Zinc reduces intimal hyperplasia in the rat carotid injury model

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Abstract

Background: The response to arterial injury following balloon dilatation is known to involve proliferative and inflammatory processes. The current widespread use of stents to maintain arterial patency not only does not eliminate but possibly exaggerates the proliferative and inflammatory phenotype and although drug-eluting stents are available, their long-term safety is yet to be determined.

Zinc is a trace element that serves as a cofactor of many enzymes. Interestingly, it has been shown to have anti-inflammatory and anti-proliferative properties. We thus sought to investigate its effect on smooth muscle cell proliferation and intimal thickening in the rat carotid artery injury model.

Methods and results: Smooth muscle cells (SMC) were cultured from carotid arteries of rats and proliferation was assessed by thymidine incorporation after exposure to different concentrations of zinc. Next, carotid artery injury was induced in rats by balloon dilatation and they were either treated with I.P injections of zinc or PBS for 2 weeks until sacrifice for assessment of neointimal formation and lumen area.

Zinc inhibited in vitro SMC proliferation in a dose-dependent manner. In vivo, zinc treatment resulted in a 50% reduction in neointimal area and a significant decrease in neointimal/media ratio with no significant change in lumen area.

Conclusion: Thus, zinc appears to reduce neointimal growth and should be tested by local delivery systems including stent coatings.

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1. Introduction

Percutaneous transluminal coronary angioplasty (PTCA) with or without stent placement is a useful approach for treating coronary vascular diseases. Unfortunately, the long-term benefit is reduced by the high incidence of restenosis [1].

The term restenosis is used to describe luminal narrowing at a site previously treated by successful PTCA [1]. The reported incidence of restenosis, differs between locations in the vasculature and between studies, and is about 30%, usually within 6 months [1]. The pathophysiology of restenosis has been extensively studied and in balloon angioplasty appears to be related to a combination of constrictive remodelling and increase in the wall mass. The increase in wall mass is a result of smooth muscle cell (SMC) proliferation and migration and excessive extracellular matrix deposition. It has been demonstrated that after angioplasty, 73% of late lumen loss is due to constrictive remodelling, and only 27% due to neointima formation [2]. Stent implantation is associated with improved safety and reduced rate of restenosis compared to balloon angioplasty [3–7]. Animal model studies and intravascular ultrasound studies proved that instant restenosis is largely a result of neointimal thickening and stenting virtually eliminates elastic recoil and negative remodelling [8].

The pathogenesis of neointimal thickening is complex. Intimal injury sustained at the time of coronary intervention results in endothelial denudation, exposure of the thrombogenic subendothelium, deep fissuring, thrombosis and inflammation [9] followed by smooth muscle cell proliferation, matrix deposition and intimal growth [9–11].
An additional contributor to the pathogenesis of restenosis is the oxygen free radical. These are generated during vascular intimal injury and increase the expression of cell adhesion molecules, activate the multifunctional transcription factor NF-κB, and modulate the expression of many genes that control cellular proliferation [9].

Zinc is an essential trace element that is a co-factor for more than 300 enzymes and involved in a variety of cellular functions. During the last decade there is an increasing number of studies pointing to the role of zinc in apoptotic [12–15] and inflammatory processes [16,17], both in vitro and in vivo. In the present study, we wished to determine whether zinc administration decreases intimal hyperplasia and in vivo. In the present study, we wished to determine whether zinc administration decreases intimal hyperplasia.

2. Materials and methods

2.1. Smooth muscle cell culture and proliferation assays

SMCs were obtained from the carotid arteries of Wistar rats by use of the collagenase and elastase digestion method. Slices of rat aorta were incubated with collagenase (1.3 U/ml), elastase (1.3 U/ml), and soybean trypsin inhibitor (1 mg/ml) for three 30 min intervals. Cell suspensions were pooled and cultured in minimum essential medium with fetal bovine serum (10%), tryptose phosphate broth (2%), glutamine (20 mM), penicillin (50 U/ml), and streptomycin (50 U/ml) in a humidified atmosphere of 5% CO2 –95% air.

For experiments with growth-arrested SMCs, cells were made quiescent by incubating in the above culture medium containing 0.2% FCS for 48 h before use. SMCs cultured in 10% FCS are hereafter referred to as growing SMCs.

Cells were grown in 12-well plates with a seeding density of 3000 cm−2. When cells reached subconfluence, they were made quiescent for 24 h in low serum conditions. Cells were then stimulated with serum and further cultured in medium containing 0.2% FCS, and the common carotid artery was harvested. The vessel was washed with PBS followed by overnight fixation in 10% buffered formalin. Blood samples were centrifuged at 3000 rpm for 15 min, and the plasma layer was sent to the chemistry laboratory for Zinc level determination.

2.2. In vivo study design

The control group included 10 rats that following carotid injury were treated for 2 weeks by two daily intra-peritoneal injections of PBS. The study group included 10 rats that were treated by 2 weeks of twice daily intra-peritoneal injections of 5 mg/kg ZnCl2.

Fourteen days after injury the rats were anesthetized. A sample of 5 ml blood was obtained from each animal and the common carotid artery was harvested. The vessel was washed with PBS followed by overnight fixation in 10% buffered formalin. Blood samples were centrifuged at 3000 rpm for 15 min, and the plasma layer was sent to the chemistry laboratory for Zinc level determination.

2.3. Animal model

A balloon-catheter denudation model of arterial injury was used to induce intimal hyperplasia in the right common carotid of male Wistar rats weighing about 350 g. Detailed procedures have been described previously [18]. Briefly, intra-peritoneal injections of ketamine hydrochloride and xylazine were used for anesthesia, followed by a longitudinal midline cervical incision that permitted exposure of the right common, external and internal carotid arteries. Distal ligation of the external carotid artery with vascular control of the common and internal carotid arteries was performed. Following arteriotomy of the external carotid artery, a 2F catheter was inserted through the arteriotomy, via the external carotid artery into the common carotid artery. Then the balloon was inflated, and passed three times without rotation, along the common carotid artery. After deflating the balloon, the catheter was withdrawn, the external carotid artery was ligated and the neck incision was closed.

2.4. Histology and morphology

The specimens were dehydrated using sequentially concentrated ethanol followed by xylene and embedded in paraffin. Five-micron cross-sections were cut and stained with hematoxylin and eosin (H and E). Intimal area, intimal area/medial area ratio and luminal area were morphometrically measured by a computer image analysis system.

2.5. Immunohistochemistry

The amin–biotin complex immunoperoxidase procedure was used to identify proliferating cells with an anti-Ki67 monoclonal antibody. Both immunohistochemistry staining for NF-κB and iNOS positive cells were also done as previously described [18].
2.6. Statistical analysis

Results of all parameters were computed employing the student’s t-test. Results are presented as mean ± S.E.M. P < 0.05 was considered significant.

3. Results

3.1. Zinc inhibits SMC proliferation in vitro

SMC appear to constitute a predominant portion of the neointima in injured rats and also the majority of restenotic lesions within stents. Thus, we first evaluated the effect of PBS in vitro on proliferation of carotid artery cultured SMC. We found that serum-stimulated thymidine incorporation into DNA was significantly reduced in SMC cultured in the presence of ZnCl2 in a dose dependent manner (Fig. 1). The observed degree of inhibition of thymidine incorporation by the VSMC at 1, 2.5, 10, 25, 50, and 100 µg/ml was respectively: 12, 21, 19, 92, 93, and 94% (Fig. 1). A statistically significant inhibition of proliferation was evident starting from final Zinc concentration of 2.5 µg/ml.

3.2. Weight and plasma zinc levels

A total of 20 rats underwent right carotid artery balloon denudation, followed by one postoperative death in the control group. All animals appeared healthy during the experiment. The average weight of the zinc-treated group was similar to the control group at baseline (356 ± 7 g versus 353 ± 7 g). After 2 weeks of treatment, the average weight of the zinc-treated group was insignificantly lower as compared to the control group (334 ± 7 g versus 349 ± 8 g).

After 2 weeks of treatment, zinc plasma levels were significantly higher in the zinc-treated group as compared to the control group (2.48 ± 0.6 µg/ml CI: 2.0–2.9 versus 1.38 ± 0.2 µg/ml CI: 1.2–1.6, P < 0.05).

3.3. The effect of zinc treatment on intimal hyperplasia

Intimal hyperplasia was quantitatively recorded by a computer-assisted morphometric analysis. As shown in Fig. 2, a 50% reduction was evident in the neointimal area in zinc treated rats as compared with PBS treated animals (mean area of 70390 ± 25150 µm² in zinc treated rats as compared to 141500 ± 36110 µm² in the controls; P < 0.05, Figs. 2A, 3A and 3B). Intimal to medial (I/M)
ratio was significantly reduced in Zinc treated rats (0.3 ± 0.11) as compared with controls (0.75 ± 0.2; P < 0.05, Fig. 2B). Zinc treatment did not significantly influence lumen area (364800 ± 38870 μm²) in the Zinc as compared with the control group (353300 ± 32910 μm², Fig. 2C). A non-injured carotid artery is shown where no evidence of neointimal growth is seen (Fig. 3C).

3.4. Immunohistochemical analysis of inflammatory and proliferative markers

To determine whether, at sacrifice, cellular markers of proliferation and inflammation were altered in the zinc treated animals, we performed immunohistochemical analysis of the injured arteries.

![Image](image_url)
4. Discussion

This study was designed to demonstrate the effect of intraperitoneal administration of zinc on intimal hyperplasia, in the rat carotid artery injury model. We chose the rat model of carotid balloon dilatation because the vessel response to injury is well characterized and has some features in common with the restenosis process in human following coronary angioplasty. However, there is no animal model that is currently optimal for simulating human restenosis, and there are several major drawbacks in the rat model that relate to the difference in the induction of the injury and the kinetics of neointimal proliferation. In the rat model, medial smooth muscle cells begin to proliferate and migrate after 24 h, medial proliferation peaks at 48 h and maximal proliferation occurs after 4 days. The intimal lesion is evident after 7 days and is well developed after 2–3 weeks, therefore the 2 week time-point was chosen for termination of the study [19]. It should be mentioned that since different methods are used for creation of the injury, the results from different operators may be difficult to compare. Since we do not use rotation with the inflated balloon within the artery, uneven neointimal growth may occur, while achieving less tissue trauma (thus more closely approximating angioplasty in humans).

Aiming to provide rational for the in vivo testing of zinc on intimal proliferation, we initially tested its influence on SMC proliferation. As measured by thymidine incorporation, we found that even a concentration of zinc of 10 μg/ml was sufficient to suppress significantly the ability of SMC to proliferate in vitro. This effect was indeed more robust as zinc concentrations increased, achieving a near complete arrest of proliferation at final levels of 100 μg/ml.

Zinc dosing in vivo were was selected based on prior studies with a matching concentration/weight ratio. After 2 weeks of intra-peritoneal administration, zinc plasma levels were indeed significantly increased in the treated group. There was a 50% decrease in the intimal area and 60% decrease in the intimal/medial ratio in the zinc-treated group as compared to the control group. The luminal area, however, was not different between the treated and control group. There are several explanations for the lack of effect on lumen patency. First, the magnitude of effect of zinc administered systemically may have been insufficient to bring about a reduction in neointima needed to significantly influence lumen area. This could be overcome in the future possibly by local delivery of zinc or alternatively, by coating it onto stents. A second explanation could be that zinc did influence significantly SMC proliferation, yet negative recoil that was not expected to change following treatment ‘compensated’ for the loss of neointima. If this explanation is correct, a stent coated with zinc will eliminate elastic
recruitment of inflammatory cells, which may have a significant role in the development of restenosis.

References


