行政院國家科學委員會專題研究計畫　成果報告

利用蛋白質晶片探討 actinomycin D 改善血管再阻塞的分子機轉

計畫類別：個別型計畫
計畫編號：NSC92-2320-B-039-018-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：中國醫藥大學生物科技學系

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 11 月 3 日

行政院國家科學委員會專題研究計畫期中報告

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摘要

關鍵詞：氣球擴張術，血管再阻塞，細胞增生

Actinomycin D 是一種化療藥物，至今尚未正式用於臨床上心血管疾病的治療。由於這類藥物能抑制細胞增生，所以本計畫主要目的即在於探討 actinomycin D 能否改善氣球擴張所造成血管再阻塞的作用，並進而了解其作用機轉。本計畫之研究結果顯示血管平滑肌細胞能被 actinomycin D 顯著的抑制生長，而這一細胞抑制作用之機轉與 PCNA、FAK、Raf 蛋白質表現量的降低有密切的關係，而另外一個蛋白質 Erk 則其表現量卻被 actinomycin D 給抑制。這些細胞層次上的作用與動物實驗相互印證，本計畫以大鼠總頸動脈接受氣球擴張的動物模型來評估 actinomycin D 的藥理作用，結果顯示血管內層增生的現象明顯受到改善。根據這些體外及體內的研究結果，actinomycin D 對於臨床上氣球擴張所造成血管再阻塞的防治應有其一定的作用。

I、Abstract

Actinomycin D is a chemotherapeutic agent that arrests cells at G1 phase in cell cycle. Thus far, it has not been clinically used for the treatment of cardiovascular diseases. Since the pathological mechanism of restenosis is primarily attributed to excessive proliferation of vascular smooth muscle cells (SMC), we tested the hypothesis whether actinomycin D could be used as a therapeutic candidate to prevent against balloon injury induced restenosis. 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 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 the carotid adventitia, the thickness of neointima was substantially reduced (45% and 55%, respectively). Thus, actinomycin D was a potent candidate in preventing restenosis. The metabolic pathway of actinomycin D on cell proliferation was further investigated. Our data suggest that down-regulation of proliferating cell nuclear antigen (PCNA), focal adhesion kinase (FAK), and Raf play a provocative role in the inhibition of SMC while up-regulation of extracellular signal-regulated kinases (Erk) indicates that Erk may be involved in regulating cell cycle arrest. Regardless, in considering the minimal toxicity and high potency, we conclude that actinomycin D may be used as a new entity for the therapeutic intervention in balloon induced restenosis.

II、Background and Specific Aims

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 (Isner et al., 1996; Serruys et al., 1988). 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 neointima 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) (Mazure et al., 1996; Bult, 2000). During which time, growth and prothrombotic factors released from platelets and white blood cells trigger the SMC cell-cycle from G1 to S phase (Kibbe et al., 2000). In theory, blocking the G1 to S phase should yield the inhibition of SMC proliferation or migration (Jin et al., 2000; Wu et al., 2001). For this reason, drugs associated with the cell cycle blocker are considered for the treatment of restenosis by oral ingestion (Grube et al., 2002).

Lately, drug-eluting stent has been introduced as a new technology to intervene the restenosis (Waksman, 2002), but the class compound that arrests cells at G1 phase in cell cycle has not been evaluated. In the present study, we test the hypothesis whether actinomycin D, a member of the chromopeptide lactone family with strong antineoplastic activity (Chang et al., 1997) via a protective mechanism involved in the cell-cycle arrest

(Jonhson et al., 1995; Tamura et al., 2000), could be used for drug-eluting stent.

We demonstrate 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. An in vivo study using rat carotid artery as a model was therefore conducted 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.

In view of our recent report showing that Ras gene is involved in the underlying mechanism for the neointima formation during the balloon injury (Jin et al., 2000; Wu et al., 2001), 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 (Zhao et al., 1998). 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.

III、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 (Wu and Yang, 1994). Apparently, the arrest was in a dose-dependent manner with an inhibitory concentration-50 (IC_{50}) of actinomycin D at 0.4 nM.

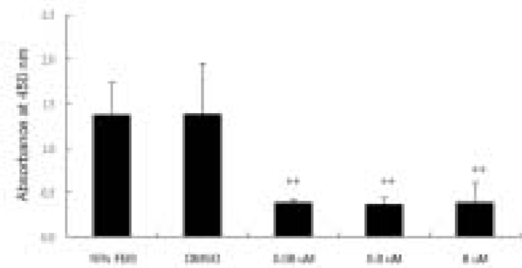

Figure 1. Effect of actinomycin D on SMC DNA synthesis. BrdU incorporation assay was used for monitoring the DNA synthesis. Cells were cultured in the presence of 15% FBS at various doses of actinomycin D. Each value represents the mean ± SD (n=6). ** $p < 0.01$ as compared to 15% FBS control group.

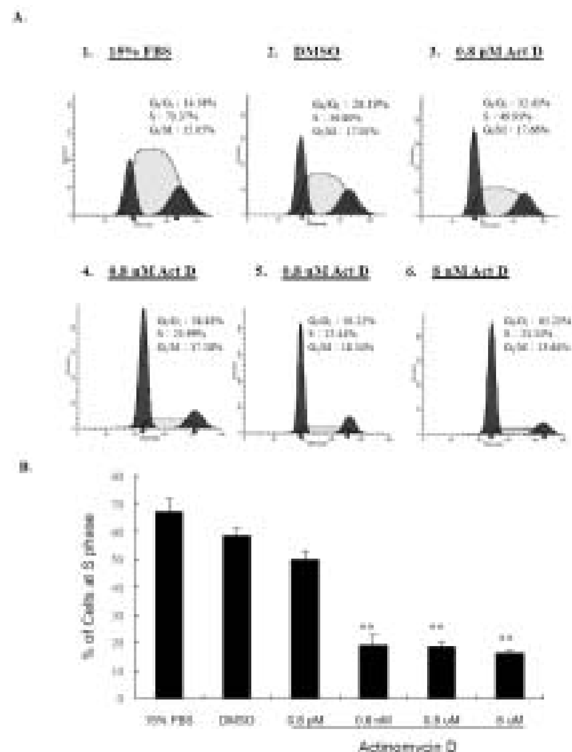

Figure 2. Flowcytometric analysis of the inhibitory effects of actinomycin D (Act D) on cell cycle. All the SMC were cultured in 15% FBS, while DMSO used for dissolving actinomycin D was used as a vehicle control. (A) Each phase (G_0/G_1, S and G_2/M) of cell cycle was determined at various doses of 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.

Cytotoxicity and efficacy of actinomycin D

The *in vitro* experiment conducted above suggests that actinomycin D was an extremely potent and effective agent to inhibit the proliferation of SMC with a mechanism involved in arresting S phase. In the next experiment we evaluated the toxicity of

actinomycin D on SMC using a lethal dose-50 (LD_{50}) as a criterion. The LD_{50} (260 uM) was about 5 orders greater than that of IC_{50} (0.4 nM) of actinomycin D (Figure 3). Thus, the toxicity of actinomycin D to SMC was considerably minimal.

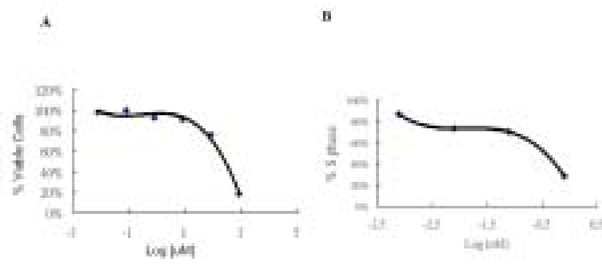

Figure 3. Cytotoxicity and efficacy of actinomycin D on SMC. Cells were cultured in 15% fetal FBS over various doses of actinomycin D for 18 h. (A) Lethal LD_{50} of actinomycin D was determined by counting the viable cells with eosin staining. (B) IC_{50} of actinomycin D was determined by analyzing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

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 neointima formation. Stenosis in vessel with balloon injury was evident as compared to that without (Figures 4A and 4B). 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 (Figures 4C and 4D). 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).

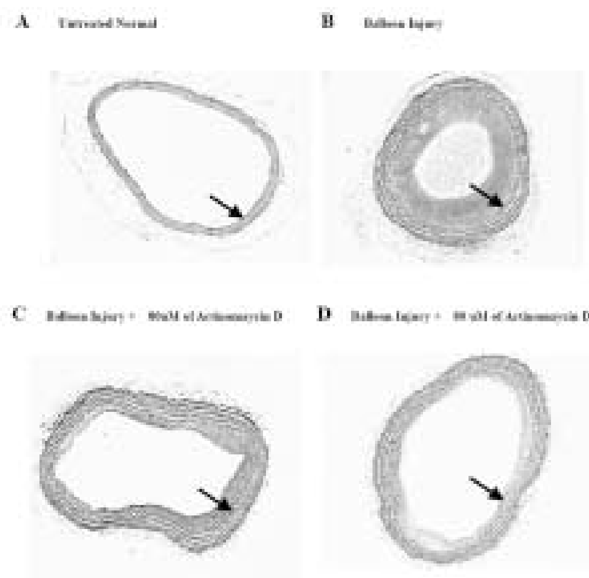

Figure 4. Cross section of carotid arteries 14 days after balloon angioplasty. (A) Normal vessel without balloon injury. (B) Balloon injured vessel. (C) Balloon injured vessel treated with 80 nM of actinomycin D. (D) Balloon injured vessel treated with 80 μM of actinomycin D. Arrow indicates the neointimal layer from the elastic laminas. All graphs were taken at 100 X magnifications.

Effects of actinomycin D on Ras activation pathway of SMC

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 80nM of actinomycin D. The expression of Erk was determined to peak at as early as 30 minutes after stimulation (Figure 6B). The effect of actinomycin D on Erk was therefore evaluated at 30 minutes 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).

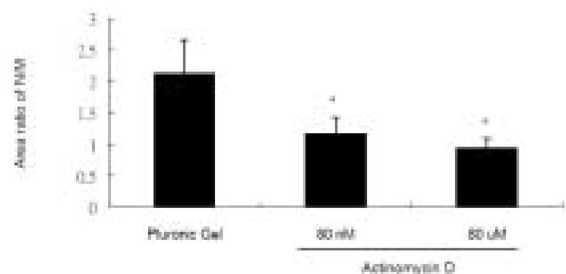

Figure 5. The inhibitory effect of actinomycin D on area ratio of neointima to media (N/M) in injured carotid arteries. Either low (80 nM) or high (80 μM) dose of actinomycin D significantly reduced the neointima as compared to the control group without actinomycin D (n=8). * p<0.05.

IV. Discussion

Actinomycin D, a potent chemotherapeutic drug, has not yet been used clinically for restenosis. One mode of actions of this

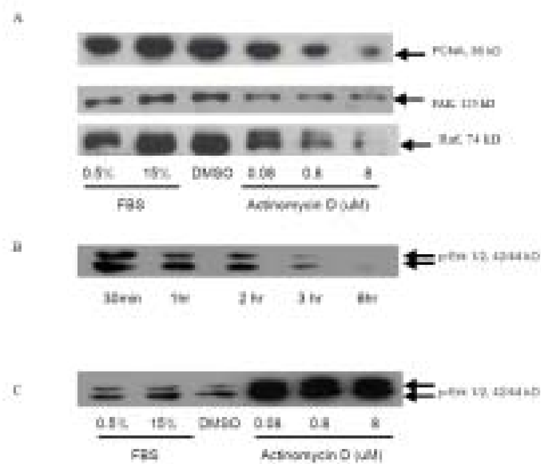

Figure 6. Western blot analysis of actinomycin D on protein expression levels. (A) Protein expression levels of PCNA, FAK and Raf. (B) Expression level of Erk. (C) Protein level of Erk was significantly increased in the presence of actinomycin D following 30 min treatment.

compound is to be an antagonist to cellular membrane-permeable SH2 domain (Kim et al., 1999) and hence attributes its inhibitory activity for cell proliferation for some cancer cells (Lu et al., 2003). 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 5 orders.

To test whether actinomycin D may effectively reduce the neointima formation caused by PTCA, 32 rats were used and divided as four groups including sham control (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. Application of pluronic gel as a drug vehicle was adapted from that developed by Indolfi et al. (1995) who utilized a mutant Ras gene released from pluronic gel into the arterial adventitia. Two weeks after balloon injury, the arteries were subjected to histological analysis. Actinomycin D was found to significantly reduce neointima formation, suggesting its potential application on clinical restenosis.

Actinomycin D is also involved in the inhibition of Shc/Grb2 interaction (Kim et al., 1999; Kim et al., 2000), in which Grb2 is a 23-25 kD protein composed of two SH3 domains and one SH2 domain (Matuoka et al., 1992). 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 (Aronheim et al., 1994). Our previous studies (Jin et al., 2000; Wu et al., 2001) demonstrated that transfection of a negatively dominant Ras gene, RasN17, effectively suppresses balloon injured neointima formation. Therefore, it is conceivable that actinomycin D that blocks Shc/Grb2 interaction should also lead a similar result. For this reason we investigated those protein expressions involved in the down stream of the Ras activation pathway.

PCNA, a cofactor of DNA polymerase-delta, was responsible for cell proliferation. FAK is a protein involved in transducing extracellular growth signal from matrix via integrin interaction. In the present study, both of these proteins were shown down regulated in the present of actinomycin D suggesting a role in cell arrest at the S-phase (Fig. 2). However, the regulation of these proteins could be in a different manner. The inhibition of actinomycin D on Raf and PCNA was dose-dependent, whereas FAK was also inhibited but at a much less dose (Fig. 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 worthy 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 (Dorafshar et al., 2003; Ghiselli et al., 2003). However, one study has shown that DNA damage can be initiated by Erk 1/2 phosphorylation (Tang et al., 2002), which may explain our present finding. 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 a DNA damage (Colman et al., 2000; Caspari, 2002). Further, ERK activation also induces $p21^{CIP1}$ (Woods et al., 1997; Sewing et al., 1997), since

pharmacologic inhibition of ERK diminishes the expression of p21^{CIP1} (Tang et al., 2002). Duff et al. (1995) 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 in SMC by actinomycin D.

Taking together, our study demonstrates that balloon induced neointima 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 a up-regulation of ERK phosphorylation. Although the precise doses of actinomycin D to reach its optimal effect remained to be determined for clinical uses, this study provides a potential usage of actinomycin D for the treatment of PTCA-induced restenosis. The compound or its analog deserves a future study as a new entity in neointima formation.

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