Constitutively Active Glycogen Synthase Kinase-3β Gene Transfer Sustains Apoptosis, Inhibits Proliferation of Vascular Smooth Muscle Cells, and Reduces Neointima Formation After Balloon Injury in Rats

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Objective—Glycogen synthase kinase (GSK)-3β is a crucial factor in many cellular signaling pathways and may play an important role in smooth muscle proliferation and apoptosis after angioplasty.

Methods and Results—To investigate the effect of GSK-3β modulation on neointima formation, smooth muscle proliferation, and apoptosis after balloon injury in vivo, we delivered adenoviral vectors expressing the constitutively active form of GSK-3β (GSK-S9A: 9th serine switched to alanine) or a control gene into rat carotid arterial segments after balloon injury with a 2F Fogarty catheter. Viral infusion mixtures (5×10⁸ pfu) were incubated in the arterial lumen for 20 minutes, and the effects of gene delivery were evaluated 3 days and 2 weeks after gene delivery with morphometry and immunohistochemical staining for proliferating and apoptotic cells. There were no significant differences in intimal, medial, and lumen areas at 3 days after the procedure. However, 2 weeks after gene delivery, the active GSK-3β gene transfer resulted in a significantly lower intima to media ratio (0.29±0.06 versus 0.86±0.09, P<0.01) and a greater lumen area (0.41±0.02 versus 0.31±0.01 mm², P<0.01) compared with the control gene transfected group. This was attributable to a significant reduction in intimal area (0.05±0.01 versus 0.15±0.02 mm², P<0.01), whereas the medial area was similar (0.17±0.01 versus 0.18±0.01 mm², P=0.21). Proliferation index was significantly reduced both at 3 days and 2 weeks in the active GSK-3β gene transferred group (2.97±0.29% versus 5.71±0.50%, P<0.01). In addition, apoptotic index, which was not significantly different between the 2 groups at 3 days, was significantly higher in the active GSK-3β gene transferred group at 2 weeks (3.14±0.68% versus 22.7±1.63%, n=10, P<0.01, for control versus active GSK-3β gene transfer).

Conclusions—In vivo delivery of the active GSK-3β gene inhibits smooth muscle proliferation, sustains apoptosis, and reduces neointima formation after balloon injury in rats and may be a future therapeutic target to limit neointima hyperplasia after angioplasty. (Arterioscler Thromb Vasc Biol. 2003;23:2330-2335.)

Key Words: glycogen synthase kinase-3β  vascular smooth muscle  neointima

Glycogen synthase kinase-3β (GSK-3β) is a protein serine/threonine kinase that was first discovered more than 20 years ago as an enzyme involved in glucose metabolism. It phosphorylates and thereby inactivates glycogen synthase, a key enzyme in the synthesis of glycogen. Since then, research has provided insight into the pleiotropic role that GSK-3β plays in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility, and cell survival. It is a crucial factor in many cellular signaling pathways and regulates several transcription factors. GSK-3β phosphorylates multiple substrates, including tau, myelin basic protein, cyclin D1, β-catenin, GATA4, c-jun, c-myc, CREB, initiation factor elf2B, heat shock factor-1, and p53. GSK-3β is also at the nodal point of various signaling pathways, such as the phosphoinositide 3 kinase pathway, the protein kinase B pathway, the mitogen-activated protein kinase cascade, and the Wnt pathway.

GSK has been suggested to play a role in several disease states, including Alzheimer disease, mood disorders, diabetes mellitus, and tumorigenesis. In the field of cardiology, GSK-3β has recently been identified as a negative regulator of cardiac hypertrophy and an important regulator of cardiac development. In addition, Hall et al showed that during neointimal formation, vascular smooth...
muscle cell apoptosis is inhibited by the upregulation of glucose metabolism and is linked to the inactivation of GSK3β. Moreover, we recently reported in vitro findings that constitutive active GSK-3β gene transfer results in a significantly reduced proliferation and migration in human aortic smooth muscle cells.34

Because GSK3β is located at the nodal point where multiple cell signals merge to control the proliferation and migration of vascular smooth muscle cells, it may represent a pharmacological target to treat restenosis after balloon injury. However, the effects of modulating GSK-3β in vivo gene transfer into rat carotid artery segments injured by balloon injury. The purpose of the study was to examine the in vivo effects of GSK-3β on vascular smooth muscle proliferation and survival in the process of neointimal formation. This study is the first to demonstrate that GSK-3β activation attenuates vascular lesion growth after acute injury.

Methods

Rat Carotid Artery Balloon Denudation Injury and Adenoviral Vector–Mediated Gene Delivery

A previously well-established rat carotid artery balloon injury model was used.35 The specific experimental protocol for the study was designed in accordance with the Guide for Experimental Animal Research from the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital. An adenoviral vector expressing the nonphosphorylatable, constitutively active mutant of GSK3β (GSK3β-S9A) where the serine residue at position 9 was mutated to alanine34 and another vector expressing β-galactosidase were used for gene delivery.

Forty adult male Sprague-Dawley rats weighing ~400 to 500 g (Daehan Biolink Co) were anesthetized with a combination anesthetic (ketamine, 70 mg/kg; xylazine, 7 mg/kg; IP; Yuhan Corp, Bayer Korea). After the left external carotid artery was exposed and heparin (35 IU) was administered systemically via the external jugular vein, a 2F Fogarty embolectomy catheter (Baxter Healthcare Corp) was introduced into an external carotid arteriotomy incision, advanced to the common carotid artery, and inflated with 0.2 mL of saline and withdrawn 3 times with rotation. The catheter was then removed, and a dwelling catheter was introduced into the arteriotomy site. After both the proximal common carotid artery and the proximal internal carotid artery were clamped, viral infusion mixtures with 5 × 105 pfu of virus containing either constitutively active GSK-3β gene (Adeno-GSK-S9A, 9th serine switched to alanine) or control gene (Adeno-β-Gal) diluted to a total volume of 100 μL were instilled via the arterial segment between the 2 clamps, and the external carotid artery was then ligated. Perfusion was restored in the common carotid artery after 20 minutes of instillation, and the neck incision was closed using 3-0 silk sutures.

Morphometric Analysis

Three days and 2 weeks after balloon injury, rats were euthanized with a lethal dose of pentobarbital, and the carotid arteries were fixed by perfusion at 120 mm Hg with 4% formaldehyde via an 18G intravenous cannula placed retrograde in the abdominal aorta. The arteries were additionally fixed by immersion in the same fixative used for perfusion. The tissues were then embedded in paraffin, and sections were stained with H&E or elastica van Gieson. The extent of neointimal formation was quantified by computed planimetry of histologically stained sections. The cross-sectional areas of the blood vessel layers including the lumen area, intimal area, and medial area were quantified at 3 different sections (proximal, middle, and distal) by using Image Pro Plus Analyzer Version 4.5 (Media Cybernetics). The intima to media (I/M) ratios were calculated from the mean of these determinations.

Immunohistochemical Staining

To identify the expression of the GSK-S9A gene and to detect proliferating cells, immunohistochemical staining against hemagglutinin antigen (the adeno-GSK-S9A included a hemagglutinin tag epitope) and proliferating cell nuclear antigen (PCNA) were performed on the virus-treated arteries. Briefly, the paraffin-embedded samples were sectioned and treated with protease K for 4 minutes. Endogenous peroxidase was quenched with methanol/peroxidase solution. The specimens were treated with 50 mmol/L Tris HCl (pH 7.6) containing 0.15 mol/L NaCl and 0.1% Tween 20 for 5 minutes, followed by incubation with either 1:100 diluted rabbit anti-hemagglutinin antibody (Santa Cruz) for hemagglutinin staining or 1:50 diluted anti-PC-10 antibody (DAKO) for PCNA staining. The specimens were then processed by incubation with 1:50 diluted 3,3-diaminobenzidine tetrahydrochloride substrate solution (DAKO) and counterstained with Mayer hematoxylin (DAKO). Proliferation was assessed by quantitating the percentage of PCNA-positive cells against total nucleated cells in 4 different sectors per tissue section. Apoptotic cells were confirmed by treating specimens with 1:200 diluted cleaved caspase-3 antibody (Cell Signaling Technology) in a similar manner.

TUNEL Staining

Detection of apoptotic cells in vivo was also performed using the TUNEL36 method with minor modifications. Briefly, 5-μm sections were deparaffinized and incubated with protease K (DAKO) 20 μg/mL for 15 minutes at room temperature. An apoptosis detection kit (Apoptag, Intergen Company) was used with the chromogen DAB. Counterstaining was done with Mayer hematoxylin (DAKO). Apoptotic cells were quantified by counting the percentage of TUNEL-positive cells against total nucleated cells in 4 different sectors per tissue section.

Western Blot Analysis

Tissue segments were harvested before and after balloon injury and 3 days after gene transfer for immunoblotting. Vessel segments were homogenized in lysis buffer, and protein concentrations were determined with protein assay kit (Pierce). Protein (25 μg) was separated by SDS-polyacrylamide electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with T-PBS (1× PBS, 0.3% Tween-20) containing 3% dry milk and incubated with primary antibody overnight at 4°C. After 3 washes with T-PBS, the membrane was reblocked and incubated with secondary antibody for 1 hour at room temperature. ECL or ECL-PLUS (Amersham) was used for detection. To reprobe the membrane, it was treated with Restore Western Blot stripping buffer (Pierce). The primary antibodies used were anti-phospho-GSK-3β(Ser9) antibody (1:750 dilution, Cell Signaling Technology) and anti-α-tubulin antibody (1:4000 dilution, Oncogene). The secondary antibodies were anti-rabbit IgG/HRP conjugate (1:2500 dilution, Promega).

Statistical Analysis

Results are reported as mean ± SEM. Mean values were compared between the active GSK-3β gene transferred group and the control gene transferred group by using the Student’s t test, and P<0.05 was considered statistically significant.

Results

Phospho-GSK Expression After Balloon Injury and Detection of Gene Expression In Vivo

Immunoblot analysis performed before and after balloon injury showed a marked increase in the expression of the inactivated phosphorylated form of GSK-3β (Figure 1A;
please see http://atvb.ahajournals.org for full-color versions of all figures). Immunoblot analysis performed on vessel segments harvested 3 days after gene transfer showed a significant reduction in the expression of the inactivated phosphorylated form of GSK-3β in vessels transduced with GSK-S9A compared with vessels transduced with control (Figure 1B). Sustained gene expression at 2 weeks after delivery was shown by immunohistochemistry using anti-hemagglutinin antibody to detect the transfer of the hemagglutinin-tagged active GSK-3β (Figure 1C). This procedure demonstrated hemagglutinin-positive cells in the luminal side of the vessel intima in the GSK-S9A group. There were no hemagglutinin-positive cells in the control gene transfected group.

**In Vivo Inhibition of Neointimal Formation**

Morphometric analysis 3 days after gene delivery showed no significant differences in lumen area or media area between the GSK-S9A group and the control group (data not shown). Little or no intima could be detected in either group. However, 2 weeks after gene delivery, the active form GSK-3β gene transfer resulted in a significantly reduced neointimal area (0.05±0.01 versus 0.15±0.02 mm², P<0.01), whereas the medial area was similar (0.17±0.01 versus 0.18±0.01 mm², P=0.21) compared with the control gene transfected group (Figure 2). This led to a significantly lower I/M ratio (0.29±0.06 versus 0.86±0.09, P<0.01) and a greater lumen area (0.41±0.02 versus 0.31±0.01 mm², P<0.01) compared with the control gene transfected group.

**Inhibition of Vascular Smooth Muscle Proliferation**

Immunohistochemical staining against PCNA was performed to examine the effects of active GSK3 gene transfer on VSMC proliferation in vivo. Staining against PCNA showed that cell proliferation was markedly reduced in the active GSK-3β–transduced group (Figure 3). Smooth muscle proliferative index (calculated as the percentage of positive cells among total nucleated cells) was significantly lower in the active GSK-3 gene transferred group both 3 days (34.1±1.49% versus 17±1.05%, n=10, P<0.01 for control versus active GSK-3β gene transfer) and 2 weeks (5.71±0.50% versus 2.97±0.29%, n=10, P<0.01 for control versus active GSK-3β gene transfer) after gene delivery (Figure 3).

**Sustained Apoptosis After Active GSK-3β Gene Transfer**

TUNEL staining was performed to examine the effects of active GSK3 gene transfer on apoptosis after balloon injury in vivo. At 3 days after balloon injury and gene delivery, both

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**Figure 1.** Phospho-GSK expression after balloon injury and detection of gene expression in vivo. A, Western blot of tissue samples before and after balloon injury showing a marked increase in the expression of phospho-GSK-3β after injury. B, Immunoblot of samples 3 days after gene delivery showing reduced expression of phospho-GSK-3β. C, Immunohistochemistry of tissue samples at 2 weeks showing expression of the GSK-S9A gene predominantly in the neointima. Immunohistochemistry was performed using primary antibody against hemagglutinin, which was an epitope tag on the transgene-encoded protein.

**Figure 2.** Reduction of neointima formation at 2 weeks. Morphometric data, intima to media (I/M) ratio (A), and H&E-stained sections of adeno-β-galactosidase and adeno-GSK-S9A transfected arterial segments (inset, high power field) (B). *P<0.01, n=10 in each group.
the control gene and active GSK-3β gene delivered arterial segments showed high activities of apoptosis, which were not significantly different (17.5 ± 1.80% versus 22.3 ± 3.91%, n = 10, \( P = 0.28 \), for control versus active GSK-3β gene transfer). However, at 2 weeks, although the apoptotic index (calculated as the percentage of TUNEL-positive cells among total nucleated cells) dropped substantially in the control group, it was sustained to a significantly higher level in the active GSK-3β gene transferred group (3.14 ± 0.68% versus 22.7 ± 1.63%, n = 10, \( P < 0.01 \), for control versus active GSK-3β gene transfer) (Figure 4).

Discussion

This study demonstrates for the first time in vivo that active GSK gene transfer results in a significant reduction of neointima formation after balloon injury in rat carotid arteries. These effects are attributable, at least in part, to the ability of GSK to inhibit smooth muscle proliferation and to promote sustained apoptosis. Our study extends recent in vitro findings that GSK plays an important role in vascular smooth muscle cell proliferation and apoptosis in the process of vascular remodeling after balloon injury.

Local gene delivery after balloon injury to rat carotid arteries is a well-established model to investigate the mechanism of restenosis and to test the therapeutic implications of various genes. In this study, we delivered a constitutively active form of GSK-3β that has been shown in vitro studies to function at the nodal point of various cell-signaling pathways. Investigations into the role of GSK-3β in cellular biology in the past few years have shown that GSK is an important modulator of cell survival and apoptosis. Several prosurvival and proproliferative pathways such as PI3K/Akt and mitogen-activated protein kinase pathways are reported to phosphorylate and thus downregulate GSK-3β activity. In addition, GSK-3β controls several downstream signals and transcription factors crucial in cell survival, including heat shock factor-1, CREB, AP-1, Myc, NFAT, CEBPα, cyclin D1, and β-catenin.

Vascular smooth muscle cell proliferation and migration are important contributors to neointima formation after balloon injury. Therefore, prior efforts to reduce the extent of restenosis have been focused on various modalities to decrease such proliferation and migration or increase apoptosis of vascular smooth muscle cells. In a recent study by Hall et al., it was shown that glucose transport and metabolism are upregulated during neointima formation and this process is mediated by the phosphorylation and inactivation of GSK-3β. They also suggested that GSK3β provides a novel mechanistic link between upregulated glucose metabolism and a prosurvival (antiapoptotic) cellular signaling pathway. Moreover, recent work from our laboratory has demonstrated that GSK-3β plays a significant role in human aortic smooth muscle cell proliferation and migration.

Active GSK-3β gene transfected human aortic smooth muscle cells showed significantly reduced proliferative activity to serum stimulation, measured by a cell proliferation assay kit used to detect viable cells by detecting the cleavage of tetrazolium salts added to the growth medium (WST-1 assay) and decreased directional and random migration measured by modified Boyden assay and agarose-cell drop assay, respectively.

In the present study, active GSK-3β gene transfer significantly reduced expression of phospho-GSK-3β (the inactivated form of GSK-3β) and reduced neointima formation by 70%. Active GSK-3 gene transfection significantly inhibited smooth muscle proliferation both at 3 days and at 2 weeks. In addition, because GSK-3β has been reported to modulate cell survival and apoptosis, we also postulated that active GSK-3β gene transfer would affect apoptosis after balloon injury. We found that apoptosis of smooth muscle cells was not significantly different between the active GSK-3β transfected group compared with the control gene transfected group at 3 days after balloon injury. However, at 2 weeks, the apoptotic index was significantly higher in the active
GSK-3β gene transfected group compared with the control gene transfected group. Previous reports from our laboratory and others on apoptosis after balloon-injury have shown that there is an initial high apoptotic activity after balloon injury that drops to lower levels at 2 weeks.50–52 As seen in our data, both the active GSK gene transferred group and the control gene transferred group showed high levels of apoptosis at 3 days, with approximately 20% of all nucleated cells being apoptotic. Considering that active GSK-3 expression may be proapoptotic, as reported in previous studies, the effects of active GSK-3β expression in the early process after balloon injury when vascular cells are exposed to a very proapoptotic milieu and the mechanical factor of balloon-injury is the key inducer of apoptosis may not be significant enough to show a difference in the level of apoptosis between active GSK-3β and control gene transferred group. At 2 weeks after balloon injury, such proapoptotic early signals disappear and the effect of active GSK-3β gene transfer on apoptosis may explain the sustained levels of apoptosis in the active GSK-3 transferred group. We believe that this sustained apoptosis along with the inhibition of smooth muscle cell proliferation resulted in a significant decrease in intimal area and I/M ratio, leading to a greater lumen area at 14 days.

The exact role of GSK-3β in the proliferation and fate of vascular smooth muscle cells is poorly understood at present. In a recent publication by Wang et al,10 it was shown that β-catenin, a classic downstream molecule of the Wnt pathway whose activity is downregulated by active GSK-3β, is a key factor in the signaling cascade in vascular remodeling. They showed β-catenin significantly inhibited apoptosis, induced activation of T-cell factor, and promoted cell-cycle progression by activating cyclin D1. Additional studies to examine the underlying pathway by which GSK-3β inhibits neointimal formation and the roles of Akt, β-catenin, and cyclin D1 in vascular smooth muscle biology are ongoing in our laboratory. In conclusion, our data show for the first time that active-form GSK-3 gene transfer inhibits neointima formation after balloon injury in rats through inhibition of proliferation and sustained apoptosis of smooth muscle cell and, thus, may be a future target for therapy to inhibit neointima hyperplasia after angioplasty.

Figure 4. Effects of in vivo active GSK-3β gene transfer on apoptosis. Apoptotic index at 3 days and 2 weeks after balloon injury and gene delivery (A), TUNEL staining at 3 days and 2 weeks (B, first and second rows), and immunohistochemistry using cleaved caspase-3 at 2 weeks (B, last row). *P<0.01, n=10 in each group.
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References


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