An additional intriguing finding in this study was a destabilizing effect of EPC transfer. We found that plaques from apoE KO mice infused with spleen cell–derived EPCs contained smaller fibrous caps and larger lipid cores. These results were also consistent with a larger number of CD3 cells within these lesions that may have contributed to plaque softening and vulnerability. In this context, 2 studies should be mentioned: the MAGIC Trial,19 in which recruitment of BM cells by granulocyte/macrophage colony-stimulating factor injections to humans was followed by increased rate of restenosis, an effect proposed to be caused by proinflammatory properties of the injected cells. Additionally, Yoon et al.27 recently observed intramyocardial calcification in rats transferred with BM cells by the intracoronary route, suggesting a direct involvement of the injected cells in this process. It is also consistent with the findings of Saha et al.,28 which showed that BM-derived cells form a significant number of smooth muscle cells that constitute the atherosclerotic plaque in experimental models.

In conclusion, we found that transfer of spleen cell–derived EPCs and BM cells accelerated atherosclerosis in apoE KO mice, whereas EPC transfer reduced markers associated with plaque stability. These findings call for caution in the dosing and scheduling protocols of cell therapy in future studies.

References


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