Effect of Epigallocatechin-3-Gallate on Intimal Hyperplasia after Vascular Grafting

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Effect of Epigallocatechin-3-Gallate on Intimal Hyperplasia after Vascular Grafting

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The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

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December 2006

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December 2006

ACKNOWLEDGEMENTS

First and the foremost, I would like to express my gratitude and appreciation to Professor Byung Chul Chang, my supervisor, for his support, guidance and encouragement from the beginning of my graduate school to the writing of my dissertation at the very end. His kindness and counsel have been overwhelming.

I would also like to thank professor Young-Hwan Park, Jong-Chul Park, Ki Dong Park, and Byeong Woo Park, Hye Yeon Lee, who have never failed help me for their support and mentorship.

I wish to acknowledge Suk Won Song, Hyun Chul Joo, Mi Hee Lee, Yeon I Woo, Young-Ah Kim, Young-Ook Kim, and my colleagues for the support and encouragement during my research.

And finally, I thank my wife, my son and my parents for providing me the inspiration to finish, with an endless love and understanding.

Han Ki Park

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ABSTRACT

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Background: Intimal hyperplasia is a major cause of stenosis after vascular grafting and intervention, and is characterized by proliferation and migration of medial smooth muscle cells with an associated deposition of extracellular connective tissue matrix in the intimal layer. Epigallocatechin-3-gallate (EGCG), the major constituent of green tea, is known to suppress the proliferation and migration of vascular smooth muscle cells (SMCs). We propose that EGCG may have a protective effect against the development of intimal hyperplasia through the suppression of vascular SMC behavior. The purpose of this study is to evaluate the effect of EGCG on the prevention of intimal hyperplasia after vascular grafting

Materials and Method: Human umbilical vein endothelial cells (HUVEC) and rat aortic smooth muscle cells (RASMC) were cultured *in vitro* and EGCG was added to the culture media (0 - 400 μM). After culturing for 3 days, cell proliferation was measured and the dose-dependent suppressive effects were studied. For the cell migration assay, cell migration speed was measured by tracing randomly selected cells under the microscope after forming a denuded area in a single layer of cells in culture media. To evaluate the effect of EGCG on intimal hyperplasia, we performed *in vivo* experiments using a canine model. We interposed an autologous jugular vein graft into the bilateral carotid arteries in 20 dogs. The vein graft was

stored for 30 minutes in normal saline (control group, n=10) or EGCG solution (EGCG group, n=10) before grafting. For the EGCG group, 100 mM of EGCG was applied in the perivascular space. After 30 days, the vein graft was retrieved and thickness of the intima and media was measured.

Results: The proliferation of RASMCs and HUVECs was suppressed by the addition of EGCG to the media (concentration of 400 and 100 μM, respectively, $p < 0.05$). The migration of RASMCs was suppressed with 200 μM of EGCG (control 38.8 μm/hour vs. EGCG group 24.3 μm/hour, $p < 0.05$), however the migration of HUVECs was not affected by EGCG treatment (control 36.9 μm/hour vs. EGCG group 34.4 μm/hour). In the *in vivo* study, the intimal thickness of the retrieved vein graft was thicker in control group than in the EGCG group (95 ± 43 vs. 47 ± 37 μm, respectively, $p < 0.05$), however there was no difference in medial thickness between the groups (247 ± 95 vs. 267 ± 224 μm, respectively, $p = 0.67$). The intima/media thickness ratio was lower in the EGCG group than in the control group (0.41 ± 0.17 vs. 0.21 ± 0.15, respectively, $p < 0.05$).

Conclusion: EGCG can suppress intimal hyperplasia after vascular grafting through the inhibition of the vascular SMC migration and proliferation. Treatment of EGCG may offer new intra-operative therapeutic modalities to reduce the development of intimal hyperplasia.

Key words: vascular disease, intimal hyperplasia, epigallocatechin-3-gallate

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I. INTRODUCTION

As the life expectancy increases, the prevalence of atherosclerotic vascular disease and the incidence of vascular surgery in elderly patients are increasing. Currently, autologous blood vessels such as the saphenous vein, and vascular prosthesis such as polyester or polytetrafluroethylene (PTFE) are used for vascular grafting. However, the use of these grafts is associated with the development of stenosis. The main cause of graft failure is the development of intimal hyperplasia[1] which is a chronic structural lesion that develops after vascular graft implantation and leads to luminal stenosis and occlusion. Intimal hyperplasia results from the response of the vessel wall to injury and is characterized by proliferation and migration of medial smooth muscle cells (SMC) to the intimal layer with an associated deposition of extracellular connective tissue matrix[2]. This process causes intimal thickening leads to luminal stenosis or restenosis and significantly complicates endovascular and open vascular procedures, which often result in clinical coronary or peripheral limb ischemia. Therefore, the control of intimal hyperplasia has important clinical applications.

Several strategies have been tested and studied in an attempt to control intimal hyperplasia, including pharmacological agents[3], irradiation[4] and gene therapy[5]. However, the modest success achieved in animal models could not be reproduced in

clinical trials.

Green tea polyphenol (GTP) or its major constituent, epigallocatechin-3-gallate (EGCG), has many biological activities. GTP has been shown to prevent cancer and inhibit cancer angiogenesis[6]. It also has been demonstrated that green tea catechins suppress vascular smooth muscle cell proliferation[7,8]. Moreover, GTP has a protective effect in endothelial cells; it prevents reactive oxygen species-induced oxidative stress and inhibits angiogenic differentiation in endothelial cells[3,9]. Based on these studies, we propose that EGCG may have a protective effect against endothelial dysfunction and the development of intimal hyperplasia after vascular grafting.

The aim of this study was to evaluate the effect of epigallocatechin-3-gallate (EGCG) on the behavior of vascular smooth cells and endothelial cells, and to investigate whether EGCG can suppress intimal hyperplasia after vascular grafting in an animal model.

II. MATERIALS AND METHODS

1. Study Design

To study the effect of EGCG on vascular smooth muscle cells and endothelial cells, rat aortic smooth muscle cells (RASMC) and human umbilical vein endothelial cells (HUVEC) were cultured *in vitro*, and different concentrations of EGCG were added to the cell culture media. The proliferation and the migration of RASMCs and HUVECs were measured and compared with the EGCG-treated and of control groups.

To study the effect on intimal hyperplasia, an *in vivo* experiment was performed using a canine vascular grafting surgery model. An autologous vein graft was harvested from the experiment animal, incubated in an EGCG solution and then implanted into the carotid artery. After 30 days, grafts were retrieved and the intimal thickness was measured and compared between the treated and control groups.

2. Materials

EGCG, a major constituent of green tea, was kindly supplied by Pharma Foods International Co. Ltd. (Kyoto, Japan), and its purity exceeded 90%.

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). Rat aorta smooth muscle cells (RASMCs) were purchased from BioBud (Seoul, Korea) and used between passages 5 to 9.

3. *In vitro* Experiment

3.1 Cell culture

HUVECs were cultured with Endothelial Cell Basal Medium-2 (EBM-2, Cambrex Bio Science Walkersville, Inc.), 1% antibiotic antimycotic solution

(gentamicin/amphotericin-B) (Sigma Co., St. Louis, MO, USA) and endothelial cell growth supplements (Cambrex) including hydrocortisone, human epidermal growth factor (hEGF), fetal bovine serum, vascular endothelial growth factor (VEGF), human fibroblast growth factor basic (hFGFB), R3-insulin like growth factor-1 (R3-IGF-1), ascorbic acid and.

RASMCs were routinely maintained in Dulbecco's modified Eagle's medium (JBI, Seoul, Korea) supplemented with 10% fetal bovine serum and a 1% antibiotic antimycotic solution at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were cultured and used between passages 5 and 9.

3.2. Cell proliferation assay

Culture wells (24-well culture plate) were inoculated with growth medium containing 2 x 10^4 cells of HUVECs and RASMCs, treated with EGCG at final concentrations of 25, 50, 100, 200, or 400 μM, and incubated for 3 days in a CO_2 incubator. The cell proliferation was quantified by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) to formazan. The extent of reduction of MTT to formazan was measured using an ELISA reader (Spectra Max 340, Molecular Device Co., Sunnyvale, CA, USA) at a wavelength of 570 nm. The absorbance for each concentration of EGCG was compared with that of an untreated control and the assay was repeated 20 times.

3.3. Cell migration assay

HUVEC and RASMCs (1 x 10^5 cells/mL) were seeded on a 4-well chambered cover-glass slide and grown to confluence overnight. Cell monolayers were wounded by using a plastic micropipette tip[10], and EGCG (200 μM) was added to the attached cells. The cells were incubated in a self-designed CO_2 mini-incubator placed on the microscope stage for 36 hours and the migration of cells into the denuded space was visualized by charge-coupled device (CCD) camera attached to the microscope as previously described[11]. In a single cell culture plate, 10 cells were randomly selected and traced to measure actual migration distance (Figure 1). The average speed of a migrating single cell was calculated by analysis of pictures taken

during the cell culture using image-processing software (MATLAB V5.3, The MathWork Inc., Natick, MA, USA). The experiments were repeated five times. Therefore, 50 cells were traced to measure the cell migration speed for each group.

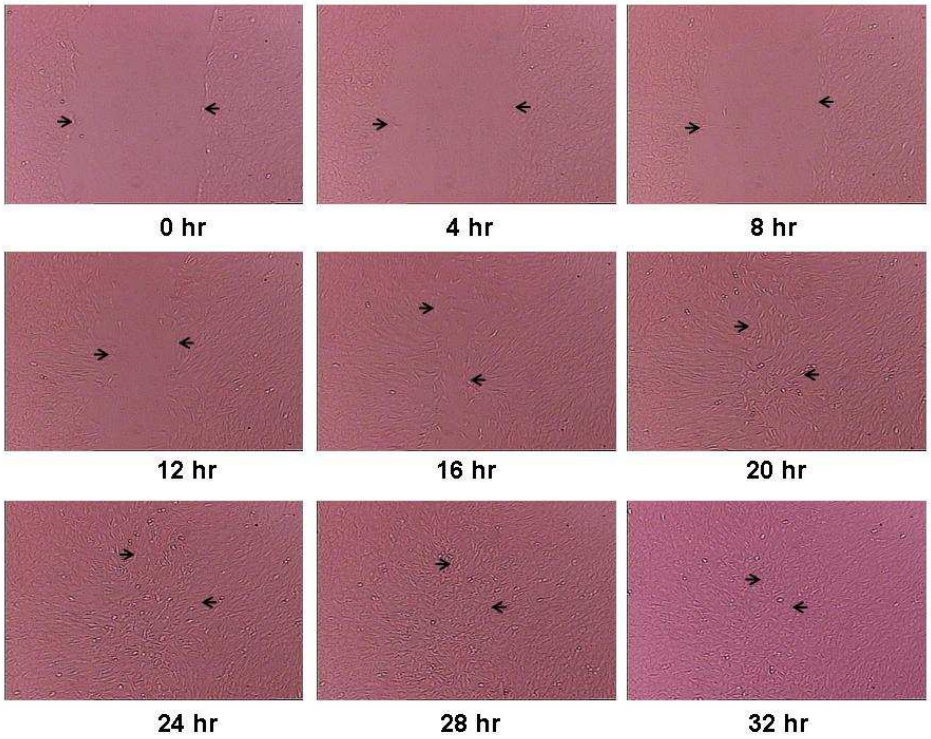

Figure 1. Cell migration assay. On a cell culture plate, randomly selected cell were traced to measure actual migration distance, and the cell migration speed was calculated.

4. *In vivo* experiment

Twenty female mongrel dogs, weighing 20 to 25 kg, were used for the *in vivo* experiment. The animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals at Yonsei University.

4.1. Vascular grafting

The animals were distributed into two groups: I (Control) and II (EGCG). The dogs were anaesthetized by an intravenous injection of Zylazine (2 mg/kg) and Zoletil (Tiletamine/Zolazepam, 1.5 mg/kg), and the anesthesia was maintained with inhalation anesthetics (Enflurane 2%).

After an intravenous administration of 2 mg/kg of heparin, two 15-mm segments of the external jugular vein were excised for use as a vascular graft. The vein grafts were stored in normal saline (group I), or 400 μM EGCG in normal saline solution (group II) for 30 minutes. The left and right carotid arteries were exposed and vein grafts were interposed into the carotid artery with running sutures of monofilament non-absorbable suture (Figure 2A). Then a sac was made with a 0.1-mm thick polytetrafluoroethylene (PTFE) patch (W. L. Gore & Associates, Inc., Flagstaff, AZ, USA) to surround the interposed vein graft (Figure 2B). The PTFE pocket was filled with 1 mL of fibrin glue (Greenplast®, Green Cross Inc, Yongin, Korea) with or without 100 mM EGCG for group II (EGCG) and group I (control) animals, respectively.

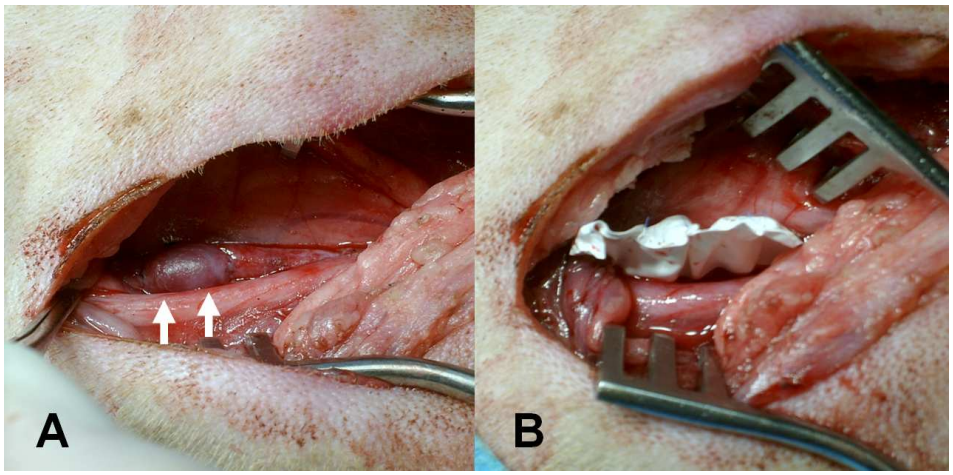

Figure 2. Vascular grafting in a canine model. A: A segment of an external jugular vein was harvested and interposed into carotid artery. Arrows indicate the interposed vein graft. B: The vein graft was wrapped with polytetrafluoroethylene membrane and the sac was filled with fibrin glue containing epigallocatechin-3-gallage (EGCG) for the ECGG group or fibrin glue alone for the control group.

4.2. Retrieval of graft and histologic examination

The implanted vein graft was retrieved 30 days after surgery. After isolation, the vein grafts were perfusion fixed *in situ* by infusion of 10% buffered formalin. Then, the graft was excised, fixed again in formalin and embedded in paraffin. The specimen was crossly sectioned at a thickness of 5 μm and the sections were stained with hematoxylin-eosin and van Gieson elastin stain. Dimensional analysis was performed by video morphometry (Innovision 150, American Innovision, Inc, USA).

The intima and media were delineated by identification of the demarcation between the crisscross orientation of the intimal hyperplastic smooth muscle cells and circular smooth muscle cells of the media, and the outer limit of the media was defined by the interface between the circular smooth muscle cells of the media and the connective tissue of the adventitia. The thickness of each layer was determined at six different points. Then, the ratio of the intimal to medial thickness was calculated.

5. Statistical analysis

The data are expressed as the mean ± SD. Data were compared between the two groups using a t-test. A value of $p < 0.05$ was considered to be statistically significant.

III. RESULTS

1. *In vitro* experiment

1.1. Effects on cell proliferation

To investigate the EGCG-induced inhibitory effects on HUVEC and RASMC proliferation, cells were treated with increasing concentrations of EGCG (25, 50, 100, 200 and 400 μM). After incubation for 3 days, cell proliferation was determined by an MTT assay. Incubating the RASMCs in the presence of 400 μM EGCG resulted in a significant ($p < 0.05$) decreases in RASMC proliferation (Figure 3).

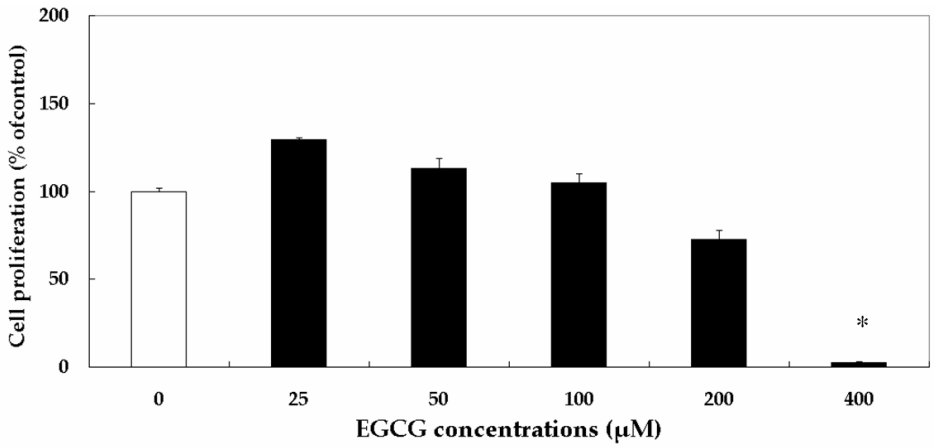

Figure 3. The proliferation of rat aorta smooth muscle cells (% of control) treated with EGCG at concentrations ranging from 0 to 400 μM. Data are expressed as the mean ± SD. * $p < 0.05$ versus control.

When the HUVECs were treated with EGCG, the MTT assay showed a dose-dependent inhibition of HUVEC proliferation with increasing concentrations of EGCG, while a statistically significant inhibition of cell proliferation was demonstrated at concentrations of 100 μM and higher ($p < 0.05$) (Figure 4).

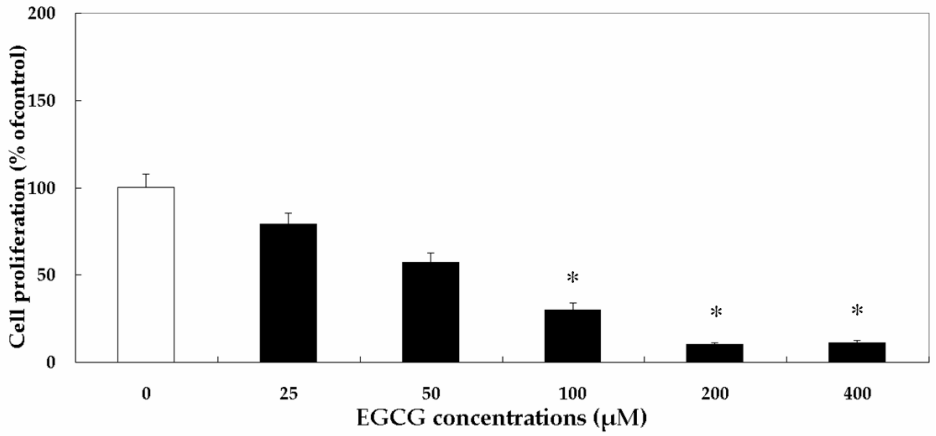

Figure 4. The proliferation of human umbilical vein endothelial cells (% of control) treated with EGCG at concentrations ranging from 0 to 400 μM. Data are expressed as the mean ± SD. * $p < 0.05$ versus control.

1.2. Effect of EGCG on cell migration

The inhibitory effects of EGCG on RASMC and HUVEC behaviors were verified by performing *in vitro* migration assays. The average migration speed of the EGCG-treated RASMCs was 24.3 ± 2.5 μm/hour, while that of the non-treated group was 38.8 ± 3.0 μm/hour. EGCG-treated RASMCs were slower in migration than non-treated RASMCs ($p < 0.05$) (Figure 5).

In contrast, EGCG did not suppress the migration of HUVECs. There was not a statistically significant difference between the average migration speed of the EGCG-treated HUVECs (34.4 ± 3.4 μm/hour) and that of the non-treated group (36.9 ± 2.0 μm/hour) (Figure 6).

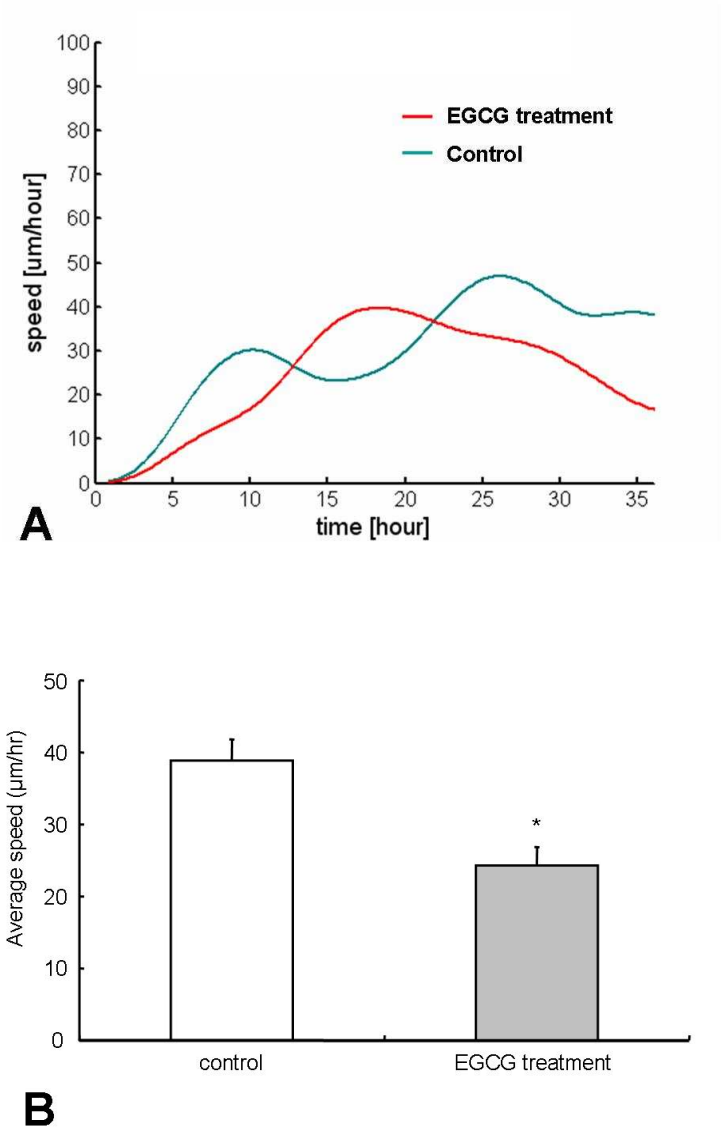

Figure 5. Migration speed of epigallocatechin-3-gallate treated and non-treated rat aortic smooth muscle cell. A: real-time migration speed. B: average speed. * $p < 0.05$

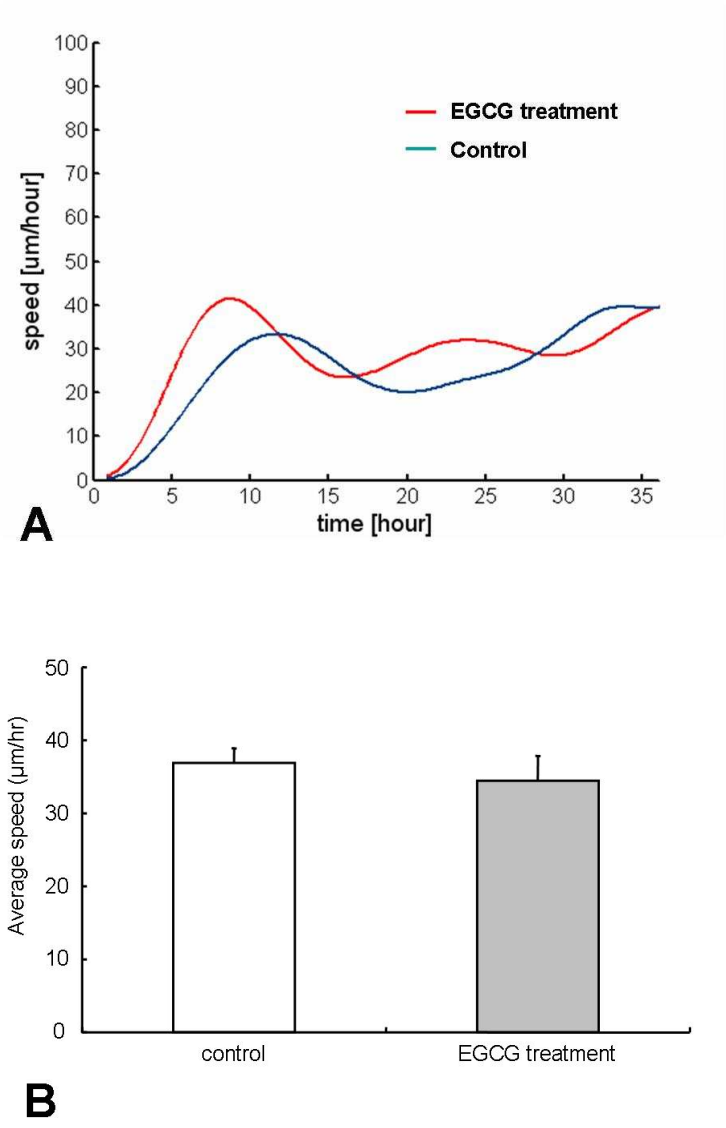

Figure 6. Migration speed of epigallocatechin-3-gallate treated and non-treated human umbilical vein endothelial cell. A: real-time migration speed. B: average speed.

2. *In vivo* experiment

2.1. Effects on intimal hyperplasia

All animals survived to 30 days and all grafts were patent at harvest. Microscopically, the luminal surfaces of the vein grafts from each group were covered with endothelial cells. A variable degree of intimal thickening was observed in Group I (control) and Group II (EGCG) (Figures 7 and 8).

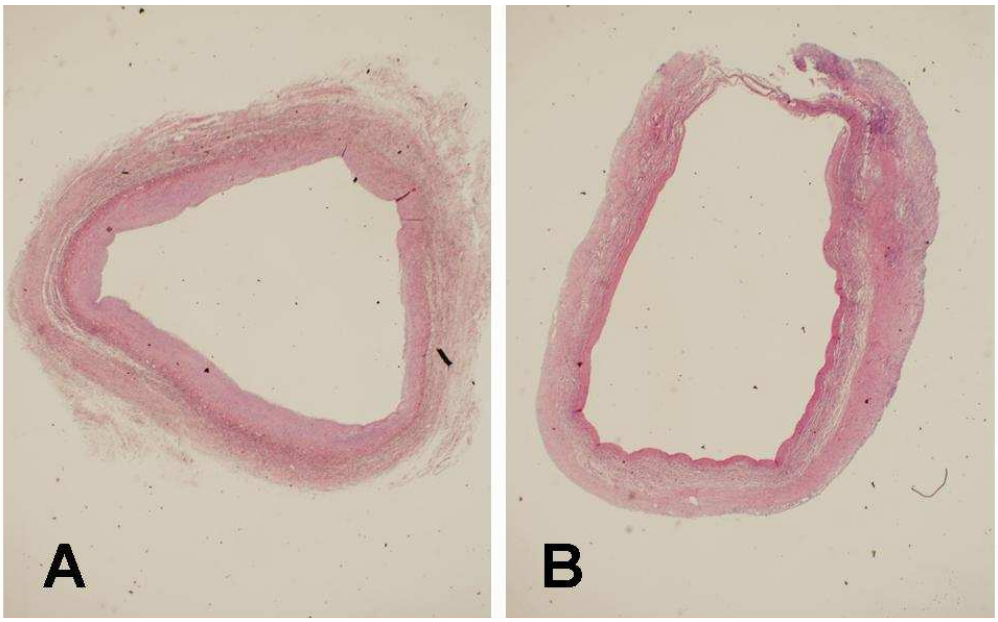

Figure 7. Histologic examination of the retrieved vein graft. A: Group I (control), B: Group II (EGCG) (van Gieson elastin stain, x40)

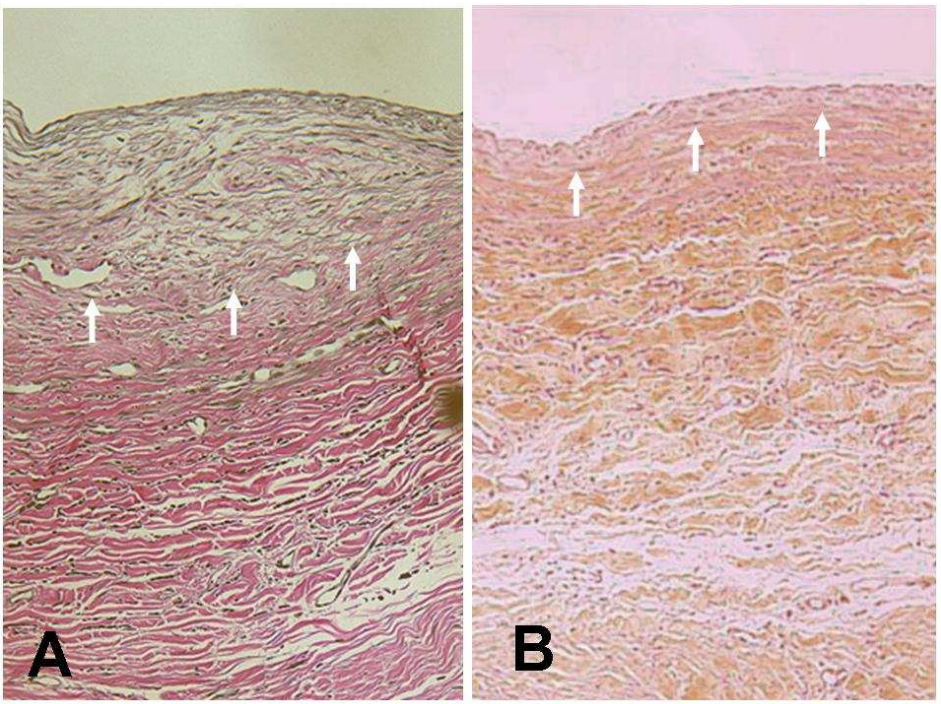

Figure 8. Histologic examination of intimal thickness of the vein grafts. A: Group I (control), B: Group II (EGCG). Arrows indicate intimal borders (van Gieson elastin stain, x100).

The mean intimal thickness was lower in Group II (EGCG) than Group I (control), but the medial thickness did not differ between the two groups. The ratio of intimal to medial thickness was lower in Group II (EGCG) than in Group I (control) (Table 1).

Table 1. Morphologic analysis of intimal and medial thickness

	Group I (control)	Group II (EGCG)
Intimal thickness (μm)	94.9 ± 43.3	46.6 ± 36.9*
Medial thickness (μm)	247.2 ± 95.0	267.3 ± 224.3
Intima/Media thickness ratio	0.41 ± 0.17	0.21 ± 0.15*

* $p < 0.05$

IV. DISCUSSION

Intimal hyperplasia is a critical initial event leading to failure of vascular grafting and vascular intervention. It occurs because the cell population increases within the innermost layer of the arterial wall, similar to what occurs during closure of the ductus arteriosus or involution of the uterus. Intimal hyperplasia also occurs in homograft transplanted organs and in veins used as arteriovenous fistulas or arterial bypass conduits[12, 13]. The underlying causes of intimal hyperplasia are migration and proliferation of vascular smooth muscle cells provoked by injury, inflammation, and stretching.

To prevent the intimal hyperplasia, several modalities have been tried, but current treatment modalities are limited in their effectiveness. In the present study, we found that local treatment with EGCG significantly inhibits intimal hyperplasia in a vein graft after vascular grafting. Based on this result, EGCG is one of the pharmacological agents that could be used to control intimal hyperplasia. However, the effect of green tea catechins on intimal hyperplasia and its mechanism is still not fully understood. Our *in vitro* data suggests that suppression of intimal hyperplasia is mediated by inhibition of vascular SMC proliferation and migration. This finding is compatible with those from recent reports which demonstrated the suppressive effect of epigalocatechin on vascular SMC proliferation[8]. The antiproliferative effect of epigalocatechin on vascular SMCs may be partly mediated through inhibition of protein tyrosine kinase activity, reducing signal transduction[7]. The inhibitory effect of EGCG on SMC migration has also been studied[14]. Catechins inhibits proteolytic enzymes derived from smooth muscle cells and this results in suppression of SMC migration across the basement membrane[15].

Until now, prevention of intimal hyperplasia by EGCG has only been demonstrated in an arterial injury model. Kim *et at*[16] applied green tea catechins to an endothelium-denuded carotid artery for 20 minutes and observed neointimal formation. They also reported that neointimal formation is inhibited by green tea catechins. There have been no reports on vein grafts after vascular grafting. In this study, we demonstrated similar results in an implanted vein graft.

Despite the demonstration of the preventive effects of EGCG on intimal hyperplasia, a safe and effective dose and application method should be clearly established before clinical application. Green tea has been consumed safely for over long time periods. But, there have not been extensive studies to determine the effective dose of EGCG for systemic administration or the systemic toxicity from high doses of EGCG. In the present study, the EGCG was applied locally around the implanted vein grafts. Local delivery is an easier way to control the cellular response after vascular injury because a high concentration of EGCG can be achieved immediately after administration. There is another theoretical advantage of local delivery. The proliferation of vascular SMCs and endothelial cells was suppressed by EGCG. If the EGCG is applied locally in the perivascular space, it will be delivered by diffusion through the vessel walls and the concentration of EGCG on the vascular SMCs could be higher than that of endothelial cells. The anti-proliferate effect of EGCG on the endothelial cell would be lower, and as a result the endothealization of the anastomosis site is less affected while SMC proliferation is suppressed.

For local delivery, there is no information about how much EGCG is effective when applied locally around the vascular grafts. At 400 μM of EGCG, the proliferation of SMC and endothelial cell is suppressed. The effects of EGCG on other cell types were not studied thoroughly, but there is a possibility that a high dose of EGCG might affect the wound healing. In this study, 100 mM of EGCG was applied locally and no deleterious effect was observed on wound healing. Further study is needed to find the optimal dose of EGCG, but this will be difficult because EGCG is water-soluble and is rapidly washed out of the vessel wall. It is thus nearly impossible to measure local concentration and change in concentration over time.

In this study, the inhibitory effect of EGCG on intimal hyperplasia was demonstrated, but further study is required to determine the optimal dose and method of administration. Further, we need to investigate the long-term morphological alterations of the implanted grafts.

V. CONCLUSION

From this experiment, EGCG showed the ability to suppress the intimal hyperplasia after vascular grafting, and I suggest EGCG can be used for new intraoperative therapeutic modalities to reduce the development of graft intimal hyperplasia and subsequent graft failure.

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ABSTRACT (IN KOREAN)

Epigallocatechin-3-Gallate의 혈관이식술 후 내막과다증식 억제효과

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박 한 기

목적 혈관내막과다증식은 혈관우회술 혹은 중재술 후 재협착의 주요한 원인으로, 혈관손상 후 중간막에 존재하는 혈관평활근세포가 내막으로 이동하며 과다증식하여, 세포외결체조직과 함께 축적되어 형성된다. 녹차추출물의 주요 성분 중 하나인 epigallocatechin-3-gallate (EGCG)는 혈관평활근세포의 이동 및 증식을 억제하는 효과가 있는 것으로 알려져 있으며, 따라서 EGCG가 혈관평활근세포의 활동을 억제함으로써 내막과다증식을 억제하는 효과가 있을 것으로 기대된다. 본 연구는 EGCG가 혈관수술 후 내막증식에 미치는 영향을 알아보기 위한 것이다.

방법 인간제대정맥내피세포(human umbilical vein endothelial cell, HUVEC)와 쥐의 대동맥평활근세포(rat aortic smooth muscle cell, RASMC)를 in vitro 환경에서 배양하고, 세포배양액에 EGCG(0 - 400 μM)를 첨가하였다. 3일간 세포배양 후 세포활성도을 측정하여 EGCG 농도에 따른 세포증식 억제효과를 비교하였다. 세포이동 측정을 위해서는 내피세포와 평활근세포가 단일층으로 배양된 배지에 세포가 없는 구역을 만들고 현미경관찰하에 세포배양하며 임의로 선택한 세포가 이동하는 것을 추적하여 세포의 이동 속도를 측정하였다. 내막증식에 미치는 효과를 확인하기 위해 개를 이용한 동물실험을 시행하였다. 20마리의 개의 양측 경동맥에 자가경정맥을 이식하였으며, 채취한 경정맥을 대조군(n=10)은 생리식염수에, EGCG군(n=10)에서는 EGCG를 포함하는

생리식염수에 30분 동안 담근 뒤 이식하였으며, EGCG군은 100 mM의 EGCG를 이식혈관주위에 도포하였다. 이식 후 30일 후에 경정맥이식편을 회수하여 내막과 중간막의 두께를 측정하였다.

결과 혈관내피세포의 증식은 EGCG의 농도가 100 μM 이상에서, 혈관평활근세포의 증식은 400 μM 이상 농도에서 대조군에 비해 억제되었다 (p < 0.05). 200 μM의 EGCG가 첨가된 세포배지에서 혈관평활근세포의 이동성은 대조군에 비해 억제되었으나 (control 38.8 μm/hour vs EGCG group 24.3 μm/hour, p < 0.05), 내피세포의 이동성은 영향을 받지 않았다 (control 36.9 μm/hour vs EGCG group 34.4 μm/hour). 동물실험 결과 회수한 정맥도관의 내막에 다양한 정도의 내막과다증식이 관찰되었으며, 대조군에서 EGCG군에 비해 내막의 두께가 두꺼웠으나 (95 ± 43 vs 47 ± 37 μm, p < 0.05), 중간막의 두께는 두 군간에 차이가 없었고 (247 ± 95 vs 267 ± 224 μm, p = 0.67), 내막/중간막의 두께비는 EGCG군에서 낮게 관찰되었다 (0.41 ± 0.17 vs 0.21 ± 0.15, p < 0.05).

결론 EGCG는 혈관우회술 후 혈관내막과다증식을 억제하는 효과가 있으며, 이는 혈관평활근세포의 증식 및 이동을 억제하여 효과를 나타낸다고 생각된다. 따라서 EGCG는 혈관이식술 및 중재술 후 발생하는 혈관내막과다증식의 억제에 효과적으로 사용될 수 있을 것으로 사료된다.

핵심되는 말 : 혈관질환, 내막증식, epigallocatechin-3-gallate