Development of a New Method for Endovascular Aortic Repair

Combination Therapy of Cell Transplantation and Stent Grafts With a Drug Delivery System

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Background—Endovascular aortic repair by stent grafts (S/Gs) has been developed as a less invasive treatment for aortic aneurysms. However, some aneurysmal cavities can remain without organization, causing re-expansion. We demonstrated previously that transplantation of a cell combination (myoblasts and fibroblasts) promoted thrombus organization in a rat model. We also developed basic fibroblast growth factor (bFGF) slow-delivery S/Gs coated with elastin and impregnated with bFGF. Here, we evaluated the effects of cell transplantation combined with bFGF slow release on canine thoracic aortic aneurysmal sacs after S/Gs repair.

Methods and Results—Thoracic aortic aneurysms were surgically created with jugular vein patches in 15 beagles. Myoblasts and fibroblasts of autologous skeletal muscle were isolated and cultured for cell transplantation. The S/Gs had 6 holes and produced endoleaks in the excluded cavities. Collagen gel (gel group, n=5) or a mixture of skeletal myoblasts and fibroblasts with collagen gel (cell group, n=5) were injected into the aneurysmal sacs excluded by the S/Gs. We also studied the effects of combined therapy of bFGF slow-release S/Gs and cell transplantation (hybrid group, n=5). After 14 days, histological analyses revealed that the excluded aneurysmal cavities of the gel group were filled with fresh thrombus, whereas the excluded cavities in the cell-transplanted groups were occupied by organized tissue. The percentages of the organized areas relative to the excluded cavities, evaluated by Masson’s trichrome staining, were 18.1±4.0%, 52.6±4.0%, and 77.1±6.9% in the gel, cell, and hybrid groups, respectively. Collagen fibers had already appeared, and increased numbers of α-smooth muscle actin–positive cells were observed in the hybrid group.

Conclusions—Cell transplantation accelerated thrombus organization. Moreover, slow release of bFGF enhanced the effects of cell transplantation. Cell transplantation into unorganized spaces may improve the outcomes of endovascular treatments of aortic aneurysms. (Circulation. 2006;114[suppl I]:I-378–I-383.)

Key Words: aorta ■ aneurysm ■ stents ■ cells ■ thrombus

Endovascular aortic repair methods have been developed as less invasive treatments for aortic aneurysms and dissections and have gained popularity for treating poor-condition patients.1–7 We reported previously the efficacy of a treatment using endovascular stent grafts (S/Gs) for aortic dissections, especially for acute onset.5–8 In successful cases, the thrombi inside the excluded aneurysmal cavity gradually become organized, followed by shrinkage of the aneurysm. However, blood flow leaks or pressure overload in the excluded aneurysmal cavity, called endoleaks or endotension, are critically important complications of the S/Gs treatment.9–11 These endoleaks or endotension inhibit thrombus organization and cause re-expansion of the aneurysm. The rate of endoleaks immediately after S/Gs repair of a thoracic aortic aneurysm has been reported to range from 4% to 30%.2,3,9

Various methods for blocking endoleaks or endotension have been reported, such as transcatheter embolization with thrombin, lipiodol, polyurethane, glue, and coils.12–15 Thrombin is the most commonly used method, although insufficient effects on clot formation in the sac and peripheral emboli have been reported for this method.12 Artificial agents seem to provide favorable results for their immediate effects; however, their long-term outcomes are still of concern.
because they are not physiological agents and may block the normal healing mechanism.

We reported previously that transplantation of a mixture of myoblasts and fibroblasts dramatically accelerated the organization of thrombi in the rat carotid arterial lumen. In the present study, we used a canine thoracic aortic aneurysm model to examine whether our method can organize the thrombi in aneurysmal sacs exposed to endoleaks after S/Gs placement.

Methods

Cell Harvest

A total of 15 adult beagles (weight, 10.4 to 13.2 kg) were used in this study. All of the animals were cared for in accordance with the guidelines approved by the Mie University Animal Experiment and Care Committee.

The dogs were sedated with an intramuscular injection of ketamine hydrochloride (10 mg/kg body weight) and atropine sulfate (0.08 mg/kg). Portions (~4 g) of the skeletal muscles of the gluteus maximus were excised and minced with scissors. Next, the muscle fragments were incubated in culture medium, consisting of DMEM containing 10% heat-inactivated FBS (ICN Biomedicals Inc), 100 µg/mL kanamycin (Invitrogen), and 100 µg/mL penicillin (Taisho Toyama Pharmaceutical Co), supplemented with 0.2% type I collagen (Sigma) for 120 minutes at 37°C in a shaking water bath. The residual muscle mass was then centrifuged at 1000 rpm for 5 minutes, and the sedimented muscle fragments were further incubated in 50 U/mL dispase (BD Biosciences) for 60 minutes at 37°C to dissociate myogenic cells. The supernatant containing the cells was collected and washed twice with culture medium.

To separate fibroblasts and myoblasts by taking advantage of their differential adherence to dishes, the obtained cells were initially plated on fibronectin-coated culture dishes (BD Biosciences) in culture medium and incubated at 37°C for 30 minutes to separate the fibroblasts. Next, the nonadherent myoblasts were collected and plated on laminin-coated culture dishes (BD Biosciences). All of the cells were grown at 37°C in 5% CO₂/95% air, and the culture medium was changed after 2 days. When the cells reached confluence, they were harvested by trypsinization, washed, and stored in a freezer until transplantation. Passages were performed when cell preservation solution (Cellbanker; Mitsubishi Chemical) in a 2-8°C freezer until transplantation. All of the cells were grown at 37°C in 5% CO₂/95% air, and the culture medium was changed after 2 days. When the cells reached confluence, they were harvested by trypsinization, washed, and stored in a freezer until transplantation.

S/Gs

The S/Gs were constructed from self-expanding Gianturco stainless steel Z stents (Cook Critical Care Inc) covered with expanded polytetrafluoroethylene (ePTFE) grafts (Impra). Each stent was composed of 2 bodies and had 6 ends at each end. The ePTFE was expanded to twice its size with a balloon catheter, and 2-mm holes were made in 6 places to create endoleaks. The outer diameters of the S/Gs were selected to be 10% to 20% larger than those of both the proximal and distal landing zones measured by preoperative aortography (AOG). The graft material was attached to the stent with 5-0 polypropylene running sutures to create an aneurysmal wall.

Aneurysm Model

The dogs were sedated using the same methods as for the cell harvest. All of the animals underwent endotracheal intubation and mechanical ventilation with 2 L/min of oxygen after induction with intravenous sodium thiopental. Anesthesia was followed by intravenous pentobarbital and pancuronium administration. Aortic patch aneurysms were constructed with the left external jugular vein in the left lateral position. The left external jugular vein was harvested and opened longitudinally to create an elliptically patulous vein patch. The patch size was ~30 × 15 mm. The descending thoracic aorta was mobilized by a left-side thoracotomy in the seventh intercostal space. Two pairs of intercostal arteries were ligated. After administration of heparin (100 U/kg), the proximal and distal regions of the aorta were clamped. A 3-cm lateral longitudinal aortotomy was created ~3 cm proximal to the diaphragm. The vein patch was sewn using 6-0 polypropylene running sutures to create an aneurysmal pouch.

Intravenous hydration with normal saline was maintained during the surgery, and antibiotics were given at the surgery and continued for 3 days. Each animal underwent a postoperative transesophageal echography (TEE) and AOG.

Image Analysis

All of the dogs were examined by means of the TEE (connected to an SSA-550A ultrasound system; Toshiba Medical Inc) and AOG (Stenoscope; GE Yokogawa Medical Systems) before and after the endovascular procedure and at sacrifice.

The AOG was performed in the following manner. The sheath catheter was inserted from the femoral artery using the Seldinger method. The dog was given an intravenous injection of heparin (50 U/kg). A total of 12 mL of contrast agent (Iopamiron 300; Nihon Schering) was injected at a rate of 6 mL/s using a 5-Fr pigtail catheter through the sheath.

Endovascular Procedure and Cell Transplantation

On the day of transplantation, the cells (myoblasts and fibroblasts) were defrosted and washed by centrifugation. The cell pellets were labeled with 5- and 6-carboxyfluorescein-diacetate-succinimidyl-ester ([CFDA] 500 µg/mL in Hanks’ balanced salt solution; Molecular Probes) for tracing, resuspended in a type I collagen gel (3.0 mg/mL, Cellmatrix Type 1-A; Nitta Gelatin Inc), and kept on ice.

The created descending thoracic aortic aneurysm was mobilized by a left-side thoracotomy in the seventh intercostal space. The infrarenal abdominal aorta was used as the access route for insertion of the S/Gs. The abdominal aorta was exposed through a midline laparotomy and mobilized. Heparin (100 U/kg) was given. The S/Gs were inserted into an 18-Fr Teflon delivery sheath (Keller-Timmermans; Cook) and deployed by quickly withdrawing the outer sheath using the pusher mandrel. This procedure was assisted by using fluoroscopy.

The gel containing the cell suspension was returned to room temperature. Under direct observation, we pricked the aneurysmal wall with a 25-gauge needle and then injected the gel with or without cells into the space between the endovascular graft wall and the aneurysm wall.

Experimental Groups

The beagles (n = 15) were divided into 3 groups. Ten beagles were excluded with elastin-coated S/Gs, followed by injection of 1.5 mL of type I collagen gel alone (gel group, n = 5) or 1.5 mL of type I collagen gel containing 1.5 × 10⁷ skeletal myoblasts and 1.5 × 10⁷ fibroblasts (cell group, n = 5). The remaining 5 beagles were excluded with bFGF-S/Gs and then injected with 1.5 mL of type I collagen gel containing 1.5 × 10⁷ skeletal myoblasts and 1.5 × 10⁷ fibroblasts (hybrid group, n = 5).

Sacrifice and Tissue Harvesting

All of the beagles were sacrificed with a lethal dose of anesthesia at 2 weeks after the endovascular procedure. The thoracic aorta was dissected, washed with saline, and fixed in 4% formalin. Specimens were divided after the metallic stent was removed. The cross-section specimens with the maximum transverse diameter were analyzed macroscopically and microscopically.
Histological Analysis
The specimens were embedded in paraffin and sectioned at 4 μm. The paraffin sections were stained with hematoxylin and eosin, Masson’s trichrome (MT), or Sirius Red. Immunohistochemistry was performed using an anti-α–smooth muscle actin (α-SMA) antibody (1A4; 1:750 dilution; Sigma), and the immunoreactivity was detected with 3,3′ diaminobenzidine.

For the MT staining, the ratio of the fibrotic and organized areas in the excluded aneurysmal cavity was calculated using the National Institutes of Health Image analysis software (version 1.61 for Macintosh) in each group. For the Sirius Red staining, the ratio of the collagen fiber area was calculated. Regarding the α-SMA staining, we examined the total number of α-SMA–positive cells per unit area in each group. We detected the CFDA-labeled cells using a fluorescence microscope.

Statistical Analysis
Data are presented as the mean±SD. For statistical analysis, the Mann–Whitney test for independent samples was selected and performed using Statview for Windows version 5.0 (SAS Institute Inc). P values of <0.05 were considered to indicate statistical significance.

We had full access to the data and take responsibility for its integrity. We have read and agree to the article as written.

Results
An aortic aneurysm developed successfully in all 15 of the beagles. The mean patch aneurysm dimensions determined by TEE were 16.6±0.67 mm (range, 15.4 to 17.8 mm). The mean diameter of the normal canine thoracic aorta was 7.6±0.39 mm (range, 7.1 to 8.3 mm). The S/G placement procedures were carried out successfully at 4 weeks after creation of the aneurysm. Endoleaks were present in all of the cases on the AOG just after the endovascular procedure (Figure 1). However, all of the endoleaks were absent at sacrifice. None of the beagles had any complications, and all were euthanized at 2 weeks after the endovascular procedure.

Organization of the Excluded Aneurysmal Cavities After Cell Transplantation in a Canine Thoracic Aortic Aneurysm
Histologically, the excluded aneurysmal cavities in the cell-transplanted groups contained large numbers of nucleated cells. In contrast, the cavities in the gel group were occupied by erythrocytes with an extremely low number of nucleated cells. The percentages of the fibrous area in the aneurysmal cavities were 18.1±4.0% for the gel group, 52.6±4.0% for the cell group, and 77.1±6.9% for the hybrid group, as evaluated by MT staining (Figure 2A-a, 2A-b, 2A-c, and 2B).

The fibrosis ratios in the cell and hybrid groups were significantly higher than that in the gel group. High-power magnification images revealed that cell transplantation accelerated fibrosis (Figure 2A-d, 2A-e, and 2A-f).

Next, we investigated the collagen contents of the aneurysmal cavities, as evaluated by Sirius Red staining. Increased Sirius Red staining was observed in the cell and hybrid groups (Figure 3). The ratio obtained for the collagen fiber–containing areas were 1.72±1.3% (gel group), 22.3±4.1% (cell group), and 52.2±5.6% (hybrid group).

Proliferation of the Transplanted Cells in the Aneurysmal Sac
CFDA, which appears as green fluorescence, was used to trace the transplanted cells and distinguish the transplanted cells from the host cells. Cells expressing green fluorescence were observed in the aneurysmal sac at 2 weeks after transplantation (Figure 4).

Immunohistological Analyses of the Organized Areas of the Excluded Aneurysmal Sac
To clarify the mechanism for the decreased luminal sizes, we characterized the cell components in the organized areas. Using immunohistological staining, α-SMA–positive cells were predominantly detected in the cell and hybrid groups. The α-SMA–positive cells were more abundant in the hybrid group than in the cell group, whereas only a tiny number of positive cells were observed in the gel group (Figure 5). The total number of α-SMA–positive cells per unit area was 0.0068±0.004 cells/μm² (gel group), 0.739±0.12 cells/μm² (cell group), and 2.73±0.13 cells/μm² (hybrid group).

Discussion
The S/Gs treatment is widely used as a safer and less invasive procedure than open surgery. However, the long-term effectiveness of this treatment in preventing aneurysm rupture and death is unknown. Recently, it has been reported that various kinds of complications related to endoleaks and endotension, in particular, can potentially occur during and after this treatment.9–11 These complications have been correlated with aneurysm rupture. An excluded aneurysmal cavity exposed to endoleaks or endotension becomes filled with fibrin and serum.11 As one of the causes of these complications, it is thought that intra-aneurysmal cavities excluded by the S/Gs become filled with unorganized thrombus.
During the organizing process of a thrombus, fibroblasts migrate into the blood clot, transform into myofibroblasts, form granulation tissue, and finally replace this tissue with solid connective tissue.\textsuperscript{17,18} We intended to accelerate this natural response for the healing process of excluded aneurysmal sac. An important point for the organization is that the migrated cells are viable, because administration of materials without viable cells appears to be associated with difficulties in promoting the organization, particularly in the long-term. Therefore, we pay a great deal of attention to the viability of our skeletal muscle cells. In our previous study, we demonstrated that simultaneous transplantation of myoblasts and fibroblasts accelerated the organization of thrombi in the arterial lumen. It is sup-

\begin{figure}
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\caption{Evaluation of the organization in the aneurysmal cavity by MT staining. A, Cross-section specimens with the maximum transverse diameter were analyzed macroscopically and microscopically at 2 weeks after the S/G insertion and cell transplantation. The majority of the aneurysmal cavity in the gel group is composed of red blood cells (a and d). The aneurysmal cavity in the cell group is partly filled with organized tissues containing cells and collagen fibers (b and e). Moreover, the whole aneurysmal cavity in the hybrid group is filled with organized tissues (c and f). B, Mean percentages of the organized area relative to the aneurysmal cavity in each group at 2 weeks after the endovascular procedure. The organization in the cell and hybrid groups is significantly more advanced than that in the gel group. *$P<0.01$.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Histological findings of the aneurysmal cavity at 2 weeks after injection of gel or cells using Sirius Red staining. Cross-section specimens with the maximum transverse diameter (a through c) and high-power magnification views (d through f) are shown. Collagen fibers stained red are present in the cell (b and e) and hybrid (c and f) groups, whereas no collagen fibers are present in the gel group (a and d). #1: aneurysmal wall; #2: aneurysmal cavity; #3: ePTFE graft. Bars=100 $\mu$m.}
\end{figure}
posed that the myoblasts enhance the phenotypic change of the fibroblasts into myofibroblasts.16

In the present study, transplantation of myoblasts and fibroblasts promoted thrombus organization in a canine aortic aneurysm model with endoleaks after S/Gs insertion. Production of collagen fibers was confirmed in the excluded aneurysmal sac at 2 weeks after the cell transplantation, and, moreover, the presence of numerous α-SMA–positive cells was confirmed. It is well known that myofibroblasts expressing α-SMA generate contraction forces and minimize the size of organized tissues.17–19 These results may be said to show the ideal tendencies, such that shrinkage of the aneurysm may be expected later.

The viability of vascular (aneurysmal) wall cells, as well as the growth factors and cytokines produced by these cells, may be important factors for organization of the thrombus. Some previous methods for preventing expansion of an aneurysm have been reported by seeding of vascular smooth muscle cells20–22 and gene introduction23 on the aneurysmal walls in animal models. In the present study, CFDA-labeled cells were observed in the aneurysmal sac at 2 weeks after the transplantation. The cellular localization was not characteristic and did not show a clear association with the vascular wall cells and organization. It is possible that the leakage flow was influenced, because we found that insertion of bFGF slow-release S/Gs in a similar aneurysmal model showed a tendency for the thrombus organization to start around the vascular walls. This may indicate that the growth factors work effectively in the presence of active cells. In real clinical cases with an arteriosclerotic aortic wall, active cell transplantation or derivation may be an alternative therapy.

Regarding clinical application of these methods, it is possible to transplant cells with a transcatheter or percutaneous puncture maneuver. The cells were mixed with gel to prevent severe bleeding after the puncture and scattering by the endoleak flow and then injected. The gel used was liquid when refrigerated, and immediately became gelatinous at body temperature. The cell/gel mixture was directly injected into the aneurysmal sac without severe bleeding in the present study. For operations with simultaneous S/G insertion, cell transplantation with a catheter is easily performed. In addition, endoleaks (at least all type I endoleaks) can be embolized through a proximal perigraft channel with catheters.13 Moreover, for re-expansion cases of post-S/G implantation, a percutaneous puncture maneuver from the back may apply to access to the excluded aneurysmal cavity under computed tomography support without open surgery. At this point in time, judging the properties of an aneurysmal cavity by MRI is important.24 Furthermore, cell transplantation under thoracoscope assistance may have an effect.

This study has some limitations. The aneurysmal walls used in our study showed no atherosclerosis. The influence of bFGF and the transplanted cells on vessel walls are unknown. Our stent graft model did not have any persistent endoleaks, and we have no precise record of when the endoleaks disappeared. We need to examine the long-term changes to

**Figure 4.** CFDA, which appears as green fluorescence, is used to trace the transplanted cells and distinguish the transplanted cells from the host cells. Cells expressing green fluorescence are observed in the aneurysmal sac at 2 weeks after the transplantation.

**Figure 5.** Immunohistological analyses of α-SMA in the organized areas of the aneurysmal cavity at 2 weeks after the transplantation. α-SMA–positive cells (brown) are predominantly detected in the cell (b) and hybrid (c) groups. The positive cells are more abundant in the hybrid group than in the cell group, whereas only a tiny number of positive cells are present in the gel group (a). Bars=100 μm.
elucidate whether this method avoids the hyperplasia that causes disorders of the aortic wall constitution. Furthermore, the exact mechanisms for the acceleration of the thrombus organization, especially the interactions between the transplanted myoblasts and fibroblasts, need to be elucidated.

In conclusion, cell transplantation accelerates thrombus organization in excluded aneurysmal cavities by S/Gs. These results show that the transplanted cells may have additional effects on endovascular aortic repair. Furthermore, S/Gs that release bFGF enhance the effects of cell transplantation.

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**Disclosures**

None.

**References**


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