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Ginkgolide A-Gold Nanoparticles Inhibit Vascular Smooth Muscle Proliferation and Migration *in Vitro* and Reduce Neointimal Hyperplasia in a Mouse Model

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Abstract

Background—Neointimal formation is mediated by phenotypic changes in vascular smooth muscle cells (SMC) and is an important mediator of restenosis following arterial reconstruction. We conjugated antioxidant Ginkgolide A (GA) to gold nanoparticles (GNP) to determine the effect of GA delivery on neointimal formation.

Materials and methods—GA was conjugated to 80nm GNP in an overnight incubation. Mouse P53LMAC01 vascular SMC were treated with various doses of GA-GNP, GA alone, GNP alone, and no treatment control. Cell proliferation and migration were analyzed, and superoxide anion levels and the phosphorylation status of ERK1/2 were determined. Mice underwent ligation of the common carotid artery along with local treatment with GNP (control) or GA-GNP. The carotid artery was harvested and subjected to immunohistochemical analysis.

Results—GA-GNP treatment significantly inhibited SMC proliferation and migration *in vitro* in comparison to GNP treatment alone, and the effect persisted for up to 72 h after treatment. Treatment with GA-GNP also reduced superoxide anion levels *in vitro*. PDGF-BB substantially induced ERK1/2 phosphorylation in GNP control cells; this PDGF-BB induced ERK1/2 phosphorylation was significantly inhibited in GA-GNP-treated cells compared to GNP only. GA-GNP significantly reduced neointimal hyperplasia after injury in mice, and proliferating cell nuclear antigen (PCNA) staining was reduced substantially in the arteries of mice treated with GA-GNP.

Conclusions—GA-GNP reduce vascular SMC proliferation and migration *in vitro* through reduced activation of ERK1/2. Local treatment with GA-GNP in areas of arterial injury reduced neointimal hyperplasia and subsequent stenosis.

Keywords

Ginkgolide A; gold nanoparticles; smooth muscle cell; cell proliferation; cell migration; neointimal hyperplasia; mouse model

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INTRODUCTION

Approaches for the surgical treatment of atherosclerotic arterial occlusive disease include arterial reconstructions such as balloon angioplasty and/or stenting, endarterectomy and open surgical bypass. However, restenosis following these interventions leads to reduced luminal diameter and ultimately occlusion and ischemia. While aggressive antiplatelet therapy and anticoagulant regimes in selected patients are often implemented, the underlying processes of neointimal hyperplasia and vascular remodeling have yet to be effectively addressed.

Restenosis due to neointimal hyperplasia is known to occur after both balloon angioplasty and stent placement [1]; this presents a substantial clinical problem in percutaneous intervention for coronary artery disease and peripheral arterial disease, especially treatment of femoropopliteal stenosis [1, 2]. In coronary artery stenting, the emergence of drug-eluting stents (DES) in the late 1990s initially promised reduced neointimal proliferation; however, subsequent studies revealed impaired endothelialization by the antiproliferative drugs (sirolimus, paclitaxel) coating the DES, leading to exposure of blood to thrombogenic stent struts [1]. Likewise, while the advent of nitinol self-expanding stents in the treatment of femoropopliteal disease have shown improved radial strength and outcomes, “exaggerated neointimal hyperplasia” and in-stent restenosis remain a significant clinical problem [3].

Several factors contribute to neointimal formation. Loss of the endothelial monolayer can occur with arterial catheterization and balloon angioplasty [4]. The endothelium is a critical factor protecting vessels from thrombosis and atherosclerosis and an important source of endothelial nitric oxide synthase (eNOS), which is a key regulator of endothelial function [5]. Denudation of the endothelium also exposes pro-thrombogenic mediators located within the subendothelial matrix, which include thrombin, thromboxane, platelet-activating factor and collagen; thrombus formation occurs, and clot formation and a pro-inflammatory signaling cascade ensue. Within a week, vascular smooth muscle cells (SMC) undergo a phenotypic transition into a synthetic phenotype with migratory capacity; subsequent migration of these cells into the intimal space, followed by SMC proliferation, are key events in early neointimal formation [4]. After several months, increased synthesis of proteoglycan-rich extracellular matrix, which essentially replaces SMC in the neointima, contributes to narrowing of the vessel lumen [6].

Oxidative stress is implicated in the pathogenesis of neointimal hyperplasia and restenosis, and delivery of antioxidants to areas of vascular injury represents a promising strategy for the prevention and treatment of neointimal hyperplasia and restenosis. In this study, we investigated the use of local delivery of antioxidants using nanoparticles to prevent the detrimental processes of neointimal hyperplasia and subsequent restenosis. Gold nanoparticles (GNP) were conjugated to ginkgolide A (GA), a terpenic lactone found in the extract of *Ginkgo biloba* leaves. Ginkgo biloba extract is a widely-used herbal dietary supplement globally. Its terpenoid component has been shown to reduce free radical formation in a rat model of cardiac ischemia; among the terpenoids tested, ginkgolide A demonstrated the strongest effect [7]. GA has also been demonstrated to induce vasorelaxation [8], and we have shown in a porcine artery model that GA can reduce endothelial dysfunction [9]. This improvement in endothelial health was mediated by an attenuation of the eNOS downregulation and free radical production induced by homocysteine, a known risk factor for cardiovascular disease [9, 10].

GNP themselves are readily internalized by human cells and are nontoxic; toxicity actually depends on the ligands conjugated to the surface [11]. Their use for the delivery of drugs has

been extensively studied, especially in cancer, and GNP are thought to be advantageous as drug vectors because they can improve solubility, *in vivo* stability, and biodistribution [12]. They have a high surface area to volume ratio, are readily modifiable and can be conjugated to a variety of ligands including small molecules, nucleic acid, lipids and peptides [11]. Drug-conjugated nanoparticles have previously been shown in a rabbit model to reduce in-stent restenosis following stent-induced injury [13]. As such, GNP represent an exciting tool for the delivery of drugs to areas in which neointimal hyperplasia and restenosis may occur.

For this investigation, we implemented a mouse model, using carotid artery ligation to induce prominent SMC proliferation and neointimal formation, which is well described [14]. We measured the effect of treatment with GNP conjugated to ginkgolide A (GA-GNP) on SMC migration and proliferation *in vitro* and investigated the antioxidant effect of GA-GNP treatment *in vivo*. The role of MAPK ERK1/2 was investigated *in vitro* as well. *In vivo*, by administering GA-GNP locally in the surgical field immediately after carotid artery ligation, we were able to compare markers of neointimal hyperplasia after treatment with GNP only (control) versus treatment with GA-GNP. We discovered that in SMC treated with GA-GNP, there was a significant reduction in SMC migration and proliferation and oxidative stress *in vitro*, which appears to be mediated by inhibition by GA-GNP of PDGF-BB induced ERK1/2 activation. In the mouse model, GA-GNP treatment reduced neointimal hyperplasia *in vivo*.

MATERIALS AND METHODS

Ginkgolide A-Gold Nanoparticles (GA-GNP)

Colloidal solution of 80 nm gold nanoparticles (GNP) was purchased from Ted Pella Inc. Ginkgolide A (GA) was incubated with equimolar ratios of GNP in 0.5x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) containing 50 mM NaCl overnight at room temperature.

Cell Cultures

Mouse P53LMAC01 vascular smooth muscle cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA) with 10% FBS at 37 °C with 5% CO₂. After reaching 70% of confluent density, cells were incubated in DMEM with 1% or 5% FBS with different doses of GA-GNP (0-50 µmol/L), Ginkgolide A (50 µmol/L) alone and one maximal volume of GNP, as a negative control. Cell proliferation and migration were analyzed by MTS and Boyden chamber assays, respectively.

Cell Proliferation

Cell proliferation was measured with CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, USA) as described in our previous study [15]. Briefly, the cells (2000 cells/well in 96-well plate) were treated in DMEM with FCS (1% and 5%) with various doses of GA-GNP (0-50 µmol/L), Ginkgolide A (50 µmol/L) alone or GNP only for 24, 48 and 72 h. Then, the cells were incubated with 20 µL MTS reagent in 100 µL of fresh culture medium for 2 h at 37°C in a humidified and 5% CO₂ atmosphere. The absorbance at 490 nm was recorded using an ELISA plate reader (EL800, Bio-Tek Instruments, Inc., Vermont, USA). A growth curve was generated from the absorbance obtained using the MTS assay; this was directly correlated with the number of metabolically active living cells. The percentage of the absorbance was calculated against untreated cells.

Cell Migration Assay

Cell migration was measured using a modified Boyden chamber assay including Fluoroblock transwell migration plates (Becton Dickinson Labware, Franklin Lakes, NJ).

Briefly, cells were seeded in the upper chamber (250 L, 1×10^5 cells/well in 24-well plate). Then, 750 μ L of 5% FBS DMEM medium with different concentrations of GA-GNP was added to the lower chamber. After incubation for 4 to 24 h, the cells were labeled with 50 nmol/L of Calcein-AM, a fluorescent dye. The fluorescence of the cells that had migrated to the lower chamber was measured from the bottom using a fluorescence microplate reader at 485/535 nm wavelength.

DHE Staining Detected by Flow Cytometry

Superoxide anion production was determined by DHE staining. DHE is freely permeable to cells. In the presence of superoxide anion, DHE is oxidized to ethidium bromide (EtBr) with red fluorescence, and it is trapped by intercalating with the DNA. EtBr is excited at 488 nm with an emission spectrum of 610 nm. Thus, the amount of EtBr detected by fluorescence measurement is correlated to the level of cellular superoxide anion. Cultured cells at approximately 80% confluence were treated in basal medium (0.2% FBS in DMEM), 5% FBS medium with or without GA-GNP (50 μ mol/L), Ginkgolide A (50 μ mol/L), or one volume of GNP for 60 min. The treatment was halted by washing cells three times with PBS. One milliliter of DHE (3 mol/L) in PBS was added into each well of six-well plates and incubated for 20 min at room temperature. The staining was then analyzed by FACS Calibure (Becton Dickinson, San Jose, CA).

MAPK Activation Detected by Western Blot

Mouse P53LMAC01 cells were treated with PDGF-BB (10 μ g/mL) in combination with GA-GNP (50 μ mol/L), or Ginkgolide A (50 μ mol/L) and free GNP. The cell lysates were collected with cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) at different time points of 0, 5, 10, 20, 45, and 90 min. Proteins were extracted and equal amount of total proteins (50 μ g) were separated by 10% SDS-polyacrylamide gel electrophoresis and then trans-blotted onto Hybond-P PVDF membrane. The membrane was incubated with the primary antibody at 4°C overnight at a dilution of 1:1000 for phosphor- and total ERK1/2. The membranes were then incubated in the horseradish peroxidase-linked secondary antibody for 50 min. Bands were visualized with ECL Plus chemiluminescent substrate (Amersham Biosciences).

Animal Model

Twelve-week-old C57BL/6J mice were obtained from Charles River Laboratories. All mice were cared for in accordance with an animal protocol approved by Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC). Animals were anesthetized by subcutaneous injections of Examine/xylazine, and animals were subjected to an exposure of left carotid arteries. The left common carotid artery was disrupted by ligating the vessel near the distal bifurcation, and prior to closure, either 20 μ L of GNP or GA-GNP (50 μ mol/L) were administered directly into the surgical bed. Animals were kept alive for 4 w after the following treatments: 1.) ligation and local GNP (20 μ L) administration (n=6); and 2.) ligation and local GA-GNP (50 μ mol/L) (1:1 volume dilution) (20 μ L) (n = 6). At the end of each experiment, mice were euthanized by an overdose of anesthetic, followed by perfusion via left ventricle with 4% paramaldehyde. The carotid arteries were taken for immunohistochemical studies.

Immunohistochemistry

Treated carotid arteries were fixed in 10% formalin and embedded in paraffin. Samples were sectioned at a 5 μ m thickness and slides were incubated in 0.3% hydrogen peroxide solution to quench endogenous peroxidase activity for 15 min. The monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:200) was applied for 30 min at room

temperature. After washing with PBS, the sections were incubated with biotinylated secondary antibody for 30 min. An avidin-biotin reaction using peroxidase enzyme was used for protein detection (ABC kit; Vector Laboratories, Burlingame, CA). Immune complexes were detected with diaminobenzidine under the microscope before counterstaining with hematoxylin for 2 min. Images were captured under microscope with an attached SPOT-RT digital camera and software (Diagnostic Instruments, Sterling Heights, Mich).

Statistical Analysis

Results were analyzed by ANOVA. P values < 0.05 were considered statistically significant. All data are expressed as mean $\pm$ SEM.

RESULTS

Ginkgolide A-GNP Inhibit Mouse SMC Proliferation *in Vitro*

Since SMC proliferation and migration are critical processes in intimal hyperplasia, we sought to determine whether Ginkgolide A could affect SMC proliferation and migration *in vitro*. First, we performed a non-radioactive colorimetric assay (MTS) to evaluate the effect of Ginkgolide A on mouse cultured SMC proliferation. Mouse P53LMAC01 cells were 80% confluent before they were treated in DMEM with 5% FBS with increasing doses (0-50 $\mu\text{mol/L}$) of GA-GNP, Ginkgolide A (50 $\mu\text{mol/L}$) alone or GNP (50 $\mu\text{mol/L}$) alone. We found that GA-GNP treatment inhibited SMC proliferation by 25% when compared to controls (Fig. 1A, $P < 0.01$, $n = 6$). A time-course study confirmed the inhibitory effect of GA-GNP on SMC proliferation (Fig. 1B), from 75% to 68% of control values after 24 to 72 h, respectively, $P < 0.01$, $n = 6$).

Ginkgolide A-GNP Inhibit Mouse SMC Migration

We likewise investigated whether Ginkgolide A affects mouse SMC migration. Cells were treated with increasing concentrations of GA-GNP (0-50 $\mu\text{mol/L}$), Ginkgolide A (50 $\mu\text{mol/L}$), GNP (50 $\mu\text{mol/L}$) alone or no treatment control in the lower chambers of a modified Boyden chamber assay. The cells that migrated through a polystyrene-membrane with 8 μm -size pores were stained with the fluorescent calcein-AM dye and measured with a fluorescence reader. As shown in Fig. 2A, GA-GNP significantly inhibited mouse SMC migration in a dose-dependent manner, with the maximum effect at 50 $\mu\text{mol/L}$; migration was reduced by 35% when compared with the non-treated control cells ($P < 0.01$, $n = 6$). To confirm these findings, we performed a time course study at 4, 8, 16 and 24 h. We observed a detectable effect of GA-GNP on cell migration as early as 4 h after cell seeding, with the most significant effect seen after 24 h (Fig. 2B). Thus, GA-GNP inhibits mouse SMC migration in a dose-dependent and time-dependent manner.

Ginkgolide A-GNP Inhibit Superoxide Anion Production in Mouse SMC

Ginkgolide A has been demonstrated to have an antioxidant effect [7, 9, 10]. We examined the effects of Ginkgolide A on superoxide anion generation under our experimental conditions in order to explore the involvement of superoxide anion levels in Ginkgolide A-inhibited mouse SMC proliferation and migration. Superoxide anion levels were determined by fluorescence staining with DHE, a compound chemically oxidized to ethidium in proportion to the amount of superoxide present; ethidium is a DNA intercalating agent detected by fluorescence [16]. Flow cytometry analysis was used to determine fluorescence. We first determined superoxide anion levels in mouse SMC after treatment with GA (50 $\mu\text{mol/L}$), GA-GNP (50 $\mu\text{mol/L}$) or GNP (50 $\mu\text{mol/L}$) alone in 5% FBS when compared to cells in the basal medium (0.2% FBS in DMEM). A representative histogram is seen in Fig. 3A. As shown in Fig. 3B, the superoxide anion level increased from 35% in basal medium

up to 55% in 5% FBS. Ginkgolide A-GNP (50 $\mu\text{mol/L}$) significantly blocked the superoxide anion increase in 5% FBS medium from 55% to 39% ($P < 0.01$, $n = 3$) after 60 min treatment. This was significantly greater than the effect of GA alone in the SMC ($P < 0.05$, $n = 3$). These data indicate that Ginkgolide A-GNP can block the superoxide anion increase seen when SMC are treated in 5% FBS; this antioxidant effect may be involved in the mechanisms whereby Ginkgolide A-GNP inhibits cell proliferation and migration in SMC *in vitro*.

Ginkgolide A-GNP Inhibit PDGF-Induced ERK1/2 Activation

We investigated the possible involvement of MAPK ERK1/2 activation with respect to the signal pathways involved in the negative effect of Ginkgolide A-GNP on mouse SMC proliferation and migration. To address this issue, mouse SMC were incubated with GA-GNP (50 $\mu\text{mol/L}$), ginkgolide A (50 $\mu\text{mol/L}$) alone or GNP alone in basal medium supplemented with PDGF-BB (10 ng/mL). Cell lysates were collected at different time points. Western blotting was used to detect phospho- and total protein of ERK1/2. As shown in Fig. 4A and Fig. 4B, PDGF-BB substantially induced phosphorylation of ERK1/2 from 5 min, which reached its peak at 45 min in GNP control cells; meanwhile, PDGF-BB-induced ERK1/2 phosphorylation was significantly inhibited in GA-GNP-treated cells.

Ginkgolide A-GNP Inhibit Neointimal Hyperplasia in a Mouse Model

Considerable neointimal hyperplasia was observed in the left carotid arteries of GNP-treated mice 4 w after ligation when compared to normal mouse left carotid arteries; meanwhile, mice that underwent GA-GNP treatment showed reduced neointimal hyperplasia (Fig. 5A). The neointimal area was decreased from $30561 \pm 6368 \mu\text{m}^2$ in the GNP-treated group to $16117 \pm 2589 \mu\text{m}^2$ in the group treated with GA-GNP (Fig. 5B, $P < 0.05$, $n = 6$). Accordingly, the lumen area was increased from $10081 \pm 3016 \mu\text{m}^2$ in the GNP-treated group to $30319 \pm 2269 \mu\text{m}^2$ in the group treated with GA-GNP (Fig. 5B, $P < 0.05$, $n = 6$).

Ginkgolide A-GNP Inhibit Neointimal Cell Proliferation in a Mouse Model

The expression of PCNA is altered during the development of neointima. To examine the expression of PCNA in response to GA-GNP treatment, we performed immunohistochemistry for PCNA on sections of arteries from the different treatment groups at 4 w after ligation. Treatment with GA-GNP was associated with markedly decreased expression of PCNA in neointimal lesions when compared to GNP treatment alone (Fig. 6).

DISCUSSION

These data demonstrate that GA-GNP treatment reduces SMC proliferation and migration and inhibits PDGF-BB-induced ERK1/2 activation *in vitro*. *In vivo*, GA-GNP treatment reduced neointimal hyperplasia in a carotid artery ligation model, as evidenced by an increase in luminal area, reduction in neointimal area and reduction in PCNA, a marker of SMC hyperplasia. These results suggest that therapeutic administration of GA-GNP or other GNP conjugates may reduce SMC migration and proliferation by interfering with oxidative stress-induced signaling, thereby reducing the neointimal hyperplasia that characterized restenosis.

SMC proliferation and migration represent key events leading to neointimal formation after vascular injury. Neointimal formation occurs as a response to injury by vascular tissue and is mediated by thrombotic and inflammatory mediators, growth factors, cytokines and, importantly, oxidative stress. While signaling by reactive oxygen species (ROS) constitutes an important normal intracellular process in healthy tissue, considerable evidence indicates that elevated levels of ROS and redox imbalance following injury contribute to restenosis

[17, 18]. Our investigation revealed that GA-GNP ameliorate oxidative stress in SMC, as administration of GA-GNP reduced superoxide anion levels in mouse SMC after stimulation with 5% FBS. In addition to the mechanical aspect of endothelial loss, the reduction in eNOS is undoubtedly an important factor in neointimal formation and restenosis, as nitric oxide is known to modulate SMC proliferation and migration [19, 20]. A variety of NO-based therapies, including inhalational NO, systemic NO donors, NOS gene therapy, NO local therapy, and NO-releasing prosthetics have been implemented to prevent neointimal hyperplasia following injury, with varying levels of success [21].

Platelet-derived growth factor (PDGF) is an important chemotactic factor for SMC migration and stimulates SMC proliferation [18]. PDGF is released by platelets in areas of vascular injury, where it facilitates neointimal formation; along with most vascular growth factors, PDGF induces SMC migration through a pathway that is ROS-dependent [16, 22]. We investigated the effect of GA-GNP on SMC after stimulation with PDGF-BB and determined that activation of extracellular signal-related kinase (ERK1/2) activation was inhibited by GA-GNP. The effects of PDGF-BB on SMC proliferation and migration are mediated in part by MAPK signaling cascades, leading to ERK1/2 phosphorylation and activation [23]. The current study demonstrates that administration of an antioxidant conjugated to GNP can reduce ERK-1/2 phosphorylation, suggesting redox sensitivity in this downstream pathway as well.

It is likely that the effects observed *in vivo* in the mouse model were mediated by the inhibitory effects of GA-GNP on SMC proliferation and migration, which is likely an effect of reduced ERK-1/2 activation in an environment of reduced oxidative stress. After treatment with GA-GNP, neointimal hyperplasia was reduced in the carotid artery ligation model; this was determined by an assessment of luminal area as well as by immunohistological analysis of PCNA staining. GA has been noted to be antiproliferative in other contexts as well; for example, some ovarian cancer cell lines demonstrate reduced cell proliferation after treatment with ginkgolide A, which appeared to be due in part to cell cycle blockage at G0/G1 [24]. In this experiment, phosphorylation and thus activation of the MAPK family members ERK-1/2, which are important downstream mediators for a variety of stimuli for neointimal hyperplasia [25], were affected by GA-GNP administration, and this is a likely mechanism for the action of GA-GNP.

Ample evidence exists that antioxidant delivery following injury may abrogate the detrimental effects of vascular remodeling that lead to neointimal hyperplasia and restenosis [26-29]. For example, treatment with the naturally occurring antioxidant α -lipoic acid has been found to inhibit neointimal hyperplasia by inhibiting apoptosis in a balloon-injured rat carotid artery animal model [26]. Antioxidants such as flavenoids have likewise been shown to block neointimal formation in balloon-injured rat arteries, as evidenced by a reduction in intima (I) to media (M) thickness (I/M) of the carotid arteries [28]. Administration of antioxidants such as alpha-tocopherol to rabbits fed a high-cholesterol diet who had polytetrafluoroethylene (PTFE) grafts implanted in the abdominal aorta also demonstrated reduced neointimal proliferation in addition to improved endothelialization when compared to rabbits fed a high-cholesterol diet only [27]. Similar results were seen in a balloon-injury model of rabbit carotid arteries, using the antioxidant 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) [29].

The current study further supports the role of antioxidant delivery in the prevention of neointimal hyperplasia after arterial intervention. We selected a model of blood-flow cessation using carotid artery ligation for our study of the effects of GA-GNP administration on neointimal hyperplasia. While it is not as physiologically relevant as mechanical injury models or vein grafts, it represents a simple procedure that reliably produces neointimal

hyperplasia despite the absence of direct endothelial injury. The carotid ligation procedure is easily reproducible, and variability between animals in this model is reduced. The robustness of the neointimal hyperplasia produced in the mouse carotid artery ligation model makes it very useful for studying neointimal formation at a molecular level [30]. And, in our model, significant neointimal hyperplasia was observed in the mouse carotid arteries that underwent ligation alone, when compared to normal carotid arteries (Figure 5). For future investigations of GA-GNP, more clinically relevant animal models should be considered.

GNP have previously been described to be excellent candidates for drug delivery due to their simple and fast preparation [31], which was indeed true for this study. As previously mentioned, the toxicity of GNP for the purpose of drug delivery depends on the conjugated ligand [11]. Also, because of their unique optical properties, the use of GNP for cancer diagnosis and detection is currently being intensely investigated [31]. In the current study, we actually found that GA-GNP administration had a greater inhibitory effect on SMC proliferation and migration and a greater reduction in superoxide anion generation than GA treatment alone *in vitro*; this may reflect the fact that conjugation to nanoparticles might allow more controlled release of the GA drug. Ginkgolide A, meanwhile, is a component of the naturally occurring *ginkgo biloba* plant, which has been used as a dietary supplement for centuries by countless people globally. We have previously demonstrated its usefulness in improving endothelial function, increasing vasoprotective eNOS expression and reducing oxidative stress in human endothelial cells [9]. In the current study, the effects of GA alone and GA-GNP on SMC were also found to be vasoprotective, limiting dysfunctional responses of SMC *in vitro*.

We examined the effect of GA-GNP on neointimal hyperplasia and PCNA expression *in vivo* in the mouse model. We showed that there was a significant reduction in neointimal formation and PCNA expression with GA-GNP in comparison to the critical control of GNP alone, proving that the concept of GNP-mediated delivery of antioxidants to areas prone to neointimal formation is feasible. The reduction in PCNA staining in the GA-GNP treated vessels in comparison to GNP only is easily observable; however, the lack of quantitative evidence of PCNA expression changes in this study represent a limitation to its interpretation. Analysis of our *in vivo* results is also limited by our lack of knowledge regarding the pharmacokinetics of GA-GNP in this model, including GA release and half-life, duration of action, and volume of distribution, all of which represent important areas for future investigation. In particular, the uptake of GA-GNP at a cellular level was not determined in this model, which would likely be investigated during the study of the kinetics of GA-GNP action in a future investigation. Finally, a comparison with GA administration alone represents an important objective for future study, both in a model of local administration similar to that of GA-GNP in this study or oral GA administration, which is especially appropriate considering the widespread use of ginkgo extract as a nutritional supplement.

In summary, GA-GNP represent a promising tool for the prevention of restenosis, by reducing SMC proliferation and migration through a reduction in oxidative stress and an attenuation of the ERK-1/2 signaling pathways, thereby reducing neointimal hyperplasia. Further investigation of the use of antioxidant-conjugated GNP for the prevention and/or treatment of restenosis due to neointimal hyperplasia will likely focus on the kinetics of drug release and specific targeting strategies, as well as a comparison with antioxidant administration alone. The efficacy of GA for the reduction of oxidative stress and SMC migration and proliferation in response to injury may be further enhanced by chemical modification of the GA compound. The use of GNP for delivery of antioxidant, antiproliferative, vasoprotective compounds represents an exciting strategy in the prevention

of restenosis and a significant advantage over currently existing strategies such as drug-eluting stents and antiplatelet therapy.

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FIG. 1.

Effect of GA-GNP on mouse SMC proliferation. **A.** Dose-dependent study by MTS assay. Mouse SMC at about 80% confluence were treated with different doses of GA-GNP (0-50 μmol/L) GA (50 μmol/L) or GNP in 5% FBS DMEM for 24 h. Then, the cell growth was determined by MTS assay as described. **B.** Time course study of mouse SMC proliferation. Mouse SMC were treated with GA-GNP (50 μmol/L), GA (50 μmol/L) or GNP. The cell proliferation activity was assessed by MTS assay after 24, 48 and 72 h. Results are expressed as percentage of the medium-alone control (100%). Each bar represents the mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ as compared to the control. $n = 6$ per group.

FIG. 2.

Effect of Ginkgolide A-GNP on mouse SMC migration. **A.** Dose-dependent study. Mouse SMC were seeded in the upper chambers of Fluoroblok transwell plate with different doses of GA-GNP (0-50 $\mu\text{mol/L}$) in the lower chambers, GA (50 $\mu\text{mol/L}$) or GNP in 5% FBS medium. After 24 h incubation, mouse SMC cells that had migrated to the lower chamber were measured by fluorescence staining and quantitation. Results are expressed as units of fluorescence. Each bar represents the mean $\pm$ SEM. $*P < 0.05$ and $**P < 0.01$ vs negative control, $n = 6$ per group. **B.** Time course study. Mouse SMC were treated with or without GA-GNP (50 $\mu\text{mol/L}$), GA (50 $\mu\text{mol/L}$) or GNP in 5% FBS, and mouse SMC migration was studied at 4, 8, 16 and 24 h. Results are expressed as percentage of the fluorescence vs 5% FBS control group. $*P < 0.05$ and $**P < 0.01$ vs 5% FBS control group, $n = 6$ per group.

FIG. 3.

Effect of Ginkgolide A-GNP on serum-induced superoxide anion production. Cells at about 80% confluence were treated in basal medium (0.2% FBS in DMEM) or 5% FBS medium with or without GA-GNP (50 $\mu\text{mol/L}$), Ginkgolide A (50 $\mu\text{mol/L}$) or GNP for 60 min. The treatment was halted by washing cells with PBS, and the cells were stained with DHE and analyzed by flow cytometry. **A.** Representative histograms of DHE staining and flow cytometry analysis. Results are expressed as percentage of positive cell staining as compared to non stained control. **B.** Quantitation of DHE staining and flow cytometry analysis. Results are expressed as percentage of positive cell staining as compared to non stained control. Each bar represents the mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$ as compared to the control. $n = 3$ per group.

FIG. 4.

Effect of Ginkgolide A-GNP on ERK1/2 phosphorylation. Mouse P53LMAC01 cells were treated with or without GA-GNP (50 µmol/L), GA (50 µmol/L) or GNP in PDGF-BB (10 µg/mL). The cell lysates were collected with cell lysis buffer after 0, 5, 10, 20, 45 and 90 min. About 50 µg of protein were used for wetern blotting with the primary antibody for phosphor- and total ERK1/2.

FIG. 5.

Effect of Ginkgolide A-GNP on neointimal hyperplasia in the mouse model. Twelve-week old mice underwent ligation of the left common carotid artery and were treated as follows: local GNP administration or local GA-GNP administration. After 4 w, the mice were sacrificed and histological analysis was performed as shown. Treatment with GA-GNP significantly reduced neointimal hyperplasia after carotid artery ligation when compared with GNP treatment only (** $P < 0.01$, $n = 6$).

FIG. 6. Effect of Ginkgolide A-GNP on neointimal cell proliferation in the mouse model. Immunohistochemical staining for PCNA was performed on the left carotid artery of mice 4 w after treatment as described.