Hepatocyte growth factor prevents intimal hyperplasia in rabbit carotid expanded polytetrafluoroethylene grafting

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Purpose: **The major cause of vascular prosthesis failure is anastomotic intimal hyperplasia caused by the proliferation and migration of smooth muscle cells. Hepatocyte growth factor (HGF) is an endothelium-specific growth factor that exerts a mitogenic action on endothelial cells. This study was designed to examine the effect of HGF on the suppression of intimal hyperplasia after small-caliber expanded polytetrafluoroethylene (ePTFE) grafting.**

Methods: **An ePTFE graft, 2 mm in diameter and 30 mm in length, was implanted in the left common carotid arteries of Japanese white rabbits, after which the animals were fed with a 1.0% cholesterol diet. HGF was infused intravenously immediately and then every day for 7 days at doses of 0.3 mg/body (the 0.3-mg HGF group; n = 20) or 1.0 mg/body (the 1.0-mg HGF group; n = 17). A control group (n = 20) underwent infusion with saline solution. The rabbits were killed on postoperative days (PODs) 1, 2, 3, 5, 7, and 28.**

Results: **The patency rates on POD 28 were 33%, 55%, and 100% in the control, the 0.3-mg HGF, and the 1.0-mg HGF groups, respectively, with a significant difference between the control and the 1.0-mg HGF group ($P < .05$). Endothelial-like cells were seen on the intraluminal surface of the graft only near the anastomotic site on POD 5 in the 1.0-mg HGF group. Intimal thickness at the distal anastomosis was 284 ± 140 μm, 106 ± 18 μm, and 67 ± 10 μm in the control, the 0.3-mg HGF, and the 1.0-mg HGF groups, respectively, with a significant difference between the control and both HGF groups ($P < .05$). The number of anti–embryonic smooth muscle antibody positive cells at the distal anastomosis was 28.6 ± 0.8, 3.8 ± 2.8, and 3.9 ± 0.9 in the control, the 0.3-mg HGF, and the 1.0-mg HGF groups, respectively, with a significant difference between the control and both HGF groups ($P < .01$).**

Conclusion: **HGF might suppress intimal thickness at the anastomotic site and improve the patency rate via rapid reendothelialization by POD 28 in a rabbit carotid ePTFE grafting model. (J Vasc Surg 2002;35:786-91.)**

The long-term results of infrainguinal arterial bypass grafting with prosthesis of less than 6 mm in diameter have been far from satisfactory. The major cause of the poor results is considered to be thrombus formation in the acute phase and obstructive intimal hyperplasia because of the proliferation and migration of medial smooth muscle cells (SMCs) in the anastomotic line during the chronic phase.[1,2] The proliferation and migration of vascular SMCs play an important role in the development of the intimal hyperplasia. A number of studies have been conducted that are related to the suppression of the proliferation and migration of SMCs after the interventions of arterial reconstruction. SMCs are known to be phenotypically modulated from a contractile state to a synthetic state. The SMCs in their synthetic state produce collagen, which leads to intimal hyperplasia.[3-5] Thus, to understand the process of phonetically modulated SMCs in the development of intimal hyperplasia, the investigation of smooth muscle (SM) phenotypes in animal models after grafting is important.

Endothelial cells (ECs) are another important component that affects the long-term patency of a graft. Once the intraluminal surface of the graft is covered with ECs, the graft becomes antithrombogenic and ECs produce several growth inhibitors for SMCs, such as prostaglandin and nitric oxide, which results in the inhibition of the proliferation and migration of SMCs.[3] For this reason, an agent that promotes the ingrowth of ECs and inhibits proliferation and migration of SMCs would be invaluable for the prevention of intimal hyperplasia after the implantation of prosthetic grafts. Hepatocyte growth factor (HGF) was originally isolated from the sera of rats after partial hepatectomy. The molecular weight of HGF is 82-85 kd, and it consists of two subunits, a 69-kd α-chain and a 34-kd β-chain. HGF is synthesized with fibroblasts and vascular SMCs. The HGF receptor, C-met, is expressed on the membrane of vascular ECs.[6,7] HGF is a multipotential cytokine that acts on ECs, stimulating migration, protease production, proliferation, and differentiation into capillary-like tubes in vitro. However, HGF does not promote the proliferation and migration of vascular SMCs.[8,9] Because of

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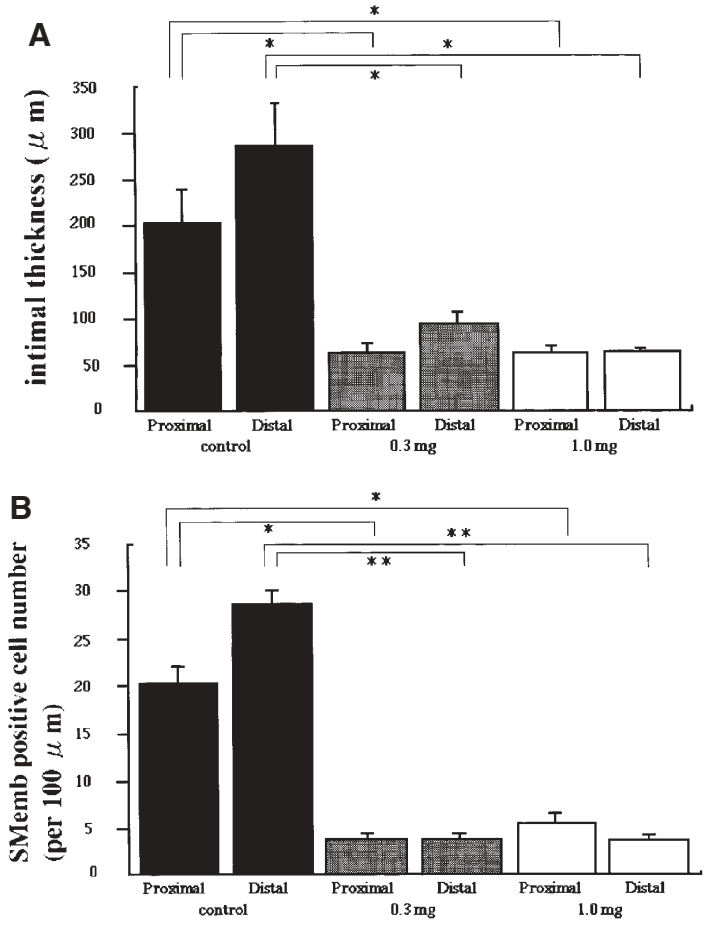

Fig 1. A, Intimal thickness on postoperative day 28 at both proximal and distal sites of anastomosis. Statistical difference was observed between control and 0.3-mg hepatocyte growth factor (HGF) groups and between control and 1.0-mg HGF groups at both anastomotic sites. Mean ± standard error, **P* < .05. **B,** Number of anti–embryonic smooth muscle antibody positive cells in proximal and distal graft intima on postoperative day 28. There were significant differences between control group and HGF-treated groups at both anastomotic sites. Mean ± standard error, **P* < .05, ***P* < .01.

its unique ability to act as a mitogen for ECs, HGF shows promising potential for the suppression of intimal hyperplasia after EC injury. We hypothesized that HGF prevents intimal hyperplasia in the anastomotic line after the implantation of prosthesis in an artery. This study was performed for the evaluation of the effect of HGF on the suppression of anastomotic intimal hyperplasia in a cholesterol-fed rabbit model of carotid artery bypass grafting with expanded polytetrafluoroethylene (ePTFE) and for the investigation of the phenotypic modulation of SMCs and the process of intraluminal healing in the early stage after bypass grafting.

METHODS

Animals. A total of 57 male Japanese white rabbits that weighed 2.5 to 3.0 kg were used in this study. After surgery, all the rabbits were fed a diet that contained 1.0% cholesterol. SMCs in their synthetic state change into foam cells under conditions of hyperlipidemia, and this foam cell is a main component in the intimal hyperplasia. So, the animals' cholesterol feed can make the evaluation of intimal thickness easy at the anastomosis. With the other method, it is hard to thicken intimal hyperplasia under normal conditions in rabbits. This experiment was reviewed by the Committee of the Ethics on Animal Experiment in Yamaguchi University School of Medicine and carried out under the control of the Guideline for Animal Experiment in Yamaguchi University School of Medicine and The Law (No. 105) and Notification (No. 6) of the Government.

The rabbits underwent anesthesia, and the left carotid artery was exposed in the anterior neck under sterile conditions. A standard walled ePTFE graft (2 mm in diameter, 30 mm in length, and 30 □m in fibril length; GORE-TEX, W.L. Gore and Associates, Inc, Flabstaff, Ariz) was interposed in an end-to-end fashion, and anastomosis was performed with 12 stitches of interrupted sutures of 8-0

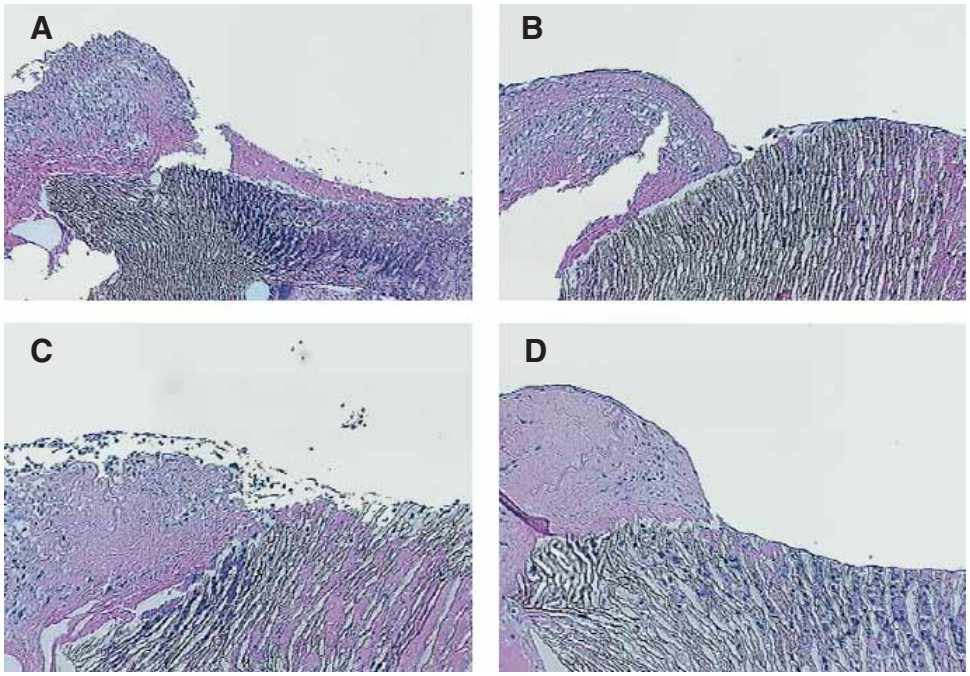

Fig 2. Light microscopic findings at distal anastomosis on postoperative day (POD) 5 and POD 7 in hepatocyte growth factor (HGF)–treated group. Endothelial-like cells were seen on POD 5 in 1.0-mg HGF group and on POD 7 in 0.3-mg HGF group (hematoxylin-eosin stain, ×20). **A,** POD 5 in 0.3-mg HGF group; **B,** POD 7 in 0.3-mg HGF group; **C,** POD 5 in 1.0-mg HGF group; **D,** POD 7 in 1.0-mg HGF group

polypropylene. The rabbits were divided into three experimental groups. The HGF or vehicle was infused immediately after surgery through the ear vein and then every day for 7 days after surgery. A control group of 20 rabbits was given 1.0 mL of saline solution intravenously as a vehicle. The remaining rabbits were divided into two groups. One group (n = 20 rabbits) underwent treatment with an intravenous injection of 0.3 mg/body HGF (0.3-mg HGF group), and another group (n = 17 rabbits) underwent treatment with 1.0 mg/body HGF in the same manner (1.0-mg HGF group). The dosages and prescribed periods were arbitrarily selected in part on the basis of previous evidence that 0.05 to 0.25 mg/kg HGF administered as a daily intravenous bolus injection five times between 6 days prevents acute renal failure after $HgCl_2$ administration.[10] This study used recombinant human HGF, which was purified from a culture medium of CHO cells transfected with an expression vector that contained deletion-type human HGF complementary DNA, and the purity of HGF exceeded 98%. The rabbits were killed on postoperative days (PODs) 1, 2, 3, 5, 7, and 28. After the rabbits had been anesthetized, the ePTFE graft was exposed and the graft patency was checked with Doppler scan signals. The ePTFE graft and the host artery were fixed antegrade, first in 500 mL of 0.9% saline solution and then in 500 mL of 4% paraformaldehyde at a pressure of 120 mm Hg, after which the ePTFE grafts were harvested.

Evaluation of intimal thickness and smooth muscle phenotype. The removed samples were embedded in paraffin, cut along the longitudinal axis, and stained with hematoxylin-eosin. The intimal thickness was measured with a computerized sketching program (Mac Scope, version 2.55, Mitani Corporation, Tokyo, Japan) at the following three positions: the anastomotic line and 100 μm and 200 μm away from the anastomotic line. These three numeric values were averaged and used as a representative value. For immunohistochemical analysis, monoclonal antibodies against human SM2 (Yamasa, Japan) and embryonic SM (SMemb; Yamasa, Japan) were used for staining myosin heavy chain isoforms in SMCs. Vascular SMCs contain the following two types of SM myosin heavy chain: SM1 and SM2, the isoforms of which are specific markers for SMC differentiation. SM2 is particularly useful as a marker of contractile-state SMCs. On the other hand, SMemb is known as a nonmuscle myosin heavy chain and is isolated from both the fetal aorta and the brain. SMemb is predominantly expressed in undifferentiated vascular SMCs and is reduced during vascular development.[4,11] SMemb is especially important for the investigation of the synthetic-state SMCs. Therefore, we measured the number of anti-SMemb antibody positive cells in the intima on POD 28. The number of anti-SMemb antibody positive cells was counted with the same computer program. We also assessed the anti-SM2 antibody positive area on POD 28.

Data analysis. The patency rate was evaluated with the Fisher exact test. The differences between means were assessed with the Bartlett test and were evaluated with the

Scheffé test. Values are expressed as means ± standard error. A *P* value of less than .05 was considered statistically significant.

RESULTS

Patency rates and intimal thickness. The patency rate on POD 7 was 100% in all the groups. However, by POD 28, the patency rates were 33% (three of nine cases) in the control group, 55% (five of nine cases) in the 0.3-mg HGF group, and 100% (seven of seven cases) in the 1.0-mg HGF group. There was a statistically significant difference between the control group and the 1.0-mg HGF group ($P < .05$). The intimal thickness at both anastomotic sites on POD 28 is shown in Fig 1,*A*. Intimal thickness at the proximal and distal anastomosis was 201 ± 90 □m and 286 ± 140 □m, respectively, in the control group, 61 ± 5 □m and 106 ± 18 □m, respectively, in the 0.3-mg HGF group, and 66 ± 7 □m and 67 ± 10 □m, respectively, in the 1.0-mg HGF group. There was a statistical difference between the control and the 0.3-mg HGF groups and between the control and the 1.0-mg HGF groups at both anastomotic sites ($P < .05$).

Microscopic findings. Fig 2 shows the microscopic findings in the HGF-treated group. Fibrin net was seen in the ePTFE graft near the anastomotic site in all the groups on POD 1 and propagated to the mid-portion of the ePTFE graft on POD 5. On the other hand, endothelial-like cells were seen only near the anastomotic site on POD 5 in the 1.0-mg HGF group and on POD 7 in the 0.3-mg HGF group (Fig 2), but were not seen in the mid-portion of the graft. The monolayer of endothelial-like cells of graft luminal surface looked like reendothelialization in the 1.0-mg HGF group on POD 7 (Fig 3,*A*), whereas no endothelial-like cells were observed in the control group within 7 days. On POD 28, the intraluminal surface of the graft only near the anastomotic site in all the groups was covered with endothelial-like cells but did not show endothelial-like cells in the mid-portion of the graft (data not shown). These are endothelial-like cells, but the various methods for the identification of endothelium in other species were tried without success in this rabbit model.

Accumulation of smooth muscle cells. On POD 5, anti-SMemb antibody positive cells were seen just beneath the EC layer of the host artery in all the groups (Fig 3,*B*). On POD 28, there were a number of anti-SMemb antibody positive cells in the intimal hyperplasic area in the control group. However, in the HGF-treated groups, the number of anti-SMemb antibody positive cells was not as prominent as that seen in the control group (Fig 4). The number of anti-SMemb antibody positive cells in the intima of the proximal site on POD 28 was 20.3 ± 3.8 in the control group, 3.3 ± 1.5 in the 0.3-mg HGF group, and 6.2 ± 0.8 in the 1.0-mg HGF group. There were significant differences between the control group and the HGF-treated groups ($P < .05$; Fig 1,*B*). The number of anti-SMemb antibody positive cells in the intima of the distal site was 28.6 ± 0.8 in the control group, 3.8 ± 2.8 in the 0.3-mg HGF group, and 3.9 ± 0.9 in the 1.0-mg HGF group. The number of anti-SMemb antibody positive cells at the distal anastomosis was significantly less in the HGF-treated groups than in the control group ($P < .01$; Fig 1,*B*). Evaluation results of the anti-SM2 antibody on POD 28 revealed that there was an anti-SM2 antibody positive area in the media of the host artery but not in the graft intima of any groups (data not shown).

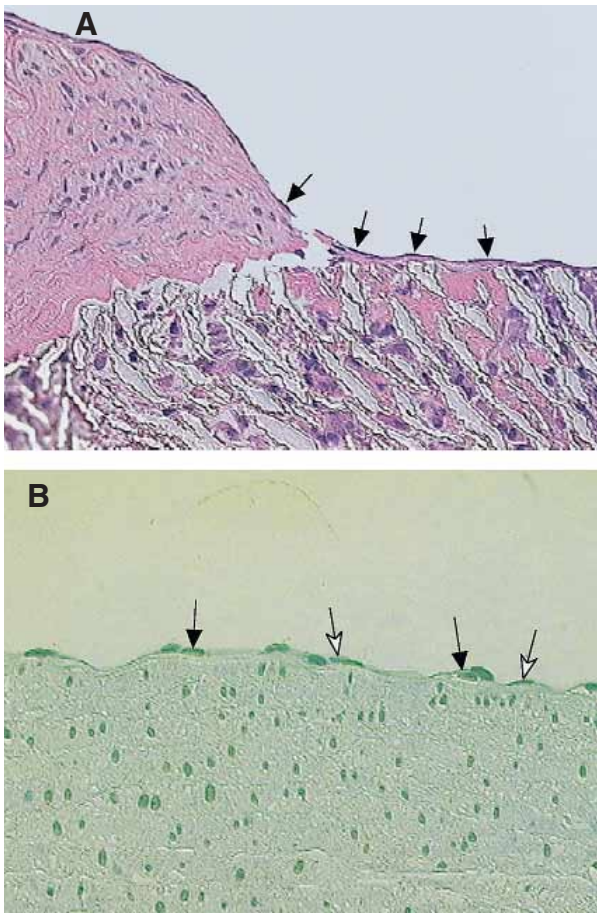

Fig 3. A, Monolayer of endothelial-like cells on graft luminal surface *(black arrow)* looked like reendothelialization in 1.0-mg hepatocyte growth factor group on postoperative day 7 (hematoxylin-eosin stain, □40). **B,** Expression of anti–embryonic smooth muscle antibody positive cells in media of distal site in 1.0-mg hepatocyte growth factor group on postoperative day 5. Anti–embryonic smooth muscle antibody positive cells, which were stained brown *(black arrow)*, were seen below endothelial cells *(white arrow)*, which indicates beginning of migration of synthetic state smooth muscle cells.

DISCUSSION

The results of this study showed that HGF significantly reduced the development of intimal hyperplasia in cholesterol-fed rabbits after carotid ePTFE grafting. Anastomotic intimal thickness is the most common cause of late graft failure after peripheral arterial reconstruction. A hypothesis has been proposed that after the implantation of prosthetic graft, vascular endothelial injuries near

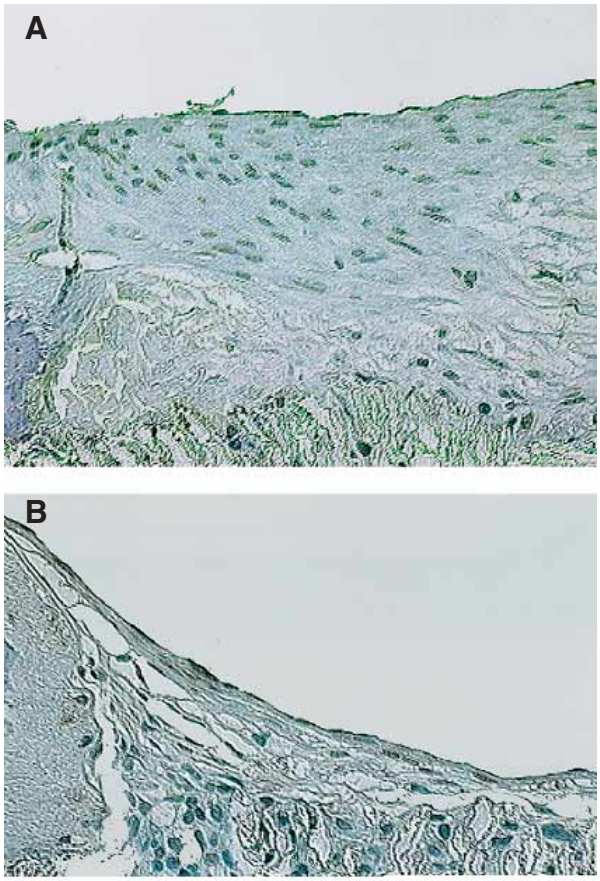

Fig 4. Expression of anti–embryonic smooth muscle (SMemb) antibody positive cells in intima of distal site on postoperative day 28. A number of anti-SMemb antibody positive cells were seen in intimal hyperplasic area in control group. However, in hepatocyte growth factor (HGF)–treated groups, number of anti-SMemb antibody positive cells was not as prominent as in control group. **A,** Control group, □40; **B,** 0.3-mg HGF group, □40; **C,** 1.0-mg HGF group, □40.

the anastomotic line and the subsequent exposure of subendothelial tissue to plasma constituents cause intimal thickness. In the initial stage of intimal thickness, endothelium near the denuded subendothelial tissue induce the migration and proliferation of SMCs and in the media of the adjacent vessel migrate into the intima.[2,12] After that, SMCs migrate to the synthetic graft, and the synthesis of connective tissue proteins, under the influence of various mitogenic factors, are now considered to be major processes involved in the formation of intimal thickness. And moreover, once the SMCs are covered with an intact endothelial monolayer, SMC proliferation returns to the base line, except at the anastomotic site.[13]

In the proliferative intimal thickness response, the synthetic phenotype of SMCs plays a major role. The phenotypic modulation of SMCs from the contractile state to the synthetic state was first described by Chamley-Campbell, Campbell, and Ross,[5] and electron microscopic study results have revealed that SMCs in their synthetic state in culture lose much myofilament and are abundant in subcellular organelles involved in protein synthesis. In fact, SMCs in their synthetic state promote the proliferation, migration, and production of connective tissue and change into foam cells during the chronic phase under conditions of hyperlipidemia.[14] The synthetic state of SMCs is stimulated with proliferative factors, such as platelet-derived growth factor, fibroblast growth factor (FGF), and transforming growth factor–□, which are released from platelets, macrophages, and ECs for paracrine and from SMCs for autocrine.[3] Once phenotypic modulation occurs, the synthetic state of SMCs keep proliferating and migrating for at least 3 months.[15] However, the proliferation of SMCs has been found to be suppressed with antiproliferative factors, such as nitric oxide, prostaglandin, and C-type natriuretic peptide released from ECs, and reendothelialization occurs after 3 to 4 weeks.[16,17] Therefore, earlier promotion and facilitation of the proliferation of ECs would control the proliferation and migration of SMCs in the acute phase, thereby preventing intimal hyperplasia in the chronic phase.

HGF, vascular endothelial growth factor, and bFGF are known to strongly stimulate the proliferation of ECs. The effect of HGF on ECs is 1.48 times stronger than that of bFGF, whereas that of vascular endothelial growth factor is 1.25 times stronger. Furthermore, bFGF stimulates the proliferation and migration of SMCs, which may cause intimal hyperplasia at the anastomotic site. However, HGF does not promote the proliferation and migration of SMCs.[15,18] With the consideration of these facts, HGF seems to be the most ideal growth factor for the prevention of intimal hyperplasia.

In this systemic prescribed study, our microscopic findings showed that endothelial-like cells started to cover the intraluminal surface of the ePTFE graft only at the anastomotic site on POD 5 in the 1.0-mg HGF group, which suggests that endothelial-like cells migrated from the host artery. Earlier reendothelialization was seen in the 1.0-mg HGF group sooner than in the 0.3-mg HGF group, which indicates that the effect of reendothelializa-

tion with systemic prescribed HGF graft might be dose-dependent. This rapid regeneration of endothelial-like cells associated with the HGF treatment suggests that it is effective in the suppression of intimal hyperplasia. The HGF-treated groups showed less intimal thickening than did the control group on POD 28, and the fact that the number of anti-SMemb antibody positive cells were significantly less in the HGF group than in the control group supports the belief that the proliferation and migration of SMCs is inhibited with the rapid regrowth of endothelial-like cells. Thus, systemic prescribed HGF was clearly effective in this implantation model.

The half-life of the HGF concentration in rats has been reported as 3.8 minutes,[19] 60% of which is metabolized in the liver. Therefore, if HGF were administered as a bolus injection, a high dose would be necessary. The process of the synthetic state of SMCs was evaluated with a histochemical stain with the anti-SMemb antibody. On POD 1, anti-SMemb antibody positive cells were seen in the media of host arteries in all the groups (data not shown), which suggests that the phenotypic modulation of SMCs was begun within 24 hours. Anti-SMemb antibody positive cells were seen in all the groups within 7 days, and by POD 28, the number of anti-SMemb antibody positive cells in the anastomotic intima was significantly less in the HGF groups than in the control groups. However, anti-SM2 antibody positive area was seen in the media of the host artery on POD 28 but not in the areas of intimal hyperplasia in all the groups. With the consideration of these facts, HGF may act as a suppressor of the proliferation and migration of SMCs, probably through rapid reendothelialization, but not as a promoter of the redifferentiation of SMCs.

The migration of SMCs in their synthetic state started below the monolayer of ECs on POD 5 in this model. In a rabbit balloon injury model, Davies and Hagen[2] reported that by 5 days after injury, SMCs were observed on the luminal side of the internal elastic lamina, which appeared to have migrated to the luminal surface through fenestrations in the internal elastic lamina. Casscells[20] reported that by 5 days after injury, SMCs had migrated into the neointima in the same model. These results indicate that the regeneration of ECs should be completed within 5 days after surgery to suppress the proliferation and migration of SMCs in both the balloon injury model and the grafting model. HGF was administered immediately after surgery and then every day for 7 days after surgery in our model. The early stage of HGF treatment was considered to be the key point for the improvement of the patency rate and for the reduction of anastomotic intimal hyperplasia in this study.

CONCLUSION

The results of this study clearly showed that HGF treatment significantly reduced intimal thickness in both the proximal and the distal anastomosis after carotid ePTFE grafting in cholesterol-fed rabbits. HGF treatment was also considered to stimulate endothelial ingrowth on the ePTFE graft. These effects may be attributable to the indirect inhibition of the migration and proliferation of SMCs via rapid reendothelialization at the anastomotic site. Thus, the significant inhibition of intimal thickness with HGF may contribute to improved long-term patency after arterial grafting.

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