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Original Article

Different Responses of Neointimal Cells to Imatinib Mesylate and Rapamycin Compared with Normal Vascular Smooth Muscle Cells

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Purpose: This study was designed to investigate whether vascular smooth muscle cells (VSMC) from the neointima showed any different response to antiproliferative agents, such as rapamycin or imatinib mesylate, compared to VSMCs from normal artery.

Materials and Methods: Intimal hyperplasia was made by carotid balloon in jury in male rats. Neointimal cells at 4 weeks after injury and normal VSMCs were extracted by enzymatic isolation method and cultured. Cell viability and proliferation were tested in VSMCs from injured left carotid artery and uninjured right carotid artery. Tests were repeated with rapamycin, imatinib mesylate or both in various concentrations.

Results: Rapamycin decreased cell viability only at a high concentration of 10^{-5} M in uninjured VSMCs. Combined drugs decreased cell viability at a lower concentration of 10^{-7} M in uninjured VSMCs, and at a higher concentration of 10^{-5} M in neointimal cells. Overall, rapamycin showed cytocidal effects at a high concentration of 10^{-5} M, whereas imatinib did not. Cell proliferation of neointima was significantly decreased along with the drug concentration. Cell proliferation of uninjured VSMCs was significantly decreased at higher drug concentrations. Combined drug therapy showed synergistic effects. Overall, neointimal cells are more susceptible to the antiproliferative effects of the drugs.

Conclusion: Neointimal cells from the injured carotid artery are more susceptible to the antiproliferative effect of imatinib and rapamycin. Both drugs can be a used for the prevention of intimal hyperplasia, which could be investigated through further in vivo studies.

Key Words: Imatinib, Sirolimus, Intimal hyperplasia, Neointimal cell, Carotid artery injury

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INTRODUCTION

Intimal hyperplasia (IH) is considered as the main cause of restenosis after vein graft bypass surgery or endovascular intervention, including angioplasty or stenting [1]. Many studies have been performed to find a way to inhibit or to treat this pathologic process. The treatment strategies for IH can be prevention of the development of IH or induction of atrophy in the established IH. However, there is no drug proven effective in clinical use [2]. The mechanism of IH is basically a response to injury, which has been widely investigated in various animal models including the rat carotid denudation model. When vascular endothelium is injured, activated vascular smooth muscle cells (VSMC) proliferate and migrate to form a neointima on top of the injured vascular endothelium [3-5].

Platelet-derived growth factor (PDGF) is one of the key regulators in proliferation, migration, survival and extracellular matrix formation of the VSMC and also in the pathologic IH after vascular injury [6,7]. Blockade of PDFG receptor is known to cause inhibition of IH and also regression of established IH [8,9]. Imatinib mesylate (Glivec; Novartis, Basel, Switzerland) is an inhibitor of PDGF receptor kinase, Bcr-Abl kinase, and c-kit receptor kinase [10], and is clinically used for the treatment of leukemia and gastrointestinal stromal tumor. In animal models, it has shown inhibitory effects on IH [11,12].

Recently, Vamvakopoulos et al. [13] reported that rapamycin and imatinib mesylate successfully inhibited IH and showed synergistic effect in a rat carotid injury model. Rapamycin (Rapamune; Pfizer, New York, NY, USA) is an mTOR inhibitor, mainly used for immunosuppression in organ transplantation. A recent report showed that introduction of stents coated with rapamycin dramatically reduced restenosis rates after coronary revascularization [14]. Prevention of restenosis with oral rapamycin has also been shown to be successful after angioplasty in animal models [15].

Because the pathogenic mechanism of IH is uncontrolled hyperplasia similar with that of cancer, some anti-cancer drugs can be used for the prevention of IH. Like cancer therapy, proliferating cells may be more susceptible to antiproliferative drugs. Therefore, we hypothesized that neointimal cells may show different responses to antiproliferative agents compared to VSMCs from normal arteries.

This study was designed to investigate whether VSMCs from the neointima showed different responses to antiproliferative agents, such as rapamycin or imatinib mesylate, compared to VSMCs from normal arteries in terms of viability and proliferation.

MATERIALS AND METHODS

1) Carotid balloon injury model in rats

Twenty male Sprague-Dawley rats weighing about 300 g were purchased from OrientBio Inc. (Seongnam, Korea), and bred for 2 weeks to allow adaptation to the environment. Carotid balloon injury was made by endothelial denudation of the carotid artery using a balloon catheter as described elsewhere [16]. Briefly, animals were anesthetized with Zoletil (Virbac, Carros, France) 10 mg/kg and xylazine (Rompun; Bayer, Leverkusen, Germany) 5 mg/kg intraperitoneally. A midline cervical incision was made and deepened down to the left carotid artery. A 2 Fr Fogarty balloon catheter (Edwards Lifesciences, Irvine, CA, USA) was introduced, advanced to the innominate artery, inflated with 0.2 mL saline, and passed 5 times in the common carotid artery (Fig. 1). After wound closure,

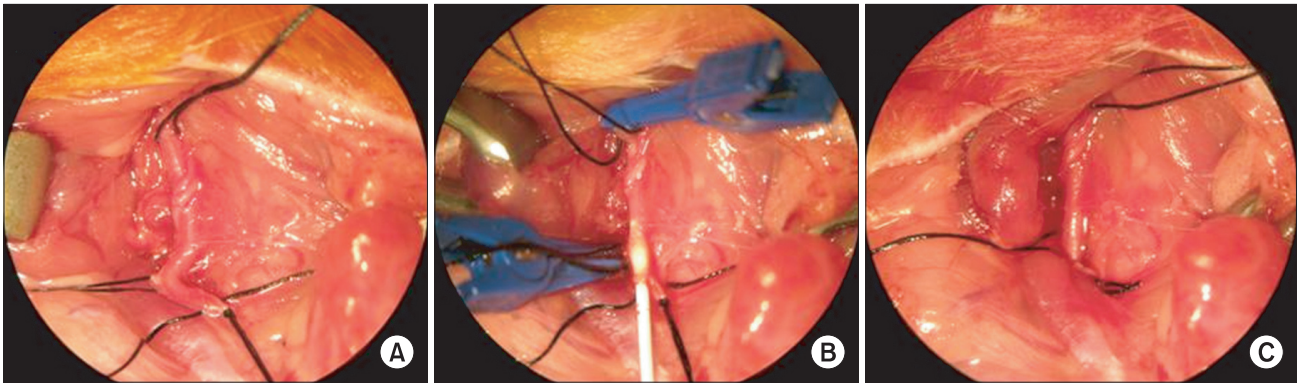

Fig. 1. Rat carotid balloon injury model. (A) After a vertical midline incision, the left carotid arteries were identified. (B) Using a 2 Fr Fogarty balloon catheter, the arterial intima was injured by pull-back and rotation techniques. (C) After repetitive injuries for 5 times, the arteriotomy site was ligated (top: caudal, bottom: cranial).

the animals were allowed to recover from anesthesia and to access freely to water and food.

Humane care was applied in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). The protocol for this study was approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (SNUH-IACUC, No. 07-0256).

2) Extraction and culture of VSMC

VSMCs from the neointima of the left carotid artery were extracted at 4 weeks after injury and cultured with the enzymatic isolation method as previously described, and the uninjured right carotid artery was also harvested for internal control [17]. Extracted VSMCs were cultured using the Dulbecco's modified eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum. VSMCs on passage 3-6 were used for the experiments.

3) Cell viability and proliferation

VSMC viability and proliferation were measured by the soluble tetrazolium salt method (WST-1; Roche Applied Science, Indianapolis, IN, USA) and bromodeoxyuridine (BrdU) assay. For the WST-1 method, VSMCs at 5×10^3 per well were cultured in 96-well plates with DMEM for 48 hours. After changing to serum-free media, various concentrations of drugs were added in the wells containing PDGF-BB 10 ng/mL. Study groups were divided into imatinib mesylate, rapamycin and combined groups. Control was treated only with PDGF-BB. After 24 hours, enzyme-linked immunosorbent assay (ELISA) absorption under 450 nm was measured in the reaction with WST-1 solution 15 μL for 4 hours. For BrdU assay, VSMCs at 5×10^3 per well were cultured in 96-well plates with DMEM for 48 hours. After cell starvation, the same concentrations of drugs as the WST-1 method were treated in the study groups and the control group. After 24 hours, ELISA absorption under 370 nm was measured in the reaction with BrdU solution 15 μL for 2 hours.

4) Statistics

All data are presented as mean±standard deviation. Comparisons between groups were performed using the Mann-Whitney U test because of small numbers. A probability value of less than 0.05 was considered statistically significant.

RESULTS

1) Establishment of neointimal hyperplasia

The degree of neointimal formation was checked serially at 3, 7, 14, and 28 days after carotid injury. Hematoxylin

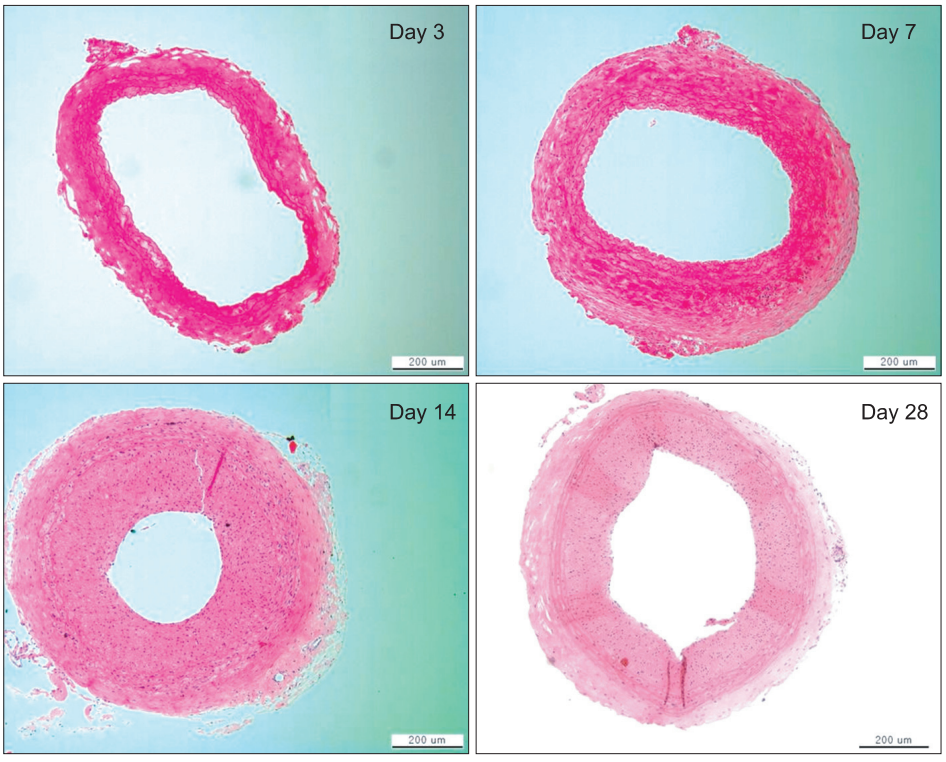

Fig. 2. Cross-sectional images of the injured carotid arteries (light microscopy; H&E stain, x100). Tissues were harvested serially at 3, 7, 14, and 28 days after injury. Intimal hyperplasia developed progressively after the injury and the neointima was largest at 28 days after injury.

and eosin stains of the cross-sectioned arteries confirmed that the neointima developed progressively after injury, being most severe at 28 days (Fig. 2). Based on this result, we decided to harvest the arterial cells at the time point of 28 days after injury.

2) VSMC viability test

Cell viability was assessed with the WST-1 assay in VSMCs from injured left common carotid artery (LCCA) and uninjured right common carotid artery (RCCA). Imatinib mesylate did not decrease the cell viability in both cells. Rapamycin decreased the cell viability only at a high concentration of 10^{-5} M in the RCCA. Combined drugs decreased the cell viability at a lower concentration of 10^{-7} M in the RCCA, and at a higher concentration of 10^{-5} M in the LCCA. Overall, rapamycin showed cytocidal effects at a high concentration of 10^{-5} M, whereas imatinib did not. Also neointimal VSMCs were more resistant to death stimuli by the drugs, whereas VSMCs from normal arteries were more susceptible (Fig. 3).

3) Proliferation assay

BrdU assay showed that cell proliferation significantly decreased along with various drug concentrations (Fig. 4). In neointimal VSMCs from the LCCA, cell proliferation was significantly inhibited by 10^{-7} M imatinib, 10^{-9} M rapamycin, or 10^{-9} M combined drugs. Interestingly, the degree of inhibition of proliferation showed a synergistic effect in the combined treatment group. In normal VSMCs from the RCCA, cell proliferation was significantly inhibited by 10^{-6} M imatinib, 10^{-6} M rapamycin, and 10^{-6} M combined drugs, which were higher concentrations than that in the LCCA. Overall, neointimal cells were more susceptible to the antiproliferative effects of the drugs.

DISCUSSION

IH is a great challenge to vascular surgeons because it is the main cause of restenosis after vascular intervention or surgery. The proliferation and migration of VSMCs is the key process, so the inhibition of VSMC activation is one of the important targets to treat or inhibit IH.

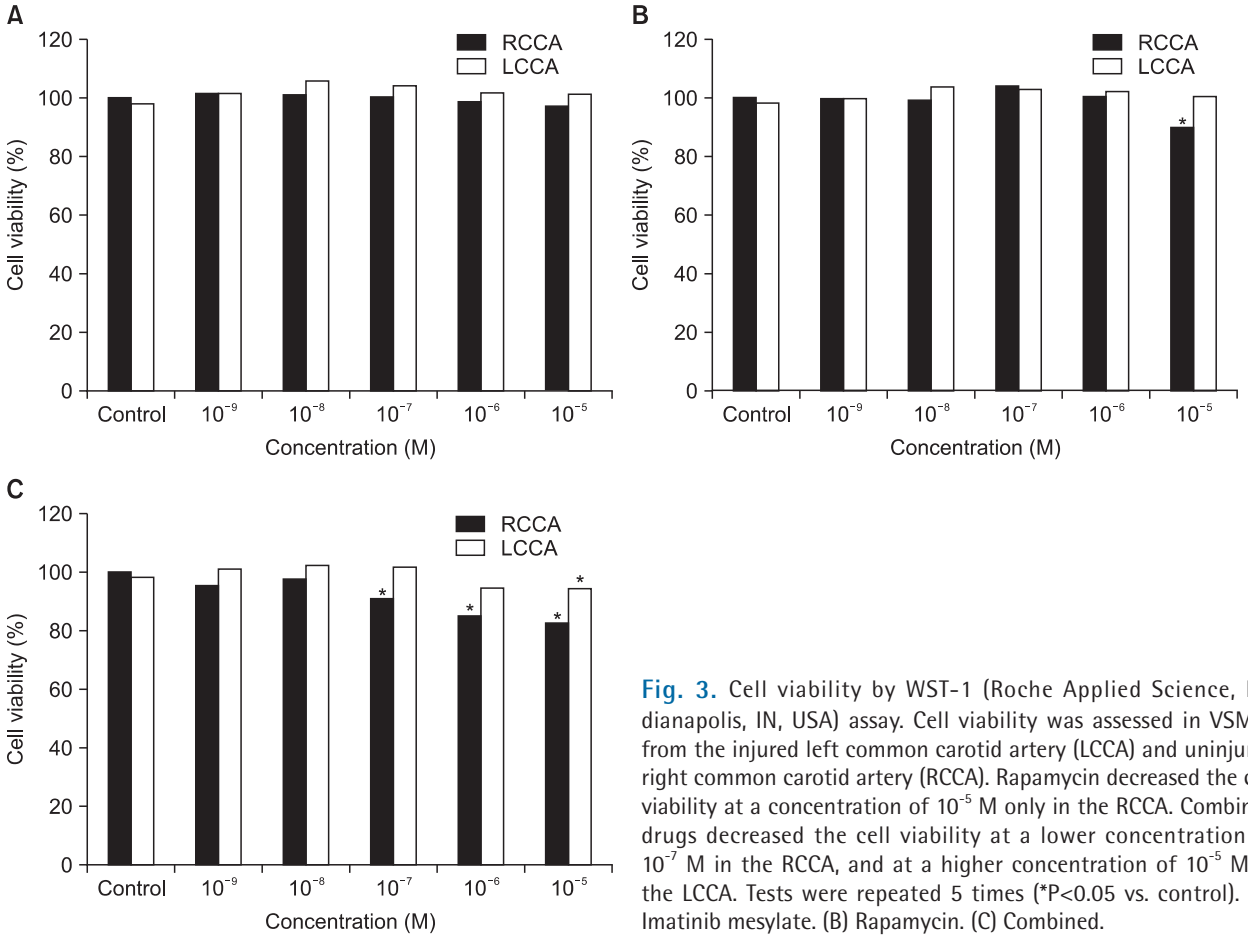

Fig. 3. Cell viability by WST-1 (Roche Applied Science, Indianapolis, IN, USA) assay. Cell viability was assessed in VSMCs from the injured left common carotid artery (LCCA) and uninjured right common carotid artery (RCCA). Rapamycin decreased the cell viability at a concentration of 10^{-5} M only in the RCCA. Combined drugs decreased the cell viability at a lower concentration of 10^{-7} M in the RCCA, and at a higher concentration of 10^{-5} M in the LCCA. Tests were repeated 5 times (*P<0.05 vs. control). (A) Imatinib mesylate. (B) Rapamycin. (C) Combined.

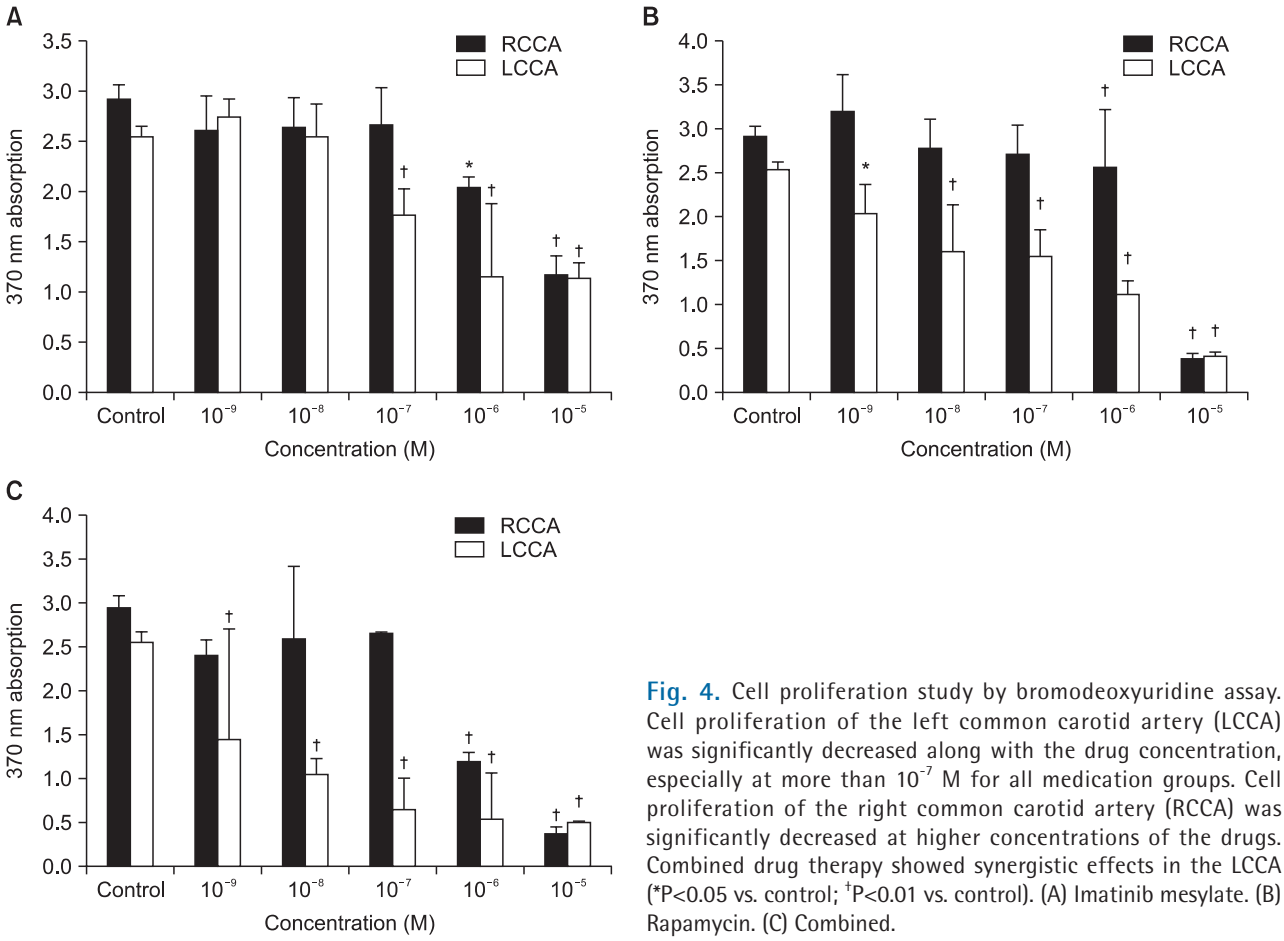

Fig. 4. Cell proliferation study by bromodeoxyuridine assay. Cell proliferation of the left common carotid artery (LCCA) was significantly decreased along with the drug concentration, especially at more than 10^{-7} M for all medication groups. Cell proliferation of the right common carotid artery (RCCA) was significantly decreased at higher concentrations of the drugs. Combined drug therapy showed synergistic effects in the LCCA (*P<0.05 vs. control; †P<0.01 vs. control). (A) Imatinib mesylate. (B) Rapamycin. (C) Combined.

In this study, neointimal cells from the injured carotid artery were more susceptible to the antiproliferative effects of imatinib and rapamycin in terms of proliferation and viability. This implies the possibility of in vivo use of these drugs for the prevention of IH without serious effects on normal VSMCs.

Another point is which cell should be used for research on the prevention or treatment of IH. Because normal quiescent VSMCs and neointimal activated VSMCs show different responses, specific cells are to be studied for the development of drugs to prevent or reduce IH.

Unfortunately we only investigated the viability and proliferation of these cells. Other important cell behaviors, such as three-dimensional migration, contraction, adhesion or apoptosis are to be investigated in future studies.

The treatment strategies for IH can be prevention of development of IH or induction of atrophy in the established IH [2]. Another study is ongoing to investigate a potential novel therapy to induce the regression of IH with rapamycin or imatinib mesylate in rat carotid injury models.

Previously, the origin of the neointimal VSMCs was thought to be the media near the injury site, but recently various origins of the neointimal cells were reported, including adventitial fibroblasts [18], circulating progenitor cells [19] and bone marrow [20]. These cells with different origin may show different responses to biologic stimuli. Unfortunately the origin of the neointimal cell is not investigated here, because it is beyond the scope of this study.

Although there are many limitations in this study, we found that imatinib and rapamycin inhibited the proliferation of both normal and neointimal VSMCs, and that combined drug therapy showed synergistic effects. Overall, neointimal cells are more susceptible to the antiproliferative effects of the drugs. In conclusion, both drugs can be used for the prevention of IH, which could be investigated through further in vivo studies.

CONCLUSION

Neointimal cells from the injured carotid artery are more susceptible to the antiproliferative effect of imatinib and rapamycin. Both drugs can be a used for the prevention of intimal hyperplasia, which could be investigated through

further in vivo studies.

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