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The molecular mechanism of actinomycin D in preventing neointimal formation in rat carotid arteries after balloon injury

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Summary

The pathological mechanism of restenosis is primarily attributed to excessive proliferation of vascular smooth muscle cells (SMC). Actinomycin D has been regarded as a potential candidate to prevent balloon injury-induced neointimal formation. To explore its molecular mechanism in regulating cell proliferation, we first showed that actinomycin D markedly reduced the SMC proliferation via the inhibition of BrdU incorporation at 80 nM. This was further supported by the G1-phase arrest using a flowcytometric analysis. Actinomycin D was extremely potent with an inhibitory concentration IC_{50} at 0.4 nM, whereas the lethal dose LD_{50} was at 260 μM. In an *in vivo* study, the pluronic gel containing 80 nM and 80 μM actinomycin D was applied topically to surround the rat carotid adventitia; the thickness of neointima was substantially reduced (45 and 55%, respectively). The protein expression levels of proliferating cell nuclear antigen (PCNA), focal adhesion kinase (FAK), and Raf were all suppressed by actinomycin D. Extracellular signal-regulated kinases (Erk) involved in cell-cycle arrest were found to increase by actinomycin D. These observations provide a detailed mechanism of actinomycin D in preventing cell proliferation thus as a potential intervention for restenosis.

Introduction

Percutaneous transluminal coronary angioplasty (PTCA), a balloon catheter-based interventional procedure, is a non-surgical modality for treating coronary artery stenosis. However, the recurrence of restenosis in 30–50% patients within 6 months following the angioplasty procedure is the major drawback of PTCA [1, 2]. The pathological process of balloon injury induced restenosis continues to be an enigmatic problem in clinical settings. The arterial remodeling resulting from balloon injury is manifested as neointimal formation with significantly loss of luminal patency. Even though certain characteristics of this adaptive response to arterial injury have been documented, the regulation of this pathological process remains elusive.

One of the causes of arterial reocclusion after PTCA, aside from mechanical stretch, has been thought to be related to the outgrowth of smooth muscle cells (SMCs) [3, 4]. During which time, growth and prothrombotic factors released from platelets and white blood cells trigger the SMC cell cycle from G1 to S phase [5]. In theory, blocking the G1 to S phase should yield the inhibition of SMC proliferation or migration [6, 7]. For this reason, drugs associated with the

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cell-cycle blocker are considered for the treatment of restenosis by oral ingestion [8]. However, the systemic side effects of this application were inevitable.

Lately, drug-eluting stent has been introduced as a new technology to locally intervene the restenosis [9]. Many pharmacological agents with antiproliferative properties, including paclitaxel, rapamycin or actinomycin D, were under clinical evaluation [10]. The results of several large-scale clinical trials including the paclitaxel-eluting stent Taxol USA (TAXUS), the ASian Paclitaxel-Eluting Stent Clinical Trial (ASPECT), sirolimus-coated Bx Velocity balloon expandable stent (SIRIUS), and the ACTinomycin Eluting Stent Improves Outcomes by Reducing Neointimal Hyperplasia (ACTION) have been reported [11, 12]. Among these studies, both paclitaxel- and sirolimus-eluting stents demonstrated a consistent results showing a significantly reduction on neointimal thickening in porcine models of restenosis [13, 14]. However, although actinomycin D, a member of the chromopeptide lactone family with strong antineoplastic activity [15, 16], showed a potent inhibition on cell proliferation by forming stable complex with DNA and interfered RNA polymerase activity [17], ACTION was not able to complete because Guidant Corporation (the sponsor for ACTION) announced that its actinomycin-D-coated stent program has an unacceptable level of adverse events [11]. Although some of these clinical results have been published recently, actinomycin D did not show a promising outcome in the prevention of restenosis [12].

In the present study, we aimed to explore the molecular mechanisms of actinomycin D on cell proliferation and cell-cycle-related protein expression at various concentrations in both *in vitro* and *in vivo* studies. These findings may provide another aspect of evaluation on the inhibitory effects of actinomycin D on balloon injury-induced neointimal formaiton. The safety and potency of actinomycin D was also evaluated.

Methods

Cell culture

A10 cells (vascular SMC) originated from rat thoracic aorta were obtained from Food Research and Development Institute (Hsinchu, Taiwan) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin,100 μg/ml streptomycin, and 1 mM sodium pyruvate. Cultured SMC were starved for 24 h followed by incubation with various doses of actinomycin D (80 nM, 800 nM, 8 μM) at 37 °C. Drug treatment was carried out for 18∼24 h. Since actinomycin D was dissolved in 0.1% DMSO, a vehicle control containing DMSO was also included.

Cell cycle and cell viability

A10 cells were subcultured at a density of 3.5×10^5 cells per well in 6-well plates. Synchronized cells with 12-h starvation were then cultured in medium containing 15% FBS with or without various doses of actinomycin D for 18 h. The cells were collected, washed with PBS, and resuspended in a DNA-staining solution containing propidium iodide and 1 mg/ml RNase. Finally, the % of cells in each phase of cell cycle was determined by a FACS Calibur flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA) using a DNA analysis software ModfitLT, version 2.0 (Verity Software, Topsham, Maine, USA). Another set of cells with the same drug treatment were trypsinized and subjected to hematocytometric analysis for the determination of viable cells.

BrdU incorporation analysis

Vascular SMC were subcultured at a density 1×10^4 cells per well in 96-wells plates. Serum-starved cells were grown in medium containing 15% FBS at various doses of actinomycin D, which was added to the cells with additional 20 h incubation. Cells were then labeled with BrdU for 4 h at 37 °C followed by removing the labeling medium to terminate the reaction. Fix-Denat solution (Boehringer mannheim, German) was added to fix the cells for 30 min at room temperature. After removing FixDenat from the plates, anti-BrdU antibody was added and incubated at room temperature for 90 min. Following the wash, a substrate solution was added and developed. The optical density, representing the cell numbers, was measured in an ELISA reader at 370 nm.

Balloon angioplasty

Thirty-two male SD rats weighing 350–400 g were purchased from National Science Council (Taipei, Taiwan). Animals were housed in a 12-h light/dark cycles with free access to food and water. All animal care followed the institutional animal ethical guidelines of China Medical University. The rats were anesthetized with 3.6% (w/v) chlorohydrate (1 ml/100 g, i.p.). Angioplasty of the carotid artery was performed using a balloon embolectomy catheter. In brief, the balloon catheter (2F Fogarty) (Becton-Dickinson, Franklin Lakes, NJ, USA) was introduced through the right external carotid artery into the aorta, and the balloon was inflated at 1.3 kg/cm^2 using an inflation device. An inflated balloon was pushed and pulled through the lumen three times to damage the vessel. Two concentrations of actinomycin D (80 nM and 80 μM suspended in 30% (w/v) of pluronic gel was coated onto arterial adventitia of balloon-injured carotid artery. Two weeks after balloon injury, rats were sacrificed with overdose of pentobarbital injection. Tissue sectioning was performed at the desktop microtone with 5 μm thickness. After staining with Weigert's method using Weigert's Iron Hematoxylin solution, Resorcin-Fuchsin solution and Van Gieson's solution to delineate the elastic fibers over which are the neointima layers, the morphological analysis of the sections was processed by means of digital computing system (Sakoi NTSC Inc., Japan). The computer program Matrox Inspector (Matrox Electronic Systems Ltd., USA) were used to measure the neointimal thickness of the vessel wall.

Western blot

The actinomycin D-treated cells were lysed using a lysis buffer (2% SDS, 50 mM DTT, 62.5 mM Tris–HCl pH 6.8) followed by an incubation at 95 °C for 5 min. Total protein was resolved in SDS-polyacrylamide gel electrophoresis followed by blotting to PVDF (Polyvinylidene fluoride) membrane. Ponceau S was used to identify the successful transferring of proteins on membrane. The membrane was then incubated in PBS buffer containing 0.2% Tween-20 and 5% non-fat milk for 1 h. Primary antibodies against PCNA, Raf, ERK and FAK reconstituted in PBS containing 0.1% Tween-20 were added and incubated overnight at 4 °C. After washed with PBS containing 0.1% Tween-20, the protein expressing level was lit on X-ray film by ECL reaction (Amersham, Arlington Height, IL, USA).

Statistics

Data are expressed as mean ± SE. Statistical analysis was conducted using unpaired-student's *t*-test. A p-value < 0.05 was considered statistically significant.

Results

The inhibitory effect of actinomycin D on SMC proliferation

Although actinomycin D is a potent therapeutic compound for cancers, its application for cardiovascular diseases remains untested. A significant inhibitory effect of actinomycin D on SMC proliferation was evidenced by a BrdU incorporation assay (Figure 1). Using a flowcytometric analysis, we demonstrate a considerable inhibition of SMC to be arrested at S phase by actinomycin D (Figure 2), which was consistent to the mode of action of actinomycin D in the other cell types [18]. The cell-cycle arrest becomes evident as concentrations of actinomycin D greater than 0.8 nM (Figure 2).

Cytotoxicity and efficacy of actinomycin D

The *in vitro* experiment conducted in the above experiment suggests that actinomycin D was an extremely potent and effective agent to inhibit the proliferation of SMC by preventing cells from getting into S phase. In the subsequent experiment, we evaluated the toxicity of actinomycin D on SMC using a lethal dose-50 (LD_{50}) as a criterion. The LD_{50} (260 μM) determined by measuring the remaining viable cells at various concentrations of actinomycin D was about five orders greater than that of IC_{50} (0.4 nM), which was calculated by measuring the percentage of cells in S phase following the treatment of actinomycin D (Figure 3). Thus, the toxicity of various concentrations of actinomycin D (0.8 pM to 80 μM) used in this study to prevent SMC proliferation was considerably minimal and potent as compared to the very low IC_{50}.

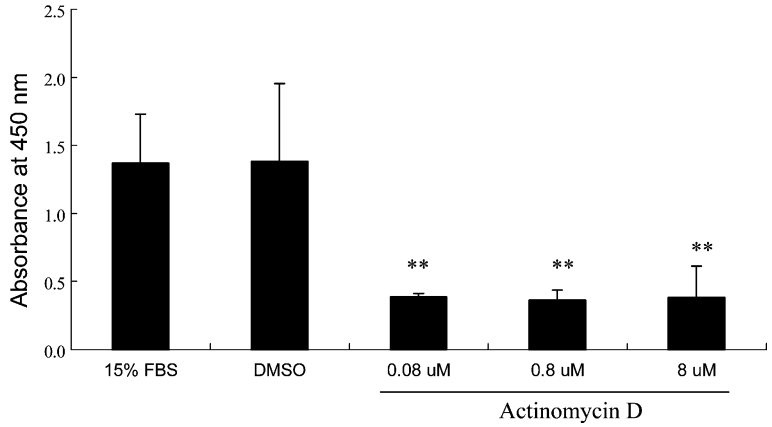

Figure 1. Effect of actinomycin D on SMC DNA synthesis. BrdU incorporation assay was used for monitoring the DNA synthesis. Cells were cultured in the presence of 15% FBS at various doses of actinomycin D. DMSO serves as a vehicle control. Each value represents the mean ± SD ($n = 6$). $^{**}p < 0.01$ as compared to 15% FBS control group.

Effect of actinomycin D on balloon-injured stenosis via pluronic gel coating on a carotid artery

In the present study, a desired concentration of actinomycin D dissolved in pluronic gel was locally applied onto the arterial adventitia of carotid artery of injured segment. After two weeks, the injured arteries were subjected for histological analysis for stenosis. Tissue section with and without balloon manipulation was then stained with Weigert's dye to visualize the neointimal formation. Stenosis in vessel with balloon injury was evident as compared to that without (Figure 4a and b). Effect of actinomycin D application on the attenuation of neointimal formation was observed. Striking protective effect by actinomycin D was seen in both of low (80 nM) and high (80 μM) dose treatment group with balloon injury (Figure 4c and d). Using computerized image analysis, the areas of intimal and media layers for each section were integrated and calculated. It demonstrates a 45% (low dose) and 55% (high dose) reduction in stenosis as compared to the balloon-injured control group (Figure 5).

Effects of actinomycin D on signaling pathway of SMC proliferation

We show that a dose-dependent inhibition by actinomycin D was found in PCNA and Raf protein expression, but not obvious in FAK (Figure 6a). It appears that the maximal inhibition for FAK was reached even as low as 80 nM of actinomycin D. The expression of Erk was determined to peak at as early as 30 min after stimulation (Figure 6b). The effect of actinomycin D on Erk was therefore evaluated at 30 min after drug treatment. Interestingly, in contrast to those seen on PCNA, Raf and FAK expression, the phosphorylated Erk was significantly up-regulated by increasing concentrations of actinomycin D (Figure 6c).

Discussion

One mode of actions actinomycin D, a potent chemotherapeutic drug, is to be an antagonist to cellular membrane-permeable SH2 domain [19] and hence attributes its inhibitory activity for cell proliferation for some cancer cells [20]. We demonstrated an inhibitory effect of actinomycin D on cultured SMC proliferation, and subsequently reveal that actinomycin D was capable of resting the cell cycle at G1 phase with an IC_{50} of 0.4 nM. The present study suggests that actinomycin D is an extremely potent agent in inhibiting cultured SMC proliferation with very minimal toxicity. Per assessment of LD_{50} and IC_{50}, the difference between them was ranged in about five orders.

An *in vivo* study using rat carotid artery as a model was conducted in the present study to evaluate if actinomycin D topically applied onto the arterial adventitia of the artery was effective in suppressing the formation of stenosis following a balloon angioplasty. Thirty-two rats were used and divided as four groups including sham control

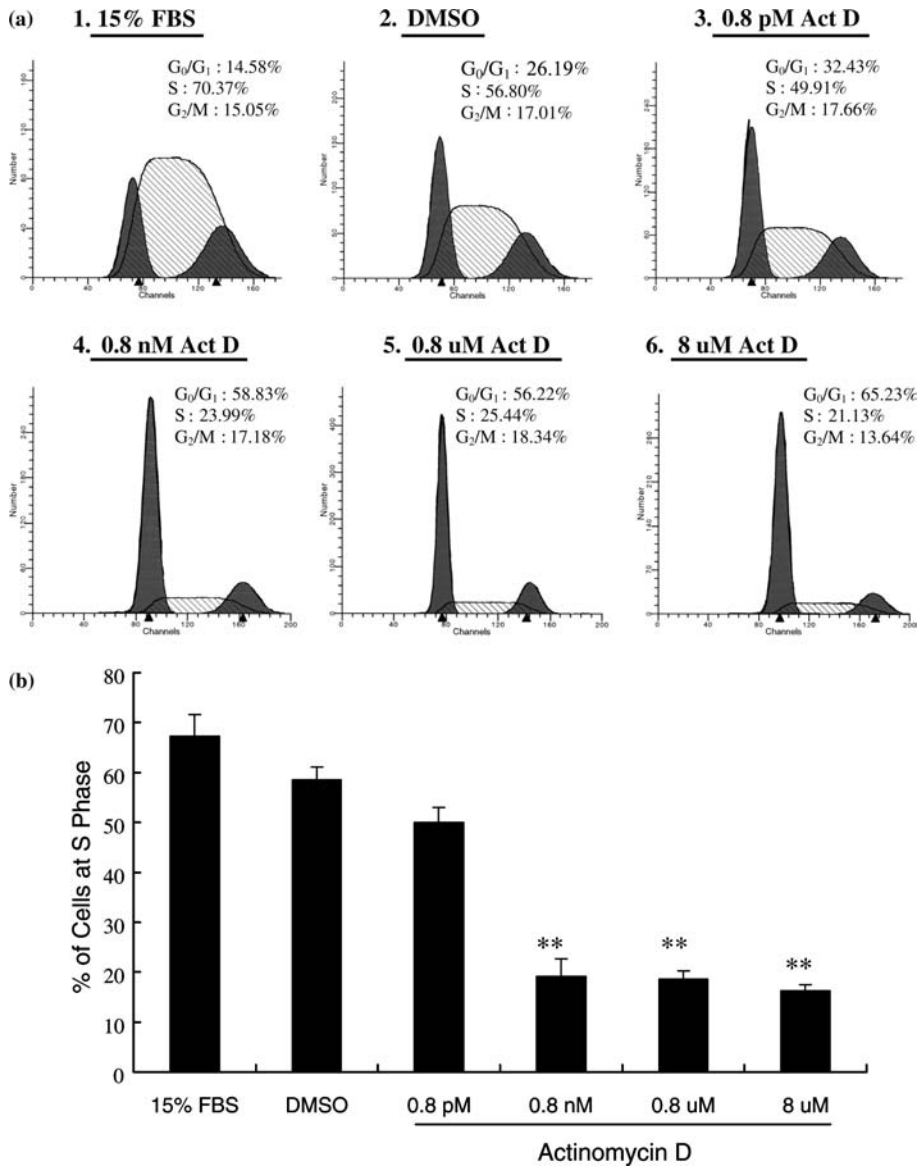

Figure 2. Flowcytometric analysis of the inhibitory effects of actinomycin D (Act D) on cell cycle. All the SMC were cultured in 15% FBS, while DMSO used for dissolving actinomycin D was used as a vehicle control. (a) Each phase (G_0/G_1, S and G_2/M) of cell cycle was determined at various doses of actinomycin D. Value in *X*-axis represents the DNA content, while the shaded area indicates the % of cells at S phase. (b) Effect of actinomycin D on the SMC arrested at S phase. Each value represents the mean ± SD ($n = 6$). $^{**}p < 0.01$ as compared to 15% FBS control group.

($n = 8$), total injury control without actinomycin D ($n = 8$), and low ($n = 8$) and high dose ($n = 8$) of actinomycin D-treated groups. Balloon was first surgically inserted into rat carotid arteries to induce injury. Two weeks after balloon injury, the arteries were subjected to histological analysis and actinomycin D was found to significantly reduce neointimal formation.

Application of pluronic gel as a drug vehicle topically applied to the surrounding of rat carotid arteries was adapted from that developed by Indolfi et al. [21] who utilized a mutant Ras gene released from pluronic gel into the arterial adventitia. Pluronic gel is an amphipathic compound composed of hydrophilic polyoxyethylene and hydrophobic polyoxypropylene [22]. A few other

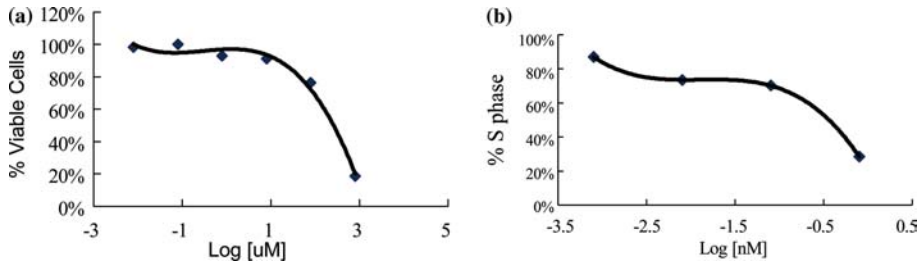

Figure 3. Cytotoxicity and efficacy of actinomycin D on SMC. Cells were cultured in 15% fetal FBS over various doses of actinomycin D for 18 h. (a) Lethal LD_{50} of actinomycin D was determined by counting the viable cells with eosin staining. (b) IC_{50} of actinomycin D was determined by analyzing the % of cells at S phase at various doses of actinomycin D via a flowcytometry assay.

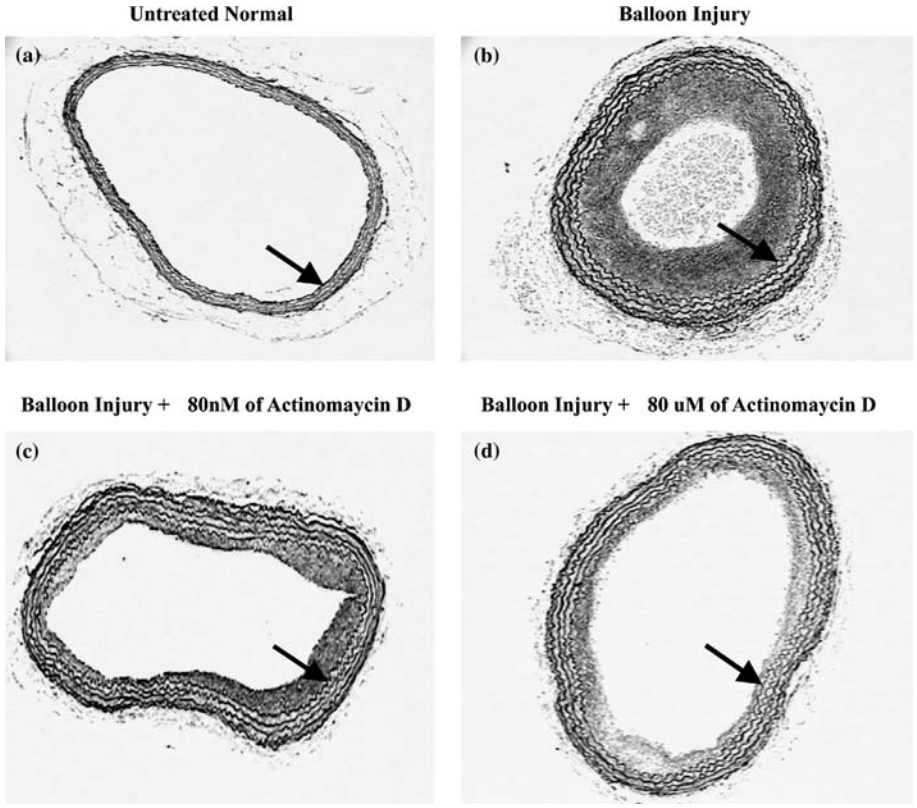

Figure 4. Cross-section of carotid arteries 14 days after balloon angioplasty. (a) Normal vessel without balloon injury. (b) Balloon-injured vessel. (c) Balloon-injured vessel treated with 80 nM of actinomycin D. (d) Balloon-injured vessel treated with 80 μM of actinomycin D. Arrow indicates the neointimal layer from the elastic laminae. All graphs were taken at 100× magnifications.

studies have also confirmed pluronic gel as an effective drug transport carriers. Villa et al. [23] have delivered antisense oligodeoxynucleotides of c-myc to balloon-injured rat arteries via pluronic gel and demonstrated a significant inhibition on neointimal formation. The drug delivery efficiency of pluronic gel was also confirmed by Siow et al. [24] who used adenovirus-mediated LacZ in pluronic gel to show an increase of enzyme activity in target tissue. Ishikawa et al. [25] using pluronic gel as a carrier for parathyroid hormone-related protein (PTHrP) surrounding the balloon-injured rat femoral arteries showed an effective prevention of neointimal formation. All these reports have affirmed pluronic gel as a powerful vehicle for drug delivery. We have thus used pluronic gel in the

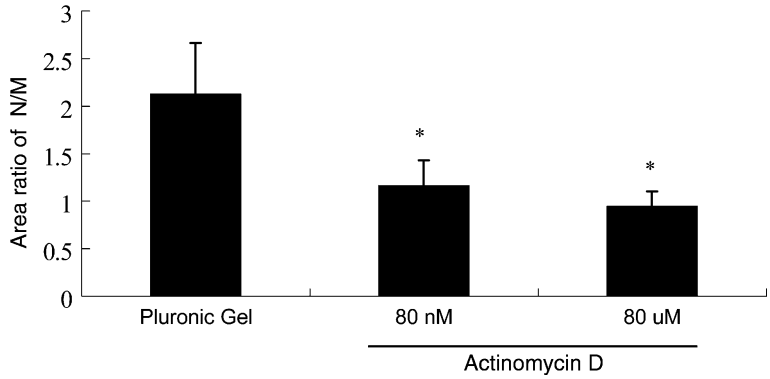

Figure 5. The inhibitory effect of actinomycin D on area ratios of neointima to media (N/M) in injured carotid arteries. Either low (80 nM) or high (80 μM) dose of actinomycin D significantly reduced the neointima as compared to the control group without actinomycin D ($n = 8$). *$p < 0.05$.

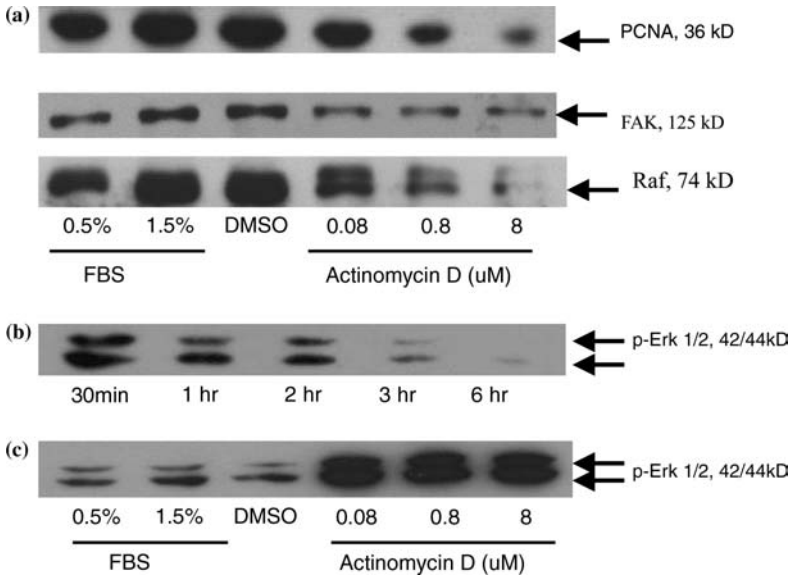

Figure 6. Western blot analysis of actinomycin D on protein expression levels. (a) Protein expression levels of PCNA, FAK and Raf. (b) Expression level of Erk. (c) Protein level of Erk was significantly increased in the presence of actinomycin D following 30 min treatment.

present study to locally deliver actinomycin D to the balloon-injured targets.

Actinomycin D is also involved in the inhibition of Shc/Grb2 interaction [19, 26], in which Grb2 is a 23–25 kD protein composed of two SH3 domains and one SH2 domain [27]. The SH2 domain binds to a specific phosphotyrosine motif of some receptor proteins or other adaptors such as Shc, whereas the SH3 domain is associated with proline-rich motif in SOS, a guanine nucleotide exchange factor for Ras proteins. Reorganization of the Grb2-SOS complexes or relocalization of SOS is then activating Ras in an early metabolic event for cell proliferation [28]. Our previous studies [6, 7] demonstrated that transfection of a negatively dominant Ras gene, RasN17, effectively suppresses balloon-injured neointimal formation. Therefore, it is conceivable that actinomycin D that blocks Shc/Grb2 interaction should also lead to a similar result. For this reason, we investigated those protein expressions involved in the down stream of the Ras activation pathway.

In view of our previous report showing that Ras gene is involved in the underlying mechanism for the neointimal formation during the balloon injury [6, 7], several proteins involved in the Ras pathway

as affected by actinomycin D were also investigated in this study. Proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase-delta, is one of the indexes for cell proliferation. FAK is a protein involved in transducing extracellular growth signal from matrix via integrin interaction. Down-regulation of FAK may result in cell-cycle arrest [29]. Raf, an important protein in the mitogen-activating protein kinase (MAPK) pathway, is responsible for signal transduction from Ras to Erk. Along the pathway, signaling of phosphorylated-Erk 1/2 is also an essential element for cell proliferation. Therefore, the protein expression level of PCNA, FAK, Raf and Erk were all evaluated in the present study to explore the mode of preventive action of actinomycin D against restenosis.

In the present study, down-regulation of PCNA translational levels by actinomycin D suggests its role in arresting cell at G0/G1 phase (Figure 2). Inhibition of FAK protein levels shows a further regulatory effect of actinomycin D on cell proliferation and migration. Suppressing Raf expression levels suggests a negative effect of actinomycin D on MAPK-mediated signaling, which is a key pathway leading to cell proliferation. However, the regulation of these proteins by actinomycin D was in different manners. The inhibition of actinomycin D on Raf and PCNA was dose-dependent, whereas FAK was also inhibited but at a much less dose (Figure 6). Whether or not, the latter event was due to the super-sensitivity of FAK on translational regulation by actinomycin D needs to be confirmed.

It is worth of mentioning that the level of phosphorylated-Erk 1/2 was up-regulated by actinomycin D in this study and was somewhat contradictory to the putative function of Erk as a critical signaling molecule leading to cell proliferation and survival [30, 31]. However, one study showing that DNA damage can be initiated by Erk 1/2 phosphorylation may explain our present finding [32]. In addition, the role of ERK in DNA-damage may manifest a different outcome. For example, activation of $p21^{CIP1}$ by over-expression of p53 can promote the cell-cycle arrest via DNA damaging [33, 34]. Several reports have demonstrated that actinomycin D can stabilize the mRNA of this CDK inhibitory protein (CKI) in culture cells [33, 35]. Further, ERK activation also induces $p21^{CIP1}$ [36, 37], since pharmacologic inhibition of ERK diminishes the expression of $p21^{CIP1}$ [32]. Duff et al. [38] have reported that actinomycin D inhibits the mRNA expression of MAP kinase phosphatase-1 (MKP-1), but prolongs the expression of phosphorylated-Erk 1/2. MKP-1 is induced by angiotensin II and selectively dephosphorylates Erk 1/2 *in vitro*. These observations further support the ERK activation resulting in inhibition of SMC proliferation by actinomycin D. To sum up, our study demonstrates that balloon-induced neointimal formation could be markedly reduced by actinomycin D. Its pharmacologic mechanism may be associated with the down-regulation of PCNA, FAK and Raf protein levels combined with up-regulation of ERK phosphorylation.

Although the outcome of the multi-center, single blind, three-arm ACTION trial failed to demonstrate the effectiveness of actinomycin D in the prevention of restenosis [12], our results in the present study detailed the molecular mechanisms of actinomycin D in preventing the smooth muscle cell proliferation either *in vitro* or *in vivo*. Several explanations can be accounted for this biased observation between that clinical trial and our study. First, the concentrations of actinomycin D used in the ACTION trial were 2.5 and 10 $\mu g/cm^2$ of metallic stent, which may be insufficient to prevent the outgrowth of vascular smooth muscle cells caused by stent disposition. The concentration of actinomycin D released from the stent surface to act on the vascular wall was not clearly defined in that trial. In the present study, we found that 80 nM and 80 μM of actinomycin D, both significantly reduced the neointimal formation in rat carotid arteries. Secondly, actinomycin D on a stent directly contact the endothelial and smooth muscle cells in the ACTION study, whereas we applied actinomycin D in pluronic gel to adventitial layer. Although the rodent models do not always translate to humans, our findings about the inhibitory effects of actinomycin D on smooth muscle cells may still shed the light into the conjunctive roles of actinomycin D with some other pharmacological agents in preventing restenosis.

Acknowledgment

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