Zoledronate Inhibits Intimal Hyperplasia in Balloon-injured Rat Carotid Artery


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KEYWORDS
Zoledronate; Bisphosphonates; Intimal hyperplasia

Abstract

Background and objective: Zoledronate has been reported to inhibit the proliferation, adhesion and migration of vascular smooth muscle cells. In the present study, we assessed whether systemic and local delivery of zoledronate would be sufficient to prevent intimal hyperplasia.

Methods: Twenty-four male Sprague-Dawley rats were assigned into four groups: non-treated group, systemic zoledronate-treated group, local collagen-treated group and local zoledronate-treated group. All four groups underwent balloon injury to the right common carotid artery. The left uninjured carotid arteries of the non-treated group were considered as normal artery samples. Twenty-one days after arterial injury and treatment, the right and left common carotid arteries were fixed, sectioned, stained and measured by computer-aided image analysis.

Results: At 3 weeks, there was a 59% reduction of the intima/media area ratio in the systemic zoledronate-treated group compared with the non-treated group (P < 0.01). There was an 87% reduction of the intima/media area ratio in the local zoledronate-treated group compared with the local collagen-treated group (P < 0.01).

Conclusions: Both systemic and local delivery of zoledronate correspond to a significant reduction in intimal hyperplasia seen at 3 weeks.

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Restenosis due to intimal hyperplasia remains the major obstacle to satisfactory long-term patency of open- and catheter-based treatment of obstructive arterial disease. Monocytes and macrophages activation and vascular smooth muscle cell proliferation and migration are thought to be central to the development of intimal hyperplasia.1,2 Many pharmacological agents, including anti-thrombotics, anti-platelet agents and angiotensin-converting enzyme inhibitors have been used in an attempt to attenuate this injury response.3

Bisphosphonates, which have been used widely in the treatment of excessive bone resorption diseases, are stable analogues of inorganic pyrophosphate. Their half-life in the circulation is short; they enter rapidly and extensively into bone, and then inactivate osteoclasts, which regulate bone
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Materials and Methods

Materials

Zoledronate was kindly provided by Novartis Pharma AG (Stein, Switzerland). Rat tail tendon collagen type I (5 mg ml\(^{-1}\)) in 0.006 N acetic acid was purchased from Shengyou Biotechnology Co., Ltd. (Hangzhou, China). The monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

Animals

Sprague-Dawley rats (male, 300–350 g) were purchased from Shanghai Laboratory Animal Centre of Chinese Academy of Sciences. Animals were fed a standard diet and water ad libitum. The animals used in this study were treated in accordance with Health Guide for the Care and Use of Laboratory Animals and the guidelines of Fudan University on the ethical use of animals.

Rat balloon-injury model

Balloon injury of the right common carotid arterial endothelium was performed in 24 male Sprague-Dawley rats as described before. Briefly, rats were anaesthetised with 10% chloral hydrate (3 ml kg\(^{-1}\), i.p.). A midline incision was made in the neck, and the right common carotid artery was exposed. A small cut was then made in the external carotid artery, and an embolectomy catheter (Fogarty 2F, Edwards Laboratories) was passed into the common carotid artery. The balloon was inflated with water until slight resistance was met to expand the common carotid artery and then slowly twisted back to the carotid bifurcation. This procedure was done three times to achieve complete denudation of the endothelium, including a mild-to-moderate injury of the inner layers of the media smooth muscle cells. Afterwards, the balloon was removed, and the external carotid artery ligated.

Preparation of zoledronate

A stock solution (10 mM) of zoledronate was prepared in calcium-free phosphate buffered saline. For every 1 ml of collagen (4.9 mg ml\(^{-1}\)) and zoledronate (100 \(\mu\)M) mixture, mix 980 \(\mu\)l of 5 mg ml\(^{-1}\) collagen, 10 \(\mu\)l of 10 mM zoledronate and 6 \(\mu\)l of 1 N NaOH. Leave the mixture on ice until ready for use.

Therapy

The 24 rats were assigned into four groups: non-treated group (group I), systemic zoledronate-treated group (group II), local collagen-treated group (group III) and local zoledronate-treated group (group IV). Groups I and III served as a control for groups II and IV, respectively. All four groups underwent balloon injury on the right carotid artery. The left uninjured carotid arteries from group I were considered as normal artery samples. For the systemic zoledronate-treated group, a dose of zoledronate (0.05 mg kg\(^{-1}\)) was injected, approximating a clinical dose, through the rat tail vein immediately after the procedure; two more doses were injected at day 7 and day 14 post procedure. For the local collagen-treated group, 200 \(\mu\)l of rat tail collagen (4.9 mg ml\(^{-1}\)) was coated around the adventitia of the injured carotid artery right after external carotid ligation. For the local zoledronate-treated group, 200 \(\mu\)l of rat tail collagen (4.9 mg ml\(^{-1}\)) and zoledronate (100 \(\mu\)M) mixture was coated around the adventitia of the injured carotid artery.

Tissue harvest and histology processing

Twenty-one days after arterial injury, rats were anaesthetised with 10% chloral hydrate (3 ml kg\(^{-1}\), i.p.). The right (injured) and left (uninjured) segments of the common carotid arteries were removed and fixed in 10% buffered formalin. A total of 24 h post fixation, the arterial segments were dehydrated and embedded in paraffin, and cut into 6-\(\mu\)m longitudinal sections. Standard haematoxylin–eosin staining was performed on serial sections.

Morphometry

The cross-sectional medial and intimal areas of the six sections from the midportion of each tissue segment were measured with Image-Pro Plus 6.0 image analysis software. Briefly, the luminal, internal elastic lamina and external elastic lamina areas were manually measured, the intimal area was calculated as the internal elastic lamina area minus luminal area and the medial area was calculated as the external elastic lamina area minus internal elastic lamina area. The ratio of the intima to media area was then calculated.

These measurements were performed by one analyst, who was blinded to the treatment.

Immunohistochemistry of PCNA expression

Immunohistochemical staining was performed on three sections from the midportion of each tissue segment as described before. Briefly, arterial sections were incubated with 0.3% hydrogen peroxide to block endogenous peroxidase,
and then with monoclonal antibodies directed against PCNA. The percentage of stained nuclei was determined as a ratio of stained nuclei to the total number of nuclei per high-power field in the section used for analysis. The mean values of these three sections were calculated for each vessel. For each group of animals, the mean values for each vessel were averaged. These examinations were performed by one analyst, who was blinded to the treatment.

Statistical analysis

Data were mean ± standard error of the mean (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni’s post hoc test and values of P < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 (San Diego, CA, USA).

Results

Histopathological changes

All the 24 rats were alive and well before sacrifice, and no major adverse effects were observed in any of the four groups. Redness and swelling at the infusion site of the rat tail were observed in group II. Incisions on the neck generally healed well in group IV.

The entire length of right (injured) and left (uninjured) common carotid arteries were obtained from the rats 3 weeks after balloon injury. Sections from the middle portion of specimens were analysed. Among the left uninjured arteries from group I, the luminal surface was smooth, the vascular wall only composed of a few layers of cells and the lumen area was enough for blood flow (data not shown). As for both the control vessels (groups I and III), large amounts of cells were present in the subendothelial space, the lumen area was reduced and the intimal area increased. Systemic and local delivery of zoledronate (groups II and IV) was found to improve the above histopathological changes and decrease the extent of neo-intima (Fig. 1).

Figure 1  Haematoxylin and eosin stained photomicrographs of rat right carotid artery sections 21 days after balloon injury at 100× magnification. The scale bar represents 100 μm (a) shows representative artery from group I; (b) shows artery from group II; (c) shows artery from group III; (d) shows artery from group IV.

Figure 2 The medial area of the right carotid artery from all four groups and the left uninjured arteries from group I. The area is calculated as mm². Data are expressed as mean ± SEM. *P < 0.05 compared with left uninjured arteries from group I.
Morphometric analysis

The average area of the left uninjured arteries was 0.096 mm², and all four groups demonstrated an increased medial area 3 weeks after balloon injury, with an average area of 0.139 mm², 0.150 mm², 0.152 mm² and 0.135 mm², respectively. However, no difference in medial area was observed between any of the four groups (Fig. 2).

Both control groups (groups I and III) developed a significant neo-intima 21 days after balloon injury, with an average area of 0.271 mm² and 0.278 mm², respectively. Both the systemic and local zoledronate-treated groups (groups II and IV) were found to have a less neo-intimal area, with an average area of 0.117 mm² and 0.034 mm², respectively (Fig. 3). Consequently, the intima/media area ratio was significantly decreased in groups II and IV compared with those in groups I and III, with a reduction by 59% and 87%, respectively (Fig. 4). No significant difference was found when the systemic zoledronate-treated group and the local zoledronate-treated group were compared.

PCNA expression

We also examined the degree of proliferation in neo-intima as assayed by immunostaining for PCNA. The dark-brown-labelled PCNA-positive nuclei were observed within the vascular neo-intima. PCNA is significantly less expressed in groups II and IV compared with groups I and III (Fig. 5). The PCNA-positive indices in the four groups were 38.73 ± 5.54, 23.14 ± 6.95, 40.89 ± 3.52 and 14.05 ± 8.64%, respectively (Fig. 6). Thus, zoledronate was associated with the reduction of the PCNA-positive indices in neo-intima, consistent with histopathological changes in neo-intima ($P < 0.01$).

Discussion

Balloon injury of the common carotid artery is a widely accepted animal model of intimal hyperplasia. The time course of neo-intimal growth following balloon injury has been established; vascular smooth muscle cells in the media begin to proliferate as they migrate to the intima. They continue to proliferate for 7–14 days to form an intimal thickening before stopping spontaneously.$^{13–15}$ In the current study, the rats were sacrificed at 3 weeks in the light of previous studies.

Many pharmacological agents have been used in an attempt to attenuate intimal hyperplasia. Bisphosphonates, besides extensive application in bone-related diseases, have been found to exhibit the effects of inhibiting development of atherosclerosis and intimal hyperplasia by transient systemic inactivation of monocytes and macrophages.$^{7,8}$ As bisphosphonates can concentrate in the arterial wall of both healthy and, especially, atheromatous rabbits for at least several days or a week,$^5$ we hypothesised that they might exert direct effects on the vascular smooth muscle cell, which is a major cell type in arterial wall. In our previous publication, we reported that zoledronate, a third generation of nitrogen-containing bisphosphonates, could inhibit the proliferation, adhesion and migration of vascular smooth muscle cells. The inhibition was more dramatic when the concentration reached up to 10–100 μM.

Zoledronate is the most potent bisphosphonate in clinical use. It is known to inhibit cell signalling through the mevalonate pathway and block the prenylation of small signalling proteins (e.g., Ras, Rac, Rab and Rho).$^{16}$ Loss of

![Figure 3](image-url) The intimal area of the right carotid artery of all four groups. The area is calculated as mm². Data are expressed as mean ± SEM. **$P < 0.01$ compared with control group.

![Figure 4](image-url) Intima/Media ratio 21 days after balloon injury of all four groups. **$P < 0.01$ compared with control group.

![Figure 5](image-url) PCNA protein expression 21 days after balloon injury at 400× magnification. Positive expression was detected by immunohistochemistry (cells stained in dark brown). The scale bar represents 50 μm (a) shows representative artery from group I; (b) shows artery from group II; (c) shows artery from group III; (d) shows artery from group IV.
prenylation consequently prevents these proteins from acting at the correct spatial location within the cell, thus ultimately affecting their normal behaviour, including cell proliferation, survival, cytoskeletal organisation and vesicular trafficking.\(^{17}\)

A peak serum concentration of zoledronate following administration of a clinical dose (4 mg over 15 min) ranges from 1 to 10 \(\text{M} \)\(^{18}\). It has been suggested, however, that the effective local concentrations of bisphosphonates at the sites of injury, especially atherosclerotic artery, may be much higher than serum levels.\(^{5}\) In our present study, we injected 0.05 mg kg\(^{-1}\) zoledronate once a week, approximately a clinical dose, to evaluate its effect on intimal hyperplasia.

As a potential method for the prevention of intimal hyperplasia, periadventitial administration of pharmacological agents directly to the injured site in the vascular has been attracting great interest.\(^{19-21}\) Periadventitial delivery may have the potential advantages of allowing a high concentration of the agent to be accumulated at the site of injured artery while minimising the likelihood of systemic side effects. Collagen is a major natural constituent of connective tissue and a major structural protein of any organ. It has long been used as a local drug delivery material, and its usefulness and safety have been verified.\(^{22}\) Therefore, in our present study, we periadventitially applied a high concentration (100 \(\mu\text{M}\)) of zoledronate mixed with collagen on an injured artery to evaluate its effects on intimal hyperplasia.

Our study shows that neo-intimal area was reduced in both the systemic and local zoledronate-treated groups compared with the related control group. However, no statistical difference, which may be due to small sample size, in the medial area was observed between any of the four groups. Consequently, the intima/media area ratio of the systemic and the local zoledronate-treated group was significantly decreased compared with the related control group, by 59% and 87%, respectively. No statistical difference, which again may be due to small sample size, was found when the systemic zoledronate-treated group and the local zoledronate-treated group were compared. The degree of proliferation in the neo-intima was assayed by immunostaining for PCNA; it was significantly less expressed in the zoledronate-treated groups as compared with the related control groups, consistent with histopathological changes in neo-intima.

As bisphosphonates have also been reported to concentrate in adventitia,\(^{6}\) is the effect of zoledronate on intimal hyperplasia due to direct inhibition on fibroblasts, which also play an important role in the development of intimal hyperplasia?\(^{23}\) Zoledronate has been demonstrated to inhibit endothelial cell adhesion, migration and survival,\(^{24}\) will zoledronate delay the process of re-endothelialisation after injury, which is beneficial to the inhibition of neo-intimal formation?\(^{25}\) Further research is necessary to optimise zoledronate to reduce intimal hyperplasia and elucidate the mechanisms in the balloon-injury model and other different models. Our promising findings of the efficacy of zoledronate at reducing intimal hyperplasia are limited to a time frame of 3 weeks. Long-term observations and optimised drug carriers are necessary before possible application to humans.

Figure 6  Effect of systemic and local delivery of zoledronate on the PCNA expression in neo-intima 21 days after balloon injury. **\(P < 0.01\), compared with control group.**
Conflict of Interest
The authors have no conflict of interest.

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