Thalidomide as a Potent Inhibitor of Neointimal Hyperplasia After Balloon Injury in Rat Carotid Artery

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Objective—Inflammation is one of the main pathogeneses of neointimal hyperplasia after coronary intervention. Thalidomide, because of its potent antiinflammatory and immunomodulatory properties, is being re-evaluated in several clinical fields. Therefore, we examined whether thalidomide therapy affects neointimal formation.

Methods and Results—In male Sprague-Dawley rats, 100 mg/kg of either thalidomide or sucrose (control) was administered daily from 3 days before injury to 2 weeks after conventional carotid artery denudation injury. Thalidomide administration resulted in a significant reduction of neointimal formation (neointima to media ratio 1.26 ± 0.29 versus 0.35 ± 0.13, P < 0.001) and proliferative activity of vascular smooth muscle cells. In addition, arterial macrophage infiltration and local expressions of tumor necrosis factor alpha (TNF-α) and basic fibroblast growth factor (bFGF) in the injured arteries as measured by immunohistochemistry and immunoblot analysis were significantly reduced by thalidomide treatment. Serum TNF-α, measured by ELISA, was also significantly reduced in the thalidomide-treated animals compared with controls after injury (856 ± 213 versus 449 ± 68 pg/mL on day 3, P = 0.001; 129 ± 34 versus 63 ± 18 pg/mL on day 14, P = 0.001), and we observed a good positive correlation between the serum TNF-α levels and the severity of neointimal growth.

Conclusions—We found that thalidomide, through its antiinflammatory and antiproliferative effects, significantly inhibits neointimal hyperplasia in balloon-injured rat carotid arteries. Our results suggest a potential role of thalidomide as a potent inhibitor of neointimal formation after angioplasty. 

Key Words: neointima ■ inflammation ■ thalidomide ■ TNF-α ■ bFGF

Despite the recent advances in strategies to prevent neointimal growth after angioplasty, it still remains the major limitation of percutaneous coronary interventions. Although the pathogenic mechanisms have not been completely resolved, there is an accumulating body of evidence that suggests that inflammation plays a key role in the development of restenosis and that its severity is also influenced by the intensity of inflammation.

Evidence that the inflammatory response activated after vessel injury induces restenosis has been presented at the cellular, molecular, and genetic levels. Mechanical injury during coronary interventions is known to activate vascular smooth muscle cells (VSMCs) and leukocytes. Activated VSMCs and leukocytes are known to release various types of cytokines and growth factors, which stimulate migration and the proliferation of VSMCs leading to neointimal hyperplasia. Moreover, genetic predispositions to restenosis have been reported in polymorphism studies on many kinds of molecules such as CD18, interleukin (IL)-1 receptor antagonist, and matrix metalloproteinases, which are important components of the inflammatory process.

Initially developed as a sedative, thalidomide has since been shown to have potent antiinflammatory and immunomodulatory properties. Presently, a wide spectrum of diseases, including cutaneous lupus, Crohn disease, rheumatoid arthritis, multiple myeloma, and graft-versus-host disease, are being treated with thalidomide. In the field of cardiovascular medicine, based on its antiinflammatory effect, thalidomide was recently administered to patients with chronic symptomatic congestive heart failure who showed reduced tumor necrosis factor (TNF)-α level and increased left ventricular ejection fraction after 6 months of thalidomide therapy.

Therefore, given these properties, we decided to investigate whether thalidomide inhibits neointimal hyperplasia after balloon angioplasty and to test its possible therapeutic use as an antirestenotic agent.

Methods

Materials and Pretreatments

Procedures involving animals were in accordance with the Guide for Experimental Animal Research from the Laboratory for Experimental Animal Research.

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Vascular Injury Model and After Treatment
Balloon injury was performed blinded of the pretreatment details. Under xylazine (5 mg/kg intraperitoneally; Yuhan Corp, Bayer Korea) and ketamine hydrochloride (50 mg/kg intraperitoneally; Yuhan Corp, Bayer Korea) anesthesia, the right external carotid arteries were exposed and the common carotid arteries were denuded of endothelium by the intraluminal passage of a 2-French arterial catheter (Baxter Healthcare Corp), which was passed to the proximal common carotid artery and withdrawn in the inflated state 5 times.

Arterial Harvest and Morphometric Analysis
Three days and 14 days after balloon injury, rats (n = 8 per time point per group) were euthanized with a lethal dose of pentobarbital. Bilateral common carotid arterial segments, ~1 cm long, were harvested and perfusion fixed with 10% neutral buffered formalin at physiological pressure. Tissues were then embedded in paraffin, and 4 to 6 sections 4-μm thick were cut from 4 equally spaced locations from the harvested arterial segments. The sections were stained with hematoxylin and eosin or Verhoeff–van Gieson stain. The luminal, neointimal, and medial areas were calculated using the Image-Pro Plus 4.5 software (Media Cybernetics Inc).

To determine the effect of thalidomide treatment on vascular remodeling, postexternal elastic lamina was defined as the circumferential length of external elastic lamina (EEL) of the injured right carotid artery at 14 days after the balloon injury, and pre-EEL as the EEL length of the uninjured right carotid artery before the procedure, assumed that the baseline size of the pre-injured right carotid artery was equal to the size of the uninjured left carotid vessel. The change in vessel size was represented as the post-EEL/pre-EEL ratio, and EEL area was the area bounded by the EEL. All sites were analyzed, and the results were averaged.

Determination of Inflammation
To understand the mechanism of thalidomide treatment in vivo, we examined inflammatory and proliferative activities of the injured carotid arteries. Another subset of rats were sacrificed at 3 days and 14 days after the injury for immunohistochemistry and Western blot analysis (n = 8 per time point per group).

Measurement of Systemic Inflammation by Serum TNF-α ELISA
To investigate the effects of thalidomide on systemic inflammation, serum TNF-α was measured on days 3 and 14 after the balloon injury as one of the systemic inflammation markers. Sham operations, in which the same procedure except the inflation of the balloon catheter was performed, were performed in 5 additional rats at the same time points. Arterial blood was collected into calcium-containing tubes from the abdominal aorta via an 18-gauge intravenous cannula at the time of vessel harvest, and TNF-α levels were measured in duplicate with a commercial ELISA kit purchased from BD PharMingen. The sensitivity of TNF-α ELISA was 13 pg/mL.

Assessment of Local Tissue Inflammation by Western Blot Analysis
TNF-α and basic fibroblast growth factor (bFGF) are expressed by activated VSMCs in response to balloon injury.6,7 To determine whether thalidomide treatment suppresses not only systemic inflammation but also local tissue inflammation, Western blot analysis was conducted to evaluate the tissue expression of TNF-α and bFGF in the harvested injured artery. Vessel tissues were homogenized in lysis buffer and protein concentrations were determined using a Micro BCA Protein Assay kit (Pierce). Twenty micrograms of protein per specimen were separated on a SDS-polyacrylamide gel, blotted onto nitrocellulose membranes, and probed with specific antibodies against TNF-α (Santa Cruz Biotechnology) and bFGF (Santa Cruz Biotechnology). β-Actin (Santa Cruz Biotechnology) was used as control.

Assessment of Local Tissue Inflammation by Immunohistochemistry
Macrophage recruitment and infiltration occur at sites of vascular injury, which is a major source of various cytokines and growth factors.2,4 Species-specific antibodies were used to immunohistochemically identify macrophages infiltration (ED-1; Serotec) and bFGF expression (Santa Cruz Biotechnology). Standard peroxidase–antiperoxidase staining procedures (avidin-biotin-peroxidase kit, Dako) were used in conjunction with heat-induced epitope retrieval. Sections were counterstained with Mayer hematoxylin, dehydrated, and mounted. The ED-1–positive cells were divided by the total number of nucleated intimal and medial cells in 4 sectors per vessel section at 14 days after injury (n = 4 per group).

Determination of Proliferative Activity
To determine whether suppressed inflammation is accompanied by the reduced proliferative activity of VSMCs, we performed immunohistochemical staining against proliferating cell nuclear antigen (PCNA) (PC10; DAKO). To quantify VSMC proliferation after balloon injury, we counted the percentage of PCNA-positive cells against total nucleated cells in 4 different sectors per vessel section (n = 3 per time point per group).

Statistical Analysis
Data are presented as mean±SD. Comparisons between the thalidomide-treated group and control group were performed using an unpaired, 2-tailed t test. SPSS 11.0 was used for all statistical calculations and P<0.05 was considered significant.

Results
Effect of Thalidomide on Neointimal Hyperplasia
Morphometric analysis 3 days after balloon injury revealed no significant differences in the neointimal or medial areas of the thalidomide-treated and control groups (n = 16) (Figure 1C and 1D). No neointima could be found in either group. However, 2 weeks after injury, bulky concentric neointimal hyperplasia was detected in the control animals (Figure 1E). In the thalidomide-treated animals, there was a 71% reduction in the neointimal area (control versus thalidomide, 0.17±0.04 versus 0.05±0.02 mm², n = 16, P<0.001), and a 72% reduction in the neointimal/media (NI/M) ratio (control versus thalidomide, 1.26±0.29 versus 0.35±0.13, P<0.001; Figure 1F and 1H) compared with the control group. Therefore, lumen area at 2 weeks was significantly greater in the thalidomide-treated group (luminal area; control versus thalidomide, 0.24±0.04 versus 0.36±0.06 mm², P<0.001). However, there were no significant differences in medial areas, EEL areas, pre-EELs, post-EELs, and post-EEL/pre-EEL ratios between the 2 groups (Figure 1G and 1H). Arteries not injured by the balloons revealed no histological differences between the control and thalidomide-treated animals (Figure 1A and 1B).

Effects of Thalidomide on Systemic Inflammation
Sham operations showed that arterial dissection and catheterization itself resulted in a significant increase in serum TNF-α levels (sham operation versus unoperated rats
531 ± 101 pg/mL versus undetectable range on day 3, n = 10, P < 0.001; 52 ± 23 pg/mL versus undetectable range on 14 day, n = 10, P < 0.001). However, balloon injury resulted in a significant additional increase in serum TNF-α levels, which was reduced by 48% and 51% on days 3 and 14 after balloon injury, respectively, in thalidomide-treated animals (control versus thalidomide 856 ± 213 versus 449 ± 68 pg/mL on day 3, n = 16, P = 0.001; 129 ± 34 versus 63 ± 18 pg/mL on day 14, n = 16, P = 0.001; Figure 2A). Furthermore, we observed a positive correlation between the serum TNF-α level and the NI/M ratio 14 days after the injury (Pearson correlation coefficient 0.915, P < 0.001, 2-tailed) (Figure 2B).

**Effect of Thalidomide on Local Inflammation**

The effect of thalidomide on the local expressions of TNF-α and bFGF in the injured vessels was confirmed by Western blot analysis. Arteries not injured by balloon angioplasty produced only faint signals, indicating that these inflammatory molecules were expressed at low levels in the basal condition, and balloon injury induced high levels of TNF-α and bFGF expression on days 3 and 14 after balloon injury in the control group. In thalidomide-treated animals, the expression of TNF-α and bFGF was significantly reduced at both time points (Figure 3A and 3B).

The local effect of thalidomide on bFGF was also demonstrated by immunohistochemistry. bFGF staining was significantly increased in the nucleus as well as the cytoplasm of the control animals (Figure 4C and 4E) after angioplasty, which was attenuated in thalidomide-treated rats (Figure 4D and 4F). With regard to bFGF staining, it is difficult to differentiate cytoplasm-positive cells from cytoplasm-negative cells because cytoplasmic boundary is somewhat indefinite and vague. So, we measured bFGF labeling index by counting bFGF-positive nuclei against the total nuclei in the neointimal and medial smooth muscle cells based on the fact that nuclear translocation of bFGF is associated with its biological activity. The bFGF labeling index was significantly lower in the thalidomide-treated group at day 3 (43.3 ± 6.9 versus 9.0 ± 2.8%, n = 3, P < 0.01 for control versus thalidomide) and at week 2 (39.8 ± 4.2 versus 15.0 ± 5.8%, n = 3, P < 0.01 for control versus thalidomide) after balloon injury (Figure 4G).
In addition, tissue macrophage infiltration was reduced by 79% in the thalidomide-treated group compared with the control group 2 weeks after balloon injury (Figure 5) (control versus thalidomide, 19.2±3.5 versus 4.0±2.1%, P=0.021).

Effect of Thalidomide on the Proliferative Activity of VSMCs

Suppressed inflammation was accompanied by a reduction in the proliferative activity of VSMCs. A remarkable decrease in the number of PCNA-positive VSMCs was demonstrated in the medial layers of arteries of thalidomide-treated rats 3 days after injury versus the control animals (Figure 6C and 6D). Moreover, this antiproliferative effect of thalidomide was sustained until 14 days after the injuries in the neointimal and medial layers of thalidomide-treated animals (Figure 6E and 6F). The percentages of PCNA-positive cells in the vessel wall at days 3 and 14 were 31.0±7.3 and 43.1±2.9% in controls and 9.4±1.9 and 7.4±1.7% in thalidomide-treated group, respectively (P<0.01).

Discussion

The activation of the inflammatory response to vessel injury has been shown in numerous studies to play a key role in neointimal growth after angioplasty. Vessel injury seems to activate VSMCs and recruit leukocytes to the site of inflammation caused by the injury, which then release various cytokines and growth factors leading to VSMC proliferation and neointimal growth. Therefore, efforts to reduce inflammation after vessel injury have been a reasonable strategy to inhibit neointimal growth.

Thalidomide, originally synthesized as a sedative, was withdrawn from widespread use in the 1960s because of teratogenicity leading to dysmelia syndrome. Recently, there has been a resurgence of interest in the drug because of its potent antiinflammatory, antiangiogenic, and immunomodulatory properties. Presently, the use of thalidomide has expanded to various clinical areas where inflammation is thought to play an important role, such as refractory cutaneous lupus, rheumatoid arthritis, Crohn disease, multiple myeloma, and chronic graft-versus-host disease.

The majority of the antiinflammatory properties of thalidomide have been attributed to change in production and release of a wide range of cytokines and growth factors. Currently, perhaps the best described effect of thalidomide is of its inhibitory action on TNF-α. In vitro studies suggest that thalidomide is a potent inhibitor of TNF-α production in...
monocytes, which is achieved in part by shortening the half-life of TNF-α mRNA. We hypothesized in the present study that thalidomide, because of its potent antiinflammatory effects, would reduce neointimal growth after vessel injury. We found for the first time to our knowledge that thalidomide administration reduced neointimal growth by 71%. Thalidomide not only reduced systemic inflammation as measured by serum TNF-α but also reduced local inflammation. Tissue expression of TNF-α, bFGF, and macrophage infiltration were all significantly attenuated by thalidomide administration.

TNF-α is known to be expressed by activated leukocytes and VSMCs in injured arteries after balloon angioplasty. Moreover, previous studies have shown that most of neointimal VSMCs express TNF-α along with its mRNA even until 10 days after injury. The local blockade of TNF-α with TNF-α antibody eluting stent reduces VSMC proliferation in saphenous vein organ culture. In other studies, tissue macrophage infiltration in the neointima and media, which is a major sources of TNF-α, was shown to be significantly increased and sustained until 14 to 28 days after injury. In a study by Zhou et al, authors reported that blocking antibody against TNF-α failed to inhibit neointimal formation despite the neutralization of tissue TNF-α activity. However, considering that TNF-α expression and macrophage infiltration is sustained at least 2 weeks in previous studies, the duration of TNF-α neutralization in Zhou et al’s study may have been insufficient because it lasted only 7 of the 21 days of the experiment.

In the present study, the inhibition of TNF-α and macrophage infiltration were sustained until day 14 after the injury. This was confirmed by ELISA and Western blot. Thalidomide treatment reduced macrophage infiltration by ≈80% (Figure 5) and the serum level of TNF-α by ≈50% (Figure 2A), which are comparable to findings from other studies. Moreover, thalidomide administration significantly suppressed local expressions of TNF-α in injured arteries (Figure 3A). Although the majority of the increase in serum TNF-α level was caused by nonspecific injury during dissection of the carotid arteries as shown by results from the sham-operated animals, there was a small but significant increase in serum TNF-α levels in animals that received balloon injury. Furthermore, we observed a positive correlation between the serum TNF-α level and the degree of neointimal hyperplasia. The interpretation of such correlation must be made with extreme caution, because it is only a correlation and not a causal relationship that we observed. However, we believe that our data show that although local inflammation is the major mechanism of neointimal growth, systemic inflammation also plays a part in its development.
Such findings are supported by other studies, which showed that nonspecific systemic inflammation can aggravate neointimal overgrowth after balloon and stent injury in the rabbit.\(^3\)

Another cytokine that may be attenuated by thalidomide is bFGF, which is a pleiotropic molecule whose mRNA is overexpressed in cytoplasms and nuclei of proliferating VSMCs and endothelial cells after balloon injury.\(^2,5\) After being released, it is internalized into the cytoplasm by low-affinity receptors and then translocated to and accumulated in the nucleus.\(^2,6,27\) In previous studies, the translocation of bFGF has been correlated to cell-cycle progression and proliferation via stimulation of protein kinase C/\(\text{PKC}\), and exposure of the VSMCs to bFGF has been shown to induce neointimal proliferation.\(^2,9\) In addition, mice treated with anti-bFGF antibody showed reduced medial muscle cell proliferation after arterial injury.\(^3,0\) Thalidomide, when used as a single agent in myeloma, myelodysplastic syndrome, and histiocytosis, was shown to significantly decrease the plasma level of bFGF.\(^1,3\)

In the present study, Western blotting (Figure 3B) showed attenuated expression of bFGF in the thalidomide-treated rats. Furthermore, immunohistochemical analysis confirmed decreased bFGF staining, identified as brown color in nuclei and cytoplasms in the artery at 3 and 14 days (Figure 4D and 4F) after injury in the thalidomide group compared with the control group (Figure 4C and 4E). Presumably, reduced macrophage infiltration and expression of TNF-\(\alpha\) and bFGF resulted in the decreased proliferative activity of VSMCs and led to the reduction of neointimal growth. We did not observe any significant differences in EEL areas (Figure 1G) and post-EEL/pre-EEL ratios (Figure 1H) between the 2 groups, suggesting that thalidomide does not affect vascular remodeling after injury.

In addition to the aforementioned effects, thalidomide has been reported to reduce the synthesis of other proinflammatory cytokines, such as IL-1, IL-6, and IL-8,\(^1,1\) and the expression of endothelial adhesion molecules, including integrin, intercellular adhesion molecule, and vascular cell adhesion molecule;\(^3,1–3,3,3\) as a consequence, the interactions between endothelial cells and circulating leukocytes are inhibited.\(^3,3\) The activation of NF-\(\kappa\)B, an important mediator of inflammation, cell proliferation, and restenosis,\(^3,4,3,5\) was also found to be blocked with thalidomide because of its inhibitory effect on I-\(\kappa\)B degradation.\(^3,6\) All these effects of thalidomide may be beneficial in the prevention of neointimal hyperplasia.

There are concerns about the safety of thalidomide since it was withdrawn from the market because of its teratogenicity. However, considering that the majority of the patients undergoing coronary interventions are those who are older than age 40 years, this should not be a big problem. Other adverse effects of thalidomide, such as peripheral neuropathy, sedation, fatigue, and dizziness, are reported to be generally very mild and do not require discontinuation of the drug. Furthermore, in a recent study, the safety and feasibility of thalidomide were confirmed in symptomatic congestive heart failure patients who used 100 to 200 mg of thalidomide daily for 6 weeks without significant adverse effects.\(^1,4\)

In conclusion, we report for the first time to our knowledge that thalidomide significantly inhibits neointimal hyperplasia in balloon-injured rat carotid arteries because of its anti-inflammatory effect and its antiproliferative action. The major effects of thalidomide may be the marked decreases in the expression of TNF-\(\alpha\) and bFGF and in macrophage infiltration. Although the results of our study remain to be confirmed in larger animal models; furthermore, in human clinical trials, the results of the present study clearly suggest the potential role of thalidomide as a potent inhibitor of neointimal growth after angioplasty.

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