Seeding of Recipient Bone Marrow Cells Reduces Neointimal Hyperplasia of De-endothelialized Rat Aortic Allograft

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SUMMARY

Transplant vasculopathy is a leading cause of graft failure and a major contributor to the lack of success with small caliber vascular allografts.

In this study we evaluate techniques of bone marrow cell seeding on small caliber vessels and assess the impact of this tactic on neointimal hyperplasia in de-endothelialized rat aortic allografts.

In a preliminary study, bone marrow cells from Lewis rats were seeded onto the chemically de-endothelialized luminal surface of the abdominal aorta of WKY rats - with or without fibrin glue. In the allograft transplantation model, de-endothelialized fresh aortic allografts of WKY rats were orthotopically transplanted into Lewis recipients either directly (n = 6) or after recipient bone marrow cell seeding (n = 6). Histological evaluation was performed at 28 days.

Bone marrow cells were able to adhere to the de-endothelialized aortic wall owing to the use of fibrin glue, but were unable to do so without fibrin glue. In the de-endothelialized allograft transplantation model, recipient bone marrow seeding led to a significant reduction of the ratio of intimal to medial area (0.40 ± 0.08 versus 0.79 ± 0.08, P = 0.0077). Some of the seeded cells remained in the intima for 4 weeks and some infiltrated the media, expressing CD31 or α-SMA.

The results suggest that recipient bone marrow cell seeding on de-endothelialized aortic allograft is feasible with the use of fibrin glue and that this technique reduces neointimal hyperplasia of the graft. (Int Heart J 2005; 46: 303-312)

Key words: Transplantation, Arterial allograft, Bone marrow cell, Seeding, Neointima

Neointimal hyperplasia due to proliferation of smooth-muscle cells (SMCs) is a major cause of transplantation-associated vasculopathy1) and, thus far, a satisfactory small caliber vascular allograft has not been developed. In cryopreserved arterial allograft transplantation, it is difficult to completely preserve the density and viability of endothelial cells (ECs) during antibiotic treatment2) and
cryopreservation, and this may result in transplant arteriosclerosis. Traditionally, intimal SMCs in transplantation-associated vasculopathy have been presumed to originate from the donor media. However, recent reports indicate that circulating progenitor cells contribute to the development of intimal hyperplasia in transplant models and that bone marrow cells (BMCs) can differentiate into ECs and SMCs. Meanwhile, other studies support the contribution of seeded recipient endothelial progenitor cells or smooth muscle cells to the reduction of transplantation-related neointimal hyperplasia. These findings encouraged us to investigate the possibility that recipient BMC seeding may reduce neointimal hyperplasia in de-endothelialized allograft transplantation.

In this study we demonstrate a technique of cell seeding on small caliber vessels and evaluate the impact of recipient bone marrow cell seeding on neointimal hyperplasia in de-endothelialized rat aortic allograft.

**METHODS**

**Animals:** Male Lewis rats, weighing 250 to 350 g, were used both as a source of syngeneic BMCs and as receivers of aortic allografts. Male WKY rats, also weighing 250 to 350 g, were used as aortic donors. Both were purchased from Charles River Japan, Inc (Yokohama, Japan). Special, humane animal-care measures were taken in compliance with the “Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo, revised 1996” and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (National Institutes of Health publication No. 85-23, revised 1985). The number of animals was limited to the minimum necessary to obtain useful results.

**Harvesting of rat abdominal aorta:** Donor animals were anesthetized with an intraperitoneal injection of pentobarbital (5 mg/100 g of body weight). The abdominal aorta of a WKY rat was exposed under a surgical microscope from the left renal vein to the iliac bifurcation, and the collaterals were cauterized. After an intravenous injection of heparin (10 IU/100 g of body weight), the abdominal aorta was harvested and irrigated with normal saline. The animals were killed humanely.

**De-endothelialization of rat aortic allografts:** Aortic allografts were infused with 0.1% Type I collagenase (Gibco BRL) diluted with Roswell Park Memorial Institute (RPMI) medium 1640, clamped, and placed in RPMI medium 1640 for 30 minutes; then, the lumens were rinsed with fetal bovine serum (Gibco BRL).

**Evaluation of de-endothelialization:** The de-endothelialization was evaluated only qualitatively, under both a light microscope and a scanning electron microscope (H-7000, Hitachi, Tokyo, Japan).
Isolation of BMCs: Bone marrow cells were collected from femora and tibias of donor rats, as described elsewhere,\textsuperscript{12} stained with PKH2 (SIGMA), and suspended in 1 mL of Dulbecco’s Modified Eagle’s Medium. The animals were killed humanely.

Cell seeding: Prior to the transplantation experiments, several methods of cell seeding were compared. (A) De-endothelialized aortic allografts were infused with cell suspension, with both ends ligated, and incubated at 37°C in RPMI medium 1640. The luminal surface of the graft was evaluated at 2 hours, 24 hours, 4 days, and 7 days. (B) Human fibrin glue (Tisseel; Baxter AG, Vienna, Austria) was used for seeding. (B-1) A de-endothelialized aortic allograft with a silicon mandrel inside (1 mm in diameter) was infused with a mixture of cell suspension and fibrinogen solution in a 1:1 proportion, and a thrombin solution was drizzled over the graft. (B-2) A de-endothelialized allograft with a silicon mandrel was infused with thrombin solution and then with a mixture of cell suspension and fibrinogen solution in a 1:1 proportion. (B-3) A de-endothelialized allograft with a silicon mandrel was infused with a mixture of cell suspension, fibrinogen solution, and thrombin solution in a proportion of 1:1:1. The 3 types of graft were placed in RPMI medium 1640 for 30 minutes, at room temperature.

Evaluation of cell seeding: Cell seeded grafts were incised longitudinally and the luminal surfaces were evaluated qualitatively, under a fluorescence microscope.

Transplantation of fresh aortic allografts: To investigate the influence of the recipients’ BMC seeding, a comparison was performed between two different sets of experiments. The first group of animals was subjected to transplantation of de-endothelialized allografts without seeding (DE; \( n = 6 \)). The other group underwent transplantation of de-endothelialized allografts with recipients’ BMC seeding (BM; \( n = 6 \)). The seeding was performed according to the B-2 technique, on both internal and external surfaces. A section of treated allograft, in each group, was examined using a scanning electron microscope. Recipients were anesthetized as described above and the abdominal aorta was exposed under a surgical microscope. After an intravenous injection of heparin (10 IU/100 g of body weight), the infrarenal aorta was transected under a segmental clamp and the allograft was transplanted orthotopically, using 9-0 Nylon monofilament interrupted sutures. No immunosuppressive or anticoagulant treatment was used.

Microscopic observation: At 28 days, the animals were deeply anesthetized and the aorta was exposed. A needle was placed in the left ventricle, the abdominal aorta was fixed \textit{in vivo} by perfusion of 10% buffered formalin for 20 minutes at a pressure of 1 mAqu (74 mmHg), and then the whole graft was excised. The middle part of the allograft was immersed in formalin for a minimum of 24 hours and embedded in paraffin for light microscopic study. Four transections (4 \( \mu \)m thick) from every 200 \( \mu \)m of each graft were stained with hematoxylin-eosin (HE) and
elastica van Gieson (EVG). The ratio of intimal to medial area (I/M ratio) was
evaluated using Photoshop 7.0 (Adobe). The averages of each set of 4 values
were subjected to comparison. Some parts of the allografts were evaluated qualita-
tively under a scanning electron microscope.
**Differentiation of seeded BMCs over time:** To observe the changes in the seeded
BMCs, the seeded allografts were taken before transplantation, immediately after
declamping following transplantation, and at 1 hour, 12 hours, 24 hours, 3 days,
7 days, 14 days, and 28 days after transplantation; three samples of each were
taken, and were frozen for fluorescence microscopic evaluation. The 4-µm thick
transections from every 200 µm were stained for CD31 and α-smooth muscle
actin (α-SMA), and nuclei were counterstained with Hoechst 33258 (Sigma), as
described elsewhere.6)

**Statistical analysis:** Morphologic quantitative results are expressed as the mean ±
SEM. Comparison between the two groups was performed using Student’s t test,
in JMP 5.0.1 J (SAS).

**RESULTS**

**De-endothelialization of aortic allograft:** To evaluate the quality of de-endothel-
ialization, both light microscopy and scanning electron microscopy were per-
formed. The luminal surface of the de-endothelialized allograft showed an
absence of ECs and conservation of the internal lamina. Scanning electron
microscopy revealed loss of the 'cobblestone' appearance after de-endothelializa-
tion (Figure 1).

**Cell seeding:** In group A, fluorescence microscopy showed that some BMCs had
seeded (Figure 2) and that there was a tendency for more cells to seed over time.
However, even at 7 days, the adhesion was so poor that seeded cells could be
flushed out with a gentle rinse (data not shown).

In group B, the number of the seeded cells did not seem different. Only in
group B-2 did the cells remain after a gentle rinse. Light microscopy revealed that
the seeded BMCs were buried in the layer of fibrin (Figure 3).

**Microscopic findings of transplanted aortic allografts:** To investigate the influ-
ence of BMC seeding on the formation of neointima, the I/M ratio was calculated
with the aid of a light microscope. The I/M ratio was smaller in the BM group
(0.40 ± 0.08) than in the DE group (0.79 ± 0.08), as shown in the Table and Figure
4. Scanning electron microscopy showed confluent endothelium with a 'cobble-
stone' appearance on the luminal surface, in both groups (Figure 5).

**Incorporation of seeded BMCs into the allograft:** Fluorescence microscopy
showed that some of the seeded cells remained in the intima for 4 weeks and that
some infiltrated the media after 7 days (Figure 6A). At 28 days the seeded cells
expressed CD 31 or α-SMA (Figure 6B).
Figure 1. Microscopic findings of the allograft before and after de-endothelialization. Arrowheads point to ECs. SEM, scanning electron microscopy.

Figure 2. Fluorescence microscopic findings of the cell suspension and the group A graft.

Table. I/M Ratio of the DE Group and the BM Group

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Figure 3. Microscopic findings of the group B graft. **A**: Fluorescence microscopy of the group B before and after rinse. **B**: The Group B-2 graft. Fluorescence microscopy showed seeded BMCs. Arrowheads indicate internal elastic laminae. Arrows indicate BMCs. F = fibrin; M = media; L = lumen.

Figure 4. I/M ratio of transplanted grafts at 28 days. Parameters are expressed as the mean ± SEM. DE = de-endothelialized grafts; BM = BMC seeded grafts. *, P = 0.0077.
Figure 5. Microscopic findings of transplanted grafts at 28 days. DE, de-endothelialized grafts; BM = BMC seeded grafts; M = media; NI = neointima; L = lumen. Arrowheads indicate internal elastic lamina.
**DISCUSSION**

In the present study, chemical de-endothelialization using Type I collagenase was applied because it can be reproduced and because the degree of injury can be easily regulated by the exposure time. In a preliminary study, two techniques for BMC seeding - with and without fibrin glue - were evaluated. In the group without fibrin glue (group A), most cells were detached by a gentle rinse even after a 7-day incubation. It is speculated that BMCs, given their round shapes and smooth surfaces, are barely able to attach to the smooth surface of the neointima. The incorporation of the seeded BMCs is shown in Figure 6.

**Figure 6.** Incorporation of the seeded BMCs. Confocal images of the seeded cell (A) at 7 days after labeling of Hoechst 33258 (blue), (B) at 28 days after immunofluorescence labeling of CD31 (red) or α-SMA (red). Arrowheads indicate internal elastic laminae and arrows BMCs. NI = neointima; M = media; L = lumen.
de-endothelialized aortic allograft even with the help of adhesion molecules. Conversely, in group B-2, BMCs suspended in fibrinogen solution could attach to the smooth lumen treated with thrombin by means of fibrin adhesion. This technique of using collagenase and fibrin glue is sufficiently simple that it can easily be applied to the clinical setting.

This study has demonstrated the ability of seeded BMCs to reduce the neointimal hyperplasia of de-endothelialized aortic allografts. Transplant atherosclerosis is considered to occur because of chronic rejection, therefore, it may be beneficial to coat the lumen of the allograft with the recipient’s cells. BMCs can give rise to either ECs or SMCs. In our study, some cells expressed CD 31 and others $\alpha$-SMA, and they were considered to contribute as reported. However, BMCs contribute not only to the healing process of injured organs but also to pathological remodeling, and it remains unclear which kind of cells derived from the bone marrow and vessel wall are important to reduce graft vasculopathy. In the mechanical vascular injury model, it is suggested that the contribution of BMCs to vascular remodeling depends on the type of arterial injury and that expression of VEGF, SDF-1, and MCP-1 is induced when the endothelium is denuded. Neointimal hyperplasia related to transplantation is considered to occur subsequent to immunoinflammatory endothelial injury and, in such circumstances, seeded BMCs may induce expression of VEGF, SDF-1, or MCP-1, correlated with recruitment of endothelial progenitor cells. To reduce transplantation-related vasculopathy, further investigation with regard to contributing cells as well as the mechanism of allograft rejection is required.

Chronic rejection of the allograft also leads to the disappearance of medial cells or to medial necrosis. Our results indicate that the seeded BMCs can infiltrate the media, suggesting a possible strategy for targeting the homing of recipient SMCs in the allograft media; in other words, long-life allografts may be realizable.

In summary, this study demonstrates that recipient BMC seeding on a de-endothelialized aortic allograft is feasible in combination with the use of fibrin glue, and that this technique reduces neointimal hyperplasia of the graft. Further studies into the differentiation and function of stem cells and techniques of cell homing will ultimately lead to the success of durable vascular and valvular allografts.

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