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Effect of Angiopeptin and Aspirin on Accelerated Graft Atherosclerosis in Transplanted Mouse Heart

In this study of the inhibitory effects of angiopeptin and aspirin on the development of accelerated graft atherosclerosis (AGAS), 22 B10.BR mice received intra-abdominal heterotopic heart transplants from B10.A mice, without immunosuppression. Group 1 (n=5) received no pharmacological intervention, Group 2 (n=6) was treated with angiopeptin, Group 3 (n=5) with aspirin, and Group 4 (n=6) with both. There was no significant difference in the incidence of AGAS among these groups. The magnitude of intimal lesion development showed less narrowing of large vessels (>100 μm in diameter) in groups 2 and 4 - i.e. the groups received angiopeptin (Group 1 = 46.9 $\pm$ 9.3%, Group 2 = 28.5 $\pm$ 9.2%, Group 3 = 44.1 $\pm$ 10.9%, Group 4 = 24.2 $\pm$ 5.9%; $p<0.01$). Comparison of the fraction of tropomyosin-positive staining cells in the intima revealed a lesser degree of staining in Group 2 ($p<0.01$). No intervention was effective in preventing smooth muscle cell proliferation in the media or inflammatory cell infiltration in the adventitia. In conclusion, our data suggest that angiopeptin is effective in the direct inhibition of intimal smooth muscle cell proliferation in relatively large vessels, whereas aspirin exhibits no inhibitory role in the progression of AGAS. Angiopeptin appears to be a potential therapeutic agent for inhibiting the progression of postoperative AGAS in clinical heart transplantation.

Key Words: *Heart transplantation; Atherosclerosis; Somatostatin; Aspirin; Transplantation, heterotopic; Mice, congenic*

Jeong Ryul Lee, Ji-Hyuk Yang, Eul Kyung Kim*, Jeong Wook Seo*

Departments of Thoracic and Cardiovascular Surgery, Pathology*, Seoul National University Children's Hospital, Seoul National University College of Medicine, Seoul, Korea

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Address for correspondence
Jeong Ryul Lee, M.D.
Department of Thoracic and Cardiovascular Surgery, Seoul National University Children's Hospital, Seoul National University College of Medicine, 28 Yongon-dong, Chongro-gu, Seoul 110-744, Korea
Tel: +82.2-760-2877, Fax: +82.2-765-7117
E-mail: jrl@plaza.snu.ac.kr

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INTRODUCTION

The development of graft atherosclerosis remains the most serious complication of long-term survivors of cardiac transplantation. It affects 30-50% of all cardiac transplantation recipients (1, 2). Although the pathogenesis of cardiac allograft vasculopathy is not clearly understood, it is generally believed to involve an immunological insult to the coronary endothelium. This results in accumulation of macrophage and lymphocyte with concomitant smooth muscle cell proliferation in intima, often affecting the entire length of the vessels. Angiopeptin, a synthetic cyclic octapeptide analogue of somatostatin, has been reported to inhibit myointimal proliferation in experimental heart transplantation models in rabbit and rat (3, 4). Vascular endothelial injuries with aggregation of platelets, formation of thrombi, and subsequent proliferation of smooth muscle cells seem to be another important factor in the accelerated graft atherosclerosis (5). Aspirin may be beneficial by inhibition of thromboxane production and platelet aggregation, as heart transplantation recipients exhibit a marked tendency towards platelet aggregation and high thromboxane level, which has been known to be resistant to low dose aspirin therapy (6-8). In this study we tested the hypothesis that angiopeptin and/or aspirin might prevent the development of AGAS in a murine heterotopic heart transplantation model.

MATERIAL AND METHODS

Animals

Adult mice (7 to 10 weeks of age) of B10.A and B10.BR strains, weighing 17-22 g, were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). B10.A and B10.BR strains differ in the D locus of the class I major histocompatibility antigen. The mice were housed under conventional conditions and fed a standard diet (Rodent laboratory chows, Ralston Purina Company, St. Louis, MO, U.S.A.) and water ad libitum. After comple-

tion of the heterotopic heart transplantation, the mice were allowed to recover with oxygen and local heat, and were then transferred to their cages 24 hr after surgery with free access to food and water.

Heterotopic heart transplantation

Cardiac allografts from B10.A mice were transplanted into B10.BR mice using standard microsurgical techniques. After adequate anesthesia of 4% chloral hydrate (0.1 mL/20 g of body weight, intraperitoneal injection) and methoxyflurane (inhalation), a sternal lid was lifted upward. The right and the left superior vena cavae were ligated with 5-0 silk. The donor heart was arrested with 0.5 mL cold heparinized saline (100 unit/ mL of saline) delivered via the inferior vena cava. The aorta and main pulmonary artery were then transected and the pulmonary veins were ligated en bloc. The donor heart was preserved in 4℃ cold saline solution until the recipient mouse was prepared.

Through a midline abdominal incision, the recipient's infrarenal abdominal aorta and inferior vena cava were dissected and controlled both proximally and distally with 5-0 silk. After a longitudinal aortic incision, an end-to-side anastomosis between the donor ascending aorta and the recipient abdominal aorta were performed, followed by an end-to-side anastomosis between the donor pulmonary artery and the recipient inferior vena cava using 10-0 nylon sutures. Subsequently, the proximal recipient aortic ligature was released first.

Experimental groups

The recipient mice were divided into four groups. Group 1 (n=5, control) underwent no pharmacological intervention. Group 2 through 4 received intraperitoneal infusions by 100 μL micro-osmotic pump (Alzet, Palo Alto, Calif.). Micro-osmotic pumps were replaced every 15 days for a total of 45 days. Group 2 (n=6) was treated with angiopeptin. Group 3 (n=5) was treated with aspirin. Group 4 (n=6) received both angiopeptin at 80 μg/kg/day and aspirin at 5 mg/kg/day. Similar micro-osmotic pump protocols were followed for groups 2 and 4.

Tissue harvesting and histological examination

All mice were sacrificed 45 days after transplantation. Hearts were excised distal to anastomotic sites, and the apex removed by transverse section. The remaining 2/3 of the heart from each animal was immersed in OCT Compound in cryomolds (Tissue Tek, Miles, Inc., Elkart, IN, U.S.A.) and snap-frozen in liquid nitrogen. Serial transverse cryosections (10 μm thickness in every 100 μm) were obtained from the mid-ventricle through the aortic anastomosis. Immunohistochemistry of the smooth muscle cell marker tropomyosin was performed on all sections using an avidin-biotin-peroxidase technique. All arteries were assessed in each of three sections spaced at 600 μm intervals, starting at the level in which mitral valve leaflets were first visible. Vessels were considered to be affected by accelerated graft atherosclerosis when cells were observed in the intima. The percentage of narrowing of vessels was calculated as 100 minus the ratio of the actual lumen area to the potential lumen area (measured from the internal elastic lamina) as previously described (9). The fraction of smooth muscle (tropomyosin positive) cells in lesions was determined by visual inspection and scored semiquantitatively on a 0 to 3 scale (0=no positive cell; 1 = positive cells present but <20% of total; 2 = 21-50% positive cells; 3 = >50% positive cells). The percentage of tropomyosin positive cells was measured in the media. The media was regarded as being intact when no other cell except smooth muscle (tropomyosin positive) cell, was observed in the media. Perivascular inflammation was scored semiquantitatively on a 0 to 3 scale (0 = no inflammation, 1 = mild increase in perivascular mononuclear cells, 2 = moderate increase, 3 = large increase in perivascular mononuclear cells or moderate increase with presence of poly-morphonuclear cells) (9).

Animal care

All animals were cared for in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Statistical analysis

The values were reported as the mean ± standard deviation. The ANOVA tests and multiple comparison with Turkey and Duncun methods were used for statistical comparisons. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

A mean number of 17.3 (±4.4) vessels were observed in each mouse. There was no statistical difference in the percentage of diseased vessels among the groups (Group 1=46.2 ± 12.5%, Group 2=37.7 ± 10.3%, Group 3=39.6

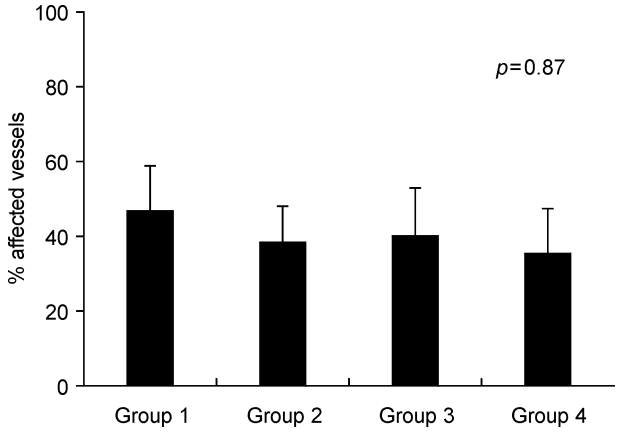

Fig. 1. Incidence of accelerated graft atherosclerosis, 6 weeks after heterotopic mouse heart transplantation. The difference is insignificant among the groups which divided by medications after transplantation (Group 1, control; Group 2, angiopeptin; Group 3, aspirin; Group 4, angiopeptin+asprin).

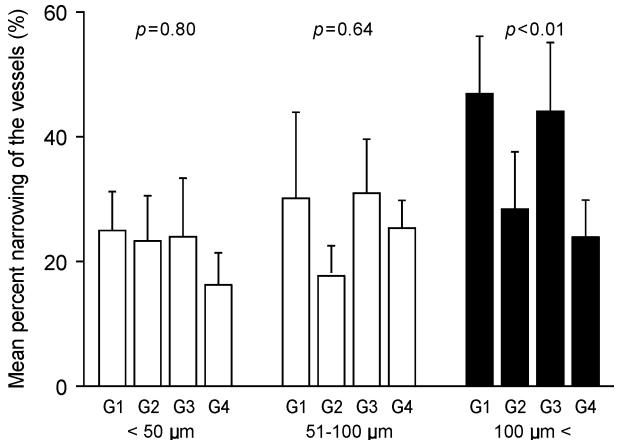

Fig. 3. Less narrowing of the lumen of affected vessels with a diameter of more than 100 μm was observed in group 2 and 4 ($p<0.01$). The percentage of narrowing of vessels was calculated as 100 minus the ratio of the actual lumen area to the potential lumen area (measured from the internal elastic lamina).

±13.3%, and Group 4=34.9±4.8%, p=0.87) (Fig. 1, 2A). The magnitude of intimal lesion development, which was compared between groups in three different subgroups (less than 50, 51-100, more than 100 μm in diameter), showed less narrowing of vessels with a diameter of more than 100 μm in both groups 2 and 4 - i.e. the groups that received angiopeptin (Group 1=46.9 ±9.3%, Group 2=28.5±9.2%, Group 3=44.1±10.9%, Group 4=24.2±5.9%; $p<0.01$) (Fig. 2B, 3). Furthermore, comparison of the fraction of tropomyosin positive cells in the intima, revealed a lesser degree of staining in Group 2 that received angiopeptin only (Group 1=1.4 ±0.2, Group 2=0.5±0.2, Group 3=1.7±0.2, Group 4=1.2±0.2; $p<0.01$) (Fig. 4). No differences were seen in the degree of medial integrity (Group 1=72.1±8.3%, Group 2=73.9±11.3%, Group 3=65.0±16.7%, Group 4=71.7±12.0%; p=0.89) (Fig. 5), or in the degree of perivascular inflammatory cell infiltration (Group 1=1.2 ±0.3, Group 2=1.2±0.3, Group 3=1.9±0.2, Group 4=1.8±0.20; p=0.09) (Fig. 6).

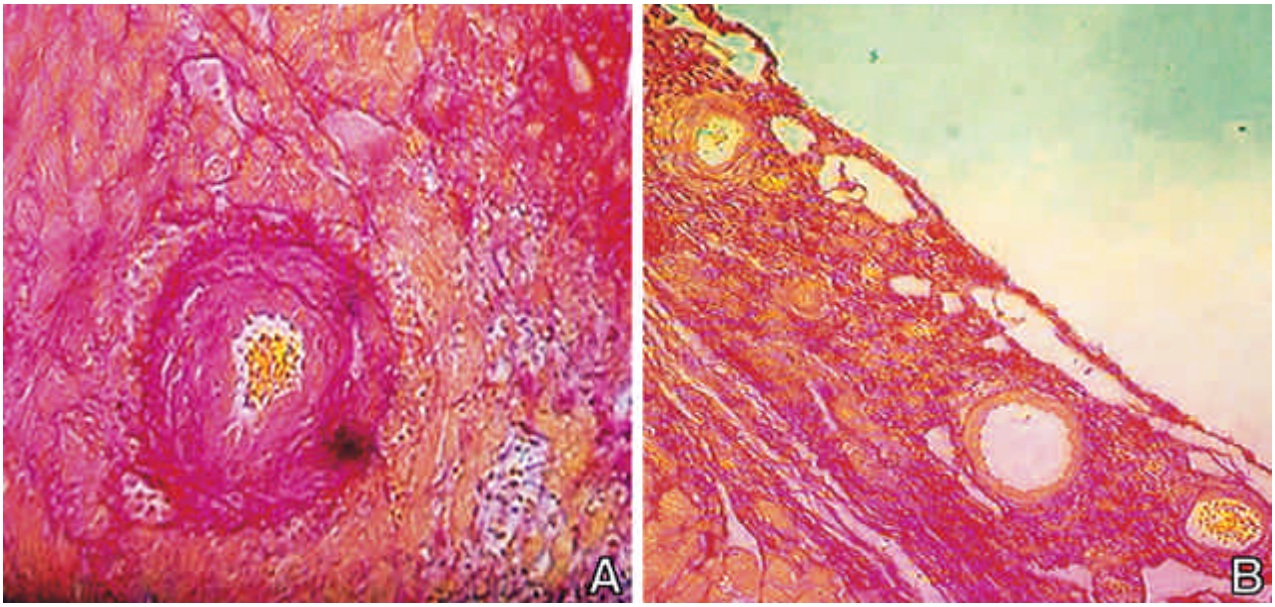

Fig. 2. Sections of B10.A cardiac allograft 45 days after heterotopic transplantation in B10.BR strain of mouse. A: Section of a coronary artery with typical features of graft atherosclerosis such as intimal proliferation and perivascular inflammatory cell infiltration. B: Section of coronary artery shows less luminal narrowing of relatively large vessels (>100 μm in diameter) in angiopeptin treated group (Sirius red, ×200).

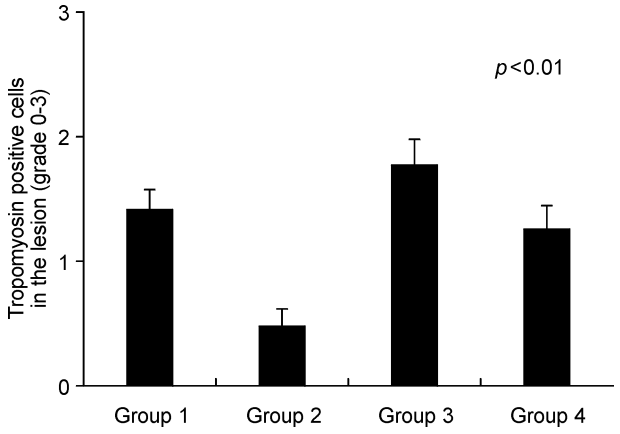

Fig. 4. Comparison of the tropomyosin-positive staining cells in the intima demonstrated lesser degree of staining in Group 2 which was treated with angiopeptin only ($p<0.01$). The fraction of the tropomyosin-positive staining cells was scored semiquantatively on a 0 to 3 scale (0, no positive cell; 1, positive staining cells present but <20% of total; 2, 21-50% positive cells; 3, >50% positive cells).

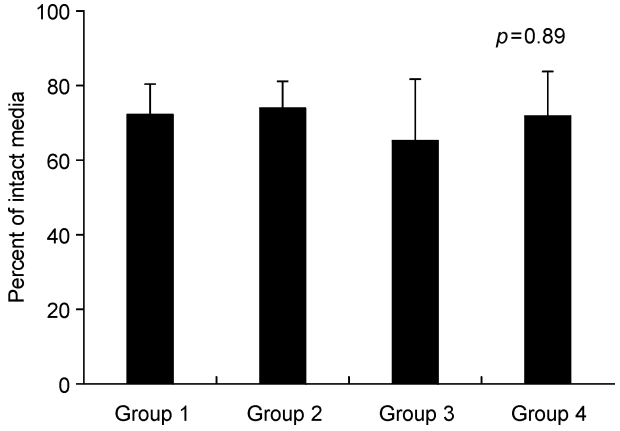

Fig. 5. Comparison of the percentage of tropomyosin-positive cells in the media shows no statistical difference.

Total ischemic time ranged between 45-65 min. Nonsurvival rate, defined as surviving for less than 24 hr, was 10%. All the donor hearts resume sinus rhythm several minutes after reperfusion.

DISCUSSION

This study demonstrates that angiopeptin, a long-acting octapeptide of somatostatin analogue, significantly reduces the extent of intimal proliferation but not the frequency of the development of accelerated graft atherosclerosis (AGAS) in a murine cardiac transplant model.

The diagnosis of AGAS is based mainly on histology from autopsy, retransplantation, or clinical diagnosis by angiography and/or intracoronary-ultrasonography (10).

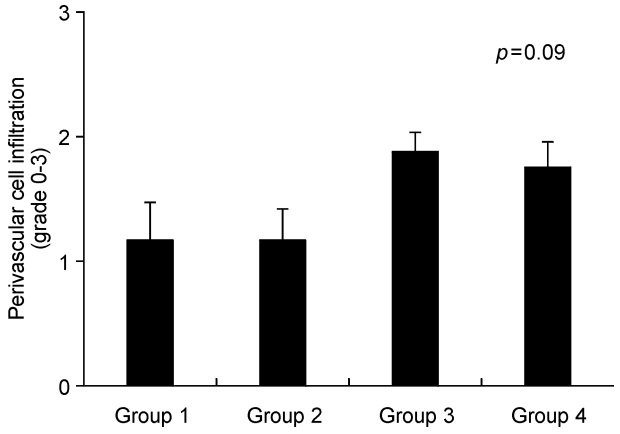

Fig. 6. Comparison of histologic grades of perivascular inflammatory cell infiltration shows no statistical difference. The perivascular inflammation was scored semiquantitatively on a 0 to 3 scale (0, no inflammation; 1, mild increase in perivascular mononuclear cells; 2, moderate increase; 3, large increase in perivascular mononuclear cells or moderate increase with presence of poly-morphonuclear cells).

An incomplete understanding of the pathogenesis of AGAS has limited our ability to develop effective treatment modalities. The pathogenesis of AGAS in humans has been investigated through study of lesions obtained from hearts removed at the time of retransplantation or at autopsy, and more recently, the development of animal models. Ardehali et al. developed a murine model of AGAS which closely resembles the disease in humans to investigate pathogenesis and potential therapy (9). In this model, vascular smooth muscle cell activation is an early and prominent feature, and smooth muscle cells contribute significantly to the cellular mass of intimal lesions. Although the mechanism is not clear, it appears that smooth muscle cell migration through the internal elastic lamina and myointimal proliferation in the walls of the muscular arteries are the major events leading to luminal narrowing of the coronary arteries after transplantation (11). This process may represent a specialized form of chronic delayed-type hypersensitivity, in which lymphocytes activated by alloantigens in the graft vessel wall induce macrophages to secrete smooth muscle cell growth factors (3, 12, 13). Several peptides (insulin-like growth factor-1, platelet-derived growth factor, epidermal growth factor, fibroblast growth factor) have been shown to have such proliferative properties (14, 15).

Angiopeptin has been demonstrated to exert an antiproliferative effect on the vascular walls of cardiac allografts in both the rabbit and rat transplant models (3, 4, 10, 16). Also, it has been shown to inhibit myointimal proliferation in the endothelium-denuded carotid artery by air-drying (15). These data suggest that angiopeptin may inhibit myointimal proliferation by a common mech-

anism in both AGAS and mechanical coronary endothelial injury. Nevertheless, its exact mechanism of action remains unknown. Possible mechanisms include a somatostatin-like action in inhibiting growth hormone and insulin-like growth factor (17, 18), direct interaction with vascular smooth muscle cells (19, 20), or alteration of smooth muscle cell receptor expression (21). Several investigators found that angiopeptin inhibits thymidine uptake in the rabbit aortic wall (22) and in the rat carotid artery (23). These results imply a direct inhibitory effect of angiopeptin on smooth muscle cell growth. In our study, the number of tropomyosin-positive staining cells in the intima, indicative of the proportion of smooth muscle cell, disclosed fewer intimal smooth muscle cells in the angiopeptin treated groups (Group 2 and 4) (Fig. 4), providing further support for angiopeptin's inhibitory effect on smooth muscle cell proliferation. The optimal dose of angiopeptin remains to be determined. Lundergan et al. observed that pre- and post-treatment of endothelium-denuded rabbit carotid artery with a dose of 100 μg/kg/day of subcutaneous angiopeptin significantly attenuated myointimal thickening as well as thymidine uptake, while doses less than 50 μg/kg/day had no effect (15); however, even a dose of 20 μg/kg/day inhibited coronary artery myointimal proliferation in cardiac allografts in the rabbit by 50% (23). In the present study, a dose of 80 μg/kg/day of angiopeptin was administered intraperitoneally and less luminal narrowing was observed in the larger (>100 μm) vessels only. This may suggest a dose dependent inhibitory effect of angiopeptin with respect to different-sized vessels. Further studies with higher doses would result in a similar effect on the smaller vessels.

de Lorgeril et al. reported that transplanted patients, as compared to healthy nontransplanted subjects, show a marked tendency for hyperaggregation of platelets (7). Aspirin, by reducing platelet aggregation, might theoretically inhibit the intimal proliferation of cardiac allograft coronary arteries. McCann et al. reported that neointimal hyperplasia was attenuated by the combination of aspirin and dipyridamole, but not by aspirin alone (24). de Lorgeril et al. showed that even high doses (500 mg/day) aspirin did not reduce platelet aggregation in cyclosporin-treated heart recipients (7). Landymore et al. observed that autologous vein grafts receiving aspirin (650 mg/day) had more significant intimal hyperplasia in the canine model (8). Although the explanation for the marked proliferative response is not clear, it may be due to the fact that high dose aspirin not only decreases platelet synthesis of thromboxane B_2, but adversely affects prostacyclin biosynthesis, reducing 6-keto PGF1α. The adverse effect of aspirin on the thromboxane/prostacyclin ratio may partly explain the marked proliferative responses in those animals receiving high dose aspirin (8). Our results, which show that a dose of 5 mg/kg/day of aspirin does not reduce AGAS, are in accordance with previous studies as well as corresponding to the clinical impression that AGAS is not affected by aspirin administration.

The mouse transplant model has several advantages in the investigation of AGAS. First, there is ample availability of congenic and mutant strains. Second, the mouse has a well-described genetic background. Third, certain combinations of strains (eg, B10.A to B10.BR) allow for long-term survival without the need for immunosuppression. Finally, a number of immunohistological and immunocytological reagents are readily available to study AGAS in this model (9).

In summary, we have demonstrated that angiopeptin inhibits myointimal proliferation in the coronary arteries of cardiac allografts in the murine transplant model. This result suggests that angiopeptin reduces the development of post-transplantation AGAS in human clinical heart transplantation. Further clinical trials are warranted to investigate this effect.

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