Fibrinogen and Vascular Smooth Muscle Cell Grafts Promote Healing of Experimental Aneurysms Treated by Embolization

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Background and Purpose—Residual necks and recurrences frequently occur after endovascular treatment of cerebral aneurysms. Addition of fibrinogen and vascular smooth muscle cells (VSMCs) to the embolic material may promote healing of embolized aneurysms by increasing neointima formation at the neck.

Methods—Bilateral carotid aneurysms were constructed with venous pouches in 31 dogs. Aneurysms were packed intraoperatively with bare Gelfoam sponges, sponges treated with fibrinogen, or fibrinogen sponges seeded with the animal’s own VSMCs or peripheral blood mononuclear cells. Animals were killed after angiography at 3 weeks, and morphometric studies were performed to measure the thickness of the neointima at the neck of treated lesions. Angiographic results and mean thickness of neointimas were compared using ANOVA. In 8 animals, 1 aneurysm was embolized with sponge seeded with VSMCs transduced by adenoviral infection to express a fluorescent protein (green fluorescent protein), and gene expression was monitored for 4, 7, 14, and 21 days by fluorescent microscopy.

Results—Aneurysms treated with sponges seeded with VSMCs had significantly thicker neointimas and were more completely obliterated at 3 weeks than control aneurysms treated with fibrinogen sponges. Peripheral blood mononuclear cells could not reproduce these findings. Sponges treated with fibrinogen alone promoted formation of a thicker neointima than bare sponges. Transduced cells transplanted into in vivo aneurysms still expressed green fluorescent protein at 3 weeks.

Conclusions—VSMC grafts can improve healing of experimental aneurysms treated by embolization. Transplantation of cells transduced to express a foreign gene opens the way for in situ gene therapy for cerebral aneurysms. (Stroke. 1999;30:1657-1664.)

Key Words: cerebral aneurysm ■ cerebrovascular disorders ■ pathology ■ muscle, smooth ■ dogs

Reurrences after embolization of cerebral aneurysms pose a potential risk of late rebleeding, and this problem is limiting the widespread application of endovascular treatment.1–4 Potential avenues to improve morphological results include strategies to increase aneurysmal thrombosis and biomolecular or cellular interventions designed to promote healing mechanisms at the neck of embolized aneurysms.5,6 There is a general pattern of wound healing in the vessel wall: Coagulation, inflammation, migration, and proliferation of vascular smooth muscle cells (VSMCs) with synthesis and deposition of extracellular matrix (ECM) are responsible for vessel integrity and vascular repair phenomena.5–11 Because the cellular events responsible for healing of aneurysms seem to follow this pattern, VSMC grafts could be considered to promote early formation of a thicker, stronger neointima, hopefully resulting in a more permanent obliteration of aneurysms after embolization.5

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We developed an aneurysm model that uses intraoperative packing with collagen sponges6,6 and have shown that complete healing of porcine aneurysms involves VSMCs, which form a thick neointima. When the same model is used in dogs, recurrence, associated with deficient neointima formation, is the rule.5,12,13 Although the exact causes of deficient healing remain speculative, we hypothesized that in situ autologous VSMC grafts may promote healing after embolization. We found support for this hypothesis by comparing the thickness of the neointima formed at the neck of grafted aneurysms with the one found in contralateral control aneurysms in the same animals. Because the technique we used to graft cells also involved a fibrinogen matrix, the potential effects of this protein per se on neointima formation are also demonstrated. To prove retention and viability of grafted cells, we transplanted cells transduced to express a reporter gene, green
fluorescent protein (GFP),\textsuperscript{14} and monitored gene expression for up to 3 weeks in vivo by fluorescent microscopy.

**Materials and Methods**

**Preparation of VSMC Grafts and Control Sponges**

VSMCs were harvested from the left femoral artery of each dog using the explant technique, cultured, and expanded as previously described.\textsuperscript{6} Gelfoam blocks measuring $15 \times 20 \times 7$ mm were cut and hydrated in the autoclave for 10 minutes in PBS and then incubated for 15 minutes in DMEM supplemented with 10% FBS containing fibrinogen (2 mg/mL). Gelfoam fragments were placed in 12-well plates with 1 mL of DMEM-fibrinogen, and $2 \times 10^6$ cells suspended in 1 mL of DMEM-fibrinogen. Plates were incubated at 37°C for 2 hours, and the gel containing cells were transferred to a 6-well plate containing an excess amount of fresh DMEM with 10% FBS. Free-floating sponges seeded with VSMCs were cultured in DMEM and 10% FBS for 1 week before in vivo transplantation. Three types of control sponges were prepared for comparison. Sponges seeded with peripheral blood mononuclear cells (PBMCs) were prepared by injection into the fibrinogen sponges of $2 \times 10^5$ PBMCs isolated by gradient centrifugation with Ficoll from 15 mL of blood collected from the same animal.\textsuperscript{15} Fibrinogen sponges were prepared with DMEM containing 2 mg/mL of fibrinogen as described above but without adding cells to the matrix. These 2 types of control sponges were prepared simultaneously with VSMC sponges and similarly kept in vitro for 1 week before transplantation. Bare Gelfoam sponges were simply hydrated for 10 minutes in PBS 2 hours before the surgical procedure.

**In Vitro Gene Transfer and Expression**

VSMCs were infected by incubation for 2 hours at 37°C with adenovirus expressing GFP for a multiplicity of infection of 150. Replication-defective adenoviral vectors that express the GFP gene were kindly supplied by Dr Bernard Massie (Biotechnology Research Institute, National Research Council of Canada).\textsuperscript{14} Transduced cells cultured in Gelfoam as previously described were transplanted into in vivo aneurysms. For each sponge implanted in vivo, 2 series of identical sponges were studied at the time of transplantation, and the other was kept in vitro until the time of killing. GFP expression was detected by fluorescence microscopy on in vitro sponges as well as on fresh in vivo specimens after axial sections of the aneurysms were obtained at 4, 7, 14, and 21 days ($n=2$ each); these were compared with sponges and aneurysms seeded with untransduced cells.

**Animal Model**

The lateral wall/venous pouch carotid aneurysm model embolized with collagen sponges has been described elsewhere.\textsuperscript{3,6} Protocols for animal experimentation were approved by the Conseil interne de protection des animaux of the University of Montreal Research Center and in accordance with the guidelines of the Canadian Council on Animal Care. We used adult mongrel dogs weighting 20 to 25 kg. All procedures were performed with the animals under general anesthesia. Dogs were sedated with an intramuscular injection of acepromazine (0.1 mg/kg), glycopyrrolate (0.01 mg/kg), and butorphanol (0.1 mg/kg), followed by intravenous injection of thiopental (15 mg/kg). After endotracheal intubation, dogs were ventilated with a Harvard respirator and kept under anesthesia with 2% isoflurane. Postoperative analgesia was provided with a Fentanyl skin patch for 3 days. A 2-cm segment of the left femoral artery was resected after proximal and distal ligation and transferred to cold DMEM supplemented with 10% FBS for primary cultures of VSMCs. Four to six weeks later, bilateral common carotid aneurysms were constructed and embolized intraoperatively with Gelfoam fragments as described.\textsuperscript{6} In brief, lateral wall aneurysms were constructed on each common carotid artery using the technique of German and Black as modified by Graves et al.\textsuperscript{16} Two segments of the same external jugular vein were harvested. After temporary occlusion of the common carotid artery, an oval 5-mm arteriotomy was created in the arterial wall, to which the open venous pouch was sutured with 8-0 prolene. One $8 \times 8$-mm Gelfoam fragment (with or without fibrinogen or cells) was inserted inside the aneurysm to completely occlude it. Treated and control sponges were placed in a random manner, and the surgeon was blinded to the content of the sponge. Angiography from the femoral route was performed immediately after surgery while the animals were still under general anesthesia to study the symmetry of morphological results of the 2 aneurysms. Dogs were allowed to consume their normal diet, and their activities were not restricted. They were anesthetized and carotid angiography was performed before killing (by barbiturate overdose) at 4 days ($n=2$) and at 1 ($n=2$), 2 ($n=2$), or 3 weeks ($n=20$) to document the degree of aneurysmal obliteration and to detect arterial stenosis. Three control animals were followed up for 3 months before angiography and killing. The common carotid artery was dissected after killing. The wall of the carotid artery was longitudinally opened opposite of the aneurysm to visualize the luminal surface of the neointima covering the neck of the aneurysm. A 2-cm axial section of the aneurysm, taken from the middle of the neck, was sliced from the specimen, examined for the presence of fluorescent cells (when appropriate), and used for neointima measurement after formalin fixation and paraffin embedding. The remainder of the aneurysm was frozen at $-70^\circ$C in Fisher’s tissue embedding medium for additional studies, including fluorescence microscopy and immunohistochemical studies of cryosections.

Healing at the neck of aneurysms was assessed in vivo by angiography at 3 weeks, and results were scored according to a classification previously described.\textsuperscript{3,5,7,17} A score of 0 indicated complete obliteration: 1, “dog ears”; 2, recurrent neck; and 3, recurrent aneurysm.

**Pathological Studies**

The neointimal layer at the neck of each aneurysm was measured (without knowledge of the nature of the sponge) in 5 locations after formalin fixation, axial sectioning, and staining with hematoxylin, phloxine, and saffron (HPS) and with Movat’s pentachrome; this method was developed and standardized in our laboratory.\textsuperscript{3,5} Immunohistochemical methods were used to characterize neointimal cells and cells inside the sponge at different time intervals after embolization using antibodies to smooth muscle $\alpha$-actin and von Wille-
brand factor. Morphometric data were analyzed using a computerized image analysis system.

### Statistics

Data were analyzed using ANOVA for repeated measures, applied to an incomplete block design with nesting: Because each dog received 2 types of treatments, applied to 2 separate aneurysms, dogs were treated as blocks, in which treatments were considered nested. Stepwise linear regression was used to investigate the relationship between angiographic score (dependent variable) and type of treatment and neointima thickness (independent variables).

### Results

#### In Vitro Studies

VSMCs were cultured from 100% of femoral artery explants in all animals. These cells were α-actin positive and proliferated to yield 6 to 8 × 10⁶ cells in 4 weeks. VSMCs could not adhere to bare Gelfoam sponges in vitro. When seeded on a collagen sponge treated with fibrinogen, VSMCs adhered to the matrix. Contraction of the matrix by VSMCs around the sponge led to formation of an organized structure that looked like an "in vitro neointima" (see Figure 1A). VSMCs were morphologically intact after adenoviral infection and still expressed α-actin (Figure 1B). Cell proliferation was unaffected by adenoviral infection, with the multiplicity of infection varying from 100 to 1000 (data not shown). GFP expression remained strong in all specimens and at all times (4 days and 1 to 4 weeks) after infection and seeding onto collagen sponges (Figure 1C and 1D).

#### In Vivo Studies

**Angiographic Results**

Angiographic results are summarized in the Table. There was no significant difference in mean angiographic scores at time 0 between aneurysms or between groups of animals. The angiographic score of lesions treated with bare sponges significantly increased at 3 weeks, confirming the tendency for recurrence of this model (P < 0.001) (Figure 2). The angiographic score of aneurysms treated with VSMCs decreased slightly at 3 weeks, but this trend did not reach statistical significance (P = 0.36). At 3 weeks, aneurysms were significantly more obliterated with VSMC grafts (score of 1.13) than with fibrinogen sponges (1.92; P = 0.01) or bare sponges (2.39; P = 0.001). Aneurysms treated with fibrinogen sponges had a significantly lower score than those treated with bare Gelfoam sponges (P = 0.05). All aneurysms treated with bare sponges and followed up for 3 months showed large recurrences (Figure 3C).

**Histological Findings**

In aneurysms treated with bare Gelfoam sponges, a very thin and incomplete neointima, consisting of VSMCs, ECM, and collagen, was consistently found at the surface of the embolic agent at 3 weeks (Figure 3). The inflammatory reaction was...
Addition of fibrinogen led to thrombus formation at the surface of the sponge (Figure 4). This thrombus was invaded by VSMCs, ECM, and collagen fibers. Inflammatory changes were more extensive. Aneurysms treated with VSMC grafts had a thick cellular neointima. Most cells were \( \alpha \)-actin positive, but inflammatory cells could also be recognized (Figure 5). Control aneurysms followed up for 3 months showed large recurrences; the neointima at the surface of the sponge, now compressed at the fundus of the aneurysm, remained thin (Figure 3C).

Measurements of the thickness of the neointima at the neck of treated aneurysms are summarized in the Table. There was a significant correlation between angiographic scores and neointima measurements at 3 weeks: the thicker the neointima, the lower the angiographic score (\( P = 0.047 \)). There was a significant difference between treatments (\( P < 0.001 \)). Aneurysms treated with a VSMC graft had the thickest neointimas (342.5 ± 7 \( \mu \)m), followed by collagen sponges treated with fibrinogen (97.6 ± 6 \( \mu \)m). PBMC grafts could not reproduce this finding (37.5 ± 15 \( \mu \)m). Aneurysms treated with bare Gelfoam sponges had the thinnest neointimas (36.1 ± 6 \( \mu \)m). These differences were statistically significant (\( P < 0.002 \)).

**In Vivo Expression of GFP**

Numerous GFP-positive cells could be detected on fresh specimens at 4 days and at 1, 2, and 3 weeks (n=2 each), whereas no fluorescence (except for background) was seen in control aneurysms of the same animals (Figure 6).

**Discussion**

**Aneurysm Models**

Animal models have been designed to study healing mechanisms after embolization of aneurysms. We developed a model that uses collagen sponges (Gelfoam) as an embolic agent.\(^5,6\) Gelfoam has been used to support vascular cell growth in vitro\(^2\) as well as to transfer engrafted cells in vivo.\(^19,20\) Fundamental histopathological findings with Gelfoam do not differ significantly from those found with the coil model.\(^5,12,21\) When the collagen sponge model is used in dogs, aneurysms do not heal at 3 weeks and the neointima formed at the neck of treated lesions is thin and incomplete. We have shown that recurrence, as assessed by angiography, is the rule at 3 months. This canine lateral wall aneurysm model may thus offer an opportunity to study factors responsible for recurrences and serve as a tool to test our therapeutic strategies in an animal that reproduces the clinical problem.

**Aneurysmal Healing, VSMC Grafts, and Neointima Thickness**

We found a significant correlation between angiographic scores and neointima thickness in this canine model. When
bare Gelfoam sponges were used to embolize aneurysms, there was a significant increase in the angiographic score from time 0 to 3 weeks, and the neointima was very thin. VSMC grafts prevented this tendency for recurrence, and the neointima at 3 weeks was significantly thicker. We previously showed that the neointima that forms at the surface of the embolic agent at the neck of treated aneurysms at 3 weeks is thick in animals prone to heal and thinner or absent in animals with a propensity for recurrences.5 Analogous to other deficient healing systems, such as skin ulcers or pseudoarthrosis, the recurrences of canine aneurysms seen at 3 months were the consequence of deficient healing early in the process. Therefore, late results could be improved by stimulating healing mechanisms in the first weeks after embolization. Autologous cell grafts allowed us to increase the thickness of the neointima formed at the neck of canine aneurysms from values associated with recurrence to levels approaching those found in pigs, animals that routinely heal.5,21 This strategy may thus favor healing after embolization, but whether it will effectively prevent long-term recurrences remains to be proven.

Figure 5. VSMC grafts in vivo. A, Axial section at the level of the neck of aneurysm embolized 3 weeks before with sponge seeded with VSMCs. Note thick neointima (arrows) (Movat’s pentachrome, magnification ×20) L indicates arterial lumen; and G, sponge infiltrated with inflammatory cells. B, Immunohistochemical study of neointima shown in A. Note numerous α-actin–positive cells (brown) in loose ECM (white) (magnification ×200). Arrow points to luminal surface of neointima.

Figure 6. GFP expression in vivo. Fluorescent microscopy of fresh specimen at the level of the neointima 1 (A) and 3 weeks (B) after embolization with sponges seeded with VSMCs transduced to express GFP (A and B), compared with contralateral aneurysm treated with untransduced cells (C). Note numerous fluorescent cells. The intensity of fluorescence decreases with time.

Thrombus, Provisional Matrix, and Neointima Formation
The neointima was significantly thickened by the addition of fibrinogen to sponges. Fibrinogen may provide a provisional matrix that is lacking in dogs. Alternatively, the fibrinogen matrix may serve as a more thrombogenic surface. In either case, the provisional fibrin or fibrinogen matrix may be essential as a physical support for cell adhesion and migration, which are basic steps in neointima formation. Thrombotic phenomena have been evoked to
explain differences in the magnitude of postangioplasty restenoses between dogs and pigs. Similarly, thrombus formation on the surface of the embolic agent may be an important factor to explain differences in neointimal thickness after embolization between these 2 species. Furthermore, fibrinogen contains peptide sequences that are involved in cellular adhesion. This property may explain why VSMCs could not be cultured on bare Gelfoam sponges but attached to the fibrinogen gel used in the formation of the grafts. The opportunity to improve morphological results of embolization by providing a more thrombogenic surface or by coating embolic agents with proteins that may favor cellular adhesion or migration is an appealing avenue for future developments.

**VSMC Grafts and Vessel Wall Healing**

Although there is still debate about the precise origin and nature of cells responsible for neointima formation, VSMCs are virtually the only cells present in the arterial media. These cells have been described as multifunctional mesenchymal cells that can reexpress different phenotypes, including cells with the ability to secrete such ECM proteins as collagen. Immunohistochemical analyses performed on different vascular cell types select for “type 2 cells” in outgrowths of medial explants. In this experimental protocol, VSMCs cells could be harvested from 100% of femoral artery explants; these cells were α-actin positive. In canine arteries, the media is composed of at least 2 cell populations. Type 1 cells are contractile VSMCs, which do not proliferate in vitro. Type 2 cells are capable of proliferating and expressing smooth muscle α-actin in culture and are probably responsible for neointima formation.

In vitro conditions select for “type 2 cells” in outgrowths of medial explants. In this experimental protocol, VSMCs cells could be harvested from 100% of femoral artery explants; these cells were α-actin positive at 2 weeks in vitro, proliferated in 10% serum, and could be grown on Gelfoam sponges treated with fibrinogen. The mechanisms responsible for increased neointima formation after VSMC grafting are not precisely known, but PBMCs did not cause the same effect. It is conceivable that grafted cells directly participated in neointima formation by proliferating and secreting ECM. However, most neointimal cells were GFP negative at 2 and 3 weeks, suggesting that either a subpopulation of cells had proliferated and progressively lost the GFP gene (which is episomal) or, more likely, that most cells composing the thicker neointima had migrated from the host rather than from the graft. It is also possible that grafts were more thrombogenic than fibrinogen sponges. Different mechanical properties of the sponges (VSMC-seeded sponges were slightly “contracted”) or a larger number of cells in VSMC sponges (which may proliferate during the 1-week incubation period) are other hypotheses that cannot be excluded. These mechanisms were not elucidated, but injection of cells with a GFP-expressing adenovirus did provide a means to monitor retention and viability of grafted cells in vivo.

**VSMCs Can Be Genetically Modified Before In Vivo Transplantation**

Vascular cells have previously been induced to express a reporter gene. The potential efficiency of adenovirus-mediated gene transfer is supported by cell culture studies: VSMCs have been infected with efficiencies approaching 100%. Adenovirus-mediated gene transfer into normal arteries may result in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. This phenomenon may interfere with protocols designed to treat proliferative diseases but may be helpful in our attempt to stimulate neointimal hyperplasia. The ex vivo cell-mediated method ensures a high efficiency of gene transfer into a specific target cell and decreases immunogenicity. Viable fluorescent cells were recovered in vivo 21 days after embolization. Such a system may thus permit development of other strategies to improve morphological results of endovascular treatment, such as cell-mediated local delivery of biologically active molecules. These could include matrix proteins, molecules involved in cell adhesion, coagulation or thrombolytic modulators, or growth factors that have been shown to favor neointima formation. We previously showed that the neointima at the neck of treated aneurysms could be significantly thickened at 2 weeks by local delivery of growth factors or platelet extracts. The fact that transduced cells still expressed a foreign gene 3 weeks after transplantation supports the hypothesis that this technique could be effective in delivering a desired peptide or protein during this critical period, if not longer. This strategy may not necessitate transplantation of a large number of cells and, as such, may be more realistically accomplished using current technologies. The fibrinogen-matrix method used to seed collagen sponges can be used to seed Guglielmi detachable coils, but a significant number of cells may be lost during coil manipulations. Inclusion of cells within a polymeric embolic agent may be more efficient for delivering cells by endovascular techniques. Of course, VSMC grafts cannot be used immediately after subarachnoid hemorrhage but may be considered for recurrences or elective treatment of unruptured aneurysms. Transplantation of genetically modified cells can, for the time being, provide a model to study the effects of overexpression of molecules that may promote or decrease neointima formation in experimental aneurysms. This investigational tool may advance our understanding of vascular healing phenomena in aneurysms treated by embolization.

**Conclusion**

Deficient healing of canine aneurysms and neointima thickness at the neck of treated lesions are linked. In aneurysms treated with bare Gelfoam sponges, the neointima is very thin and angiographic scores tend to increase even at 3 weeks. Deficient healing is associated with insufficient thrombosis and can be partly compensated by adding fibrinogen to the embolic agent. The neointima at the neck of experimental aneurysms could be significantly increased by VSMC grafts. Autotransplantation of VSMCs could significantly improve angiographic results at 3 weeks. Transplantation of transduced cells is feasible, and gene expression persists at least 3 weeks, opening the way for increased neointima formation after VSMC grafting.
to gene therapy for cerebral aneurysms. This strategy could also help us to understand healing phenomena after embolization of aneurysms.

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References
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22. Raymond et al. Cellular Grafts and Healing of Aneurysms
Clinically, the methods described in this article may not be very relevant, because it appears that aneurysm obliteration with Guglielmi detachable coils is more efficacious than with other methods. Nevertheless, the authors describe important principles that may someday be clinically applicable. Protein coating has already been reported for Guglielmi detachable coils, but this study reached farther. Not only can viable cells be incorporated in the gelfoam, they can also be genetically altered in vitro before implantation.

I believe inducing endothelial growth is actually more important than enhancing the thrombogenicity of the implants. So far, I have seen more problems due to late (1 week) thrombus spreading from the implant than aneurysm recurrence or rebleeding, and with early reendotheliazation of the aneurysm neck, such a complication could obviously be prevented.

The debate about the preferred treatment of cerebral aneurysms is far from settled. Although operative treatment has remained more or less the same for the past 20 years, endovascular treatment is still in full development and furthermore, advances can be expected. This article provides a glimpse into possible future, exciting directions.

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Reference
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