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Inhibitory effect of ligustrazine on proliferation of rabbit vascular smooth muscle cells after arterial injury[1]

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KEY WORDS vascular smooth muscle; cultured cells; thoracic aorta; balloon dilatation; ligustrazine; cell division

ABSTRACT

AIM: To study the inhibitory effect of ligustrazine (Lig) on growth of cultured rabbit aortic vascular smooth muscle cells (VSMC) after balloon injury. **METHODS**: Twenty New Zealand white rabbits were subjected to arterial injury with a balloon catheter (810 kPa for three consecutive inflations, 1 min each time). The uptake of [^{3}H]thymidine in primary cultural VSMC incubated with rabbit serum, which obtained from the animals treated without or with Lig (40 mg·kg^{-1}·d^{-1}, iv) for 21 d (7 d before and 14 d after the injury procedure) was determined. The determination was performed in direct addition of TMP to culture as well. And histological cross-sections of the blood wall were also analyzed. **RESULTS**: After balloon injury the intimal thickening (77 ± 23) μm and lumen diameter narrowing (877 ± 118) μm in dilated sites were increased significantly than the normal adjacent wall [(41 ± 13) μm, $P < 0.01$; (1033 ± 175) μm, $P < 0.05$, respectively]. Treatment with Lig decreased both intimal thickening (56 ± 16) μm ($P < 0.05$) and lumen diameter narrowing (1023 ± 157) μm ($P < 0.05$). Lig inhibited [^{3}H]thymidine uptake in VSMC incubated with the serum obtained from these rabbits. Direct addition of Lig inhibited [^{3}H]thymidine uptake in cultured VSMC in a dose-dependent manner (40 - 4000) μg/well. **CONCLUSION**: Lig shows a pronounced inhibitory effect on VSMC proliferation after balloon injury.

INTRODUCTION

The success of percutaneous transluminal coronary angioplasty (PTCA) is limited by late restenosis, which occurred in 30% - 50% of all cases, chiefly within the first six months after the intervention[1]. Restenosis is due to the proliferation of vascular smooth muscle cells (VSMC) at the injury site and constrictive remodelling of the whole artery. The arterial response to injury appears to be an important factor in restenosis[2]. There were many attempts to prevent restenosis based on the pathophysiology, but so far no successful method has been found. Clinical studies using alternative instrumental devices have not convincingly demonstrated a benefit in reducing the incidence of restenosis. Few drugs have been clinically effective in preventing restenosis, although studies frequently report successful suppression of neointimal proliferation in animal models of balloon vascular injury[2, 3]. It seems that one or more pharmacologic approaches will be required. Development of suitable pharmacological agents modulating VSMC proliferation is critical for further investigation of vascular hyperplasia and its prevention[2].

Ligustrazine (Lig), is a constituent of the herb *Lingusticum wallichii* Franchat, has been isolated, purified, and chemically synthesized. It has been widely used to treat cardiovascular diseases. Its major therapeutic effects are likely to be via its action as a vasodilator, a free radical scavenger, and an anti-platelet aggregation agent[4-6]. Recently, Tang *et*

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al[7] have reported that Lig has the influence on growth and cleavage of primary cultural VSMC, indicating that this pharmacological action of Lig may be related to the prevention of restenosis.

Although Lig has been claimed to inhibit proliferation of VSMC, there is uncertainty as to the mechanisms of anti-proliferation of Lig involved. Moreover, *in vivo* investigations of the inhibitory effects of Lig suffer from the lack of knowledge on VSMC after arterial injury. The purpose of the present study is to examine whether Lig inhibit proliferation of VSMC from aortic artery after balloon injury. And in this paper we further evaluate the possibility of Lig as a potential agent in preventing recurrent stenosis after PTCA.

MATERIALS AND METHODS

Drugs and reagents Lig 20 g·L^{-1}, sterile injection, purity 98.5 %, was purchased from Beijing 4th Pharmaceutical Co, China. Dulbecco's modified Eagle's medium (DMEM) and trypsin were obtained from Gibco. Fetal bovine serum (FBS), benzylpenicillin, and streptomycin sulphate were obtained from Flow Laboratories. [^{3}H] Thymidine (specific activity, 851 TBq·mol^{-1}) was obtained from Shanghai Institute of Nuclear Research, China. All other chemicals were of the best reagent grade commercially available. Lig was prepared in distilled water, and sterilised with 0.2 μmol·L^{-1} filters before used for assay of cell growth.

Experimental procedure New Zealand white rabbits [♂, n = 21, supplied by Experimental Animal Center of Sun Yat-sen University of Medical Sciences, China, (2.17 ± 0.16) kg in weight] were used. All rabbits were offered normal rabbit chow daily supplemented with greens twice a week, water was allowed *ad lib*. The rabbits were randomly divided into two groups except one for cell culture (see below). Lig group (n = 10) received Lig (40 mg·kg^{-1}·d^{-1} iv). Control group (n = 10) received equal volumes of normal saline. Treatment was started 7 d before balloon injury. Balloon injury of aortic artery was then performed. Treatment was continued, and the rabbits were killed 14 d after injury.

Rabbits of the two groups were subjected to arterial injury with a balloon catheter as previously described[8]. In brief, under general anesthesia (sodium pentobarbitone 30 mg·kg^{-1} iv), a longitudinal incision was made across the right upper thigh and groin area and the superficial femoral artery mobilized. A 3-mm diameter noncompliant (polyethylene) angioplasty balloon was advanced about 7 cm into the thoracic aorta via a superficial femoral arteriotomy. Three consecutive inflations to a pressure of 810 kPa, with each inflation lasting 1 min, were carried out. After removal of the balloon the arteriotomy site was tied off proximally. Balloon-injured rabbits were killed by air embolism via the marginal ear vein and thoracic aortae were retrieved. Two 5-mm segments were cut from both dilated and normal adjacent portion of each aorta for morphometric analysis (10 segments each group). Cross-sectional areas of the intima were measured using a computerized morphometry system (Neuroscience Inc, Guangzhou Medical College, China).

Blood was collected from rabbits for each period (before and after treatment), after overnight fasting, by heart puncture without anesthesia and allowed to stand at 37 ℃ for 1 h, then centrifuged at 1000 × g for 10 min. Serum obtained was heated at 56 ℃ for 30 min and sterilized by filtration. Batches were stored at 4 ℃ until used, at which time they were refiltered, and aliquots taken for autoradiograph.

Cell culture VSMC were obtained from the normal rabbit. The aorta was excised aseptically (3 – 4 cm of the thoracic aorta above the diaphragm) and the adventitia was removed. After longitudinal incision of the vessel, the intima was carefully stripped off and the medial tissue was divided into 0.5 – 1 mm^3 segments. The segments were nourished with DMEM supplemented with 20 % FBS, benzylpenicillin (50 000 U·L^{-1}), streptomycin sulphate (50 mg·L^{-1}). Cells were incubated at 37 ℃ in humidified atmosphere of 95 % air + 5 % CO_2. The medium was changed daily until cells began to migrate from the explants, then the cultures were fed twice a wk. For subculture, the cells were trypsinized with 0.05 % trypsin at 37 ℃ for 20 min, centrifuged at 250 × g, resuspended in complete medium, and split 1∶2. Cell outgrowth was generally visible with 1 wk and the cells grew almost to confluency by 10 – 14 d. VSMC were characterised by their typical "hill and valley" morphology under the phase contrast microscope.

They contained bundles of myofilaments with fusiform dense-body attachments as shown by electron microscopy. These features are markers of SMC. Studies were conducted on VSMC (passages 5) that had achieved confluence in 20 % FBS. Then, the cells were incubated in a defined serum-free medium for 2 d before addition of drugs.

Cell growth assay VSMC were trypsinized with 0.05 % trypsin at 37 ℃ for 20 min, centrifuged at 250 × g, resuspended in DMEM supplemented with 20 % NRS (normal rabbit serum). With the aid of an ocular grid, cells were counted and diluted up to density of 2×10^9 cells·L^{-1}. Cells were split into 14 plastic flasks (30 mL) and were incubated at 37 ℃ in humidified atmosphere of 95 % air + 5 % CO_2. The cell counts were taken on d 1 – 7 (two flasks each day). VSMC were seeded separately at densities of 1.25×10^5, 2.5×10^5, 5×10^5, 1×10^6, 2×10^6 cells/well in DMEM and 20 % NRS (40 μL/well) in 96-well plates (in a total volume of 200 μL/well). Culture plates were incubated at 37 ℃ for 96 h in a 5 % CO_2 incubator. The radioactivity was counted as described below.

VSMC were seeded at a density of 1×10^6 cells/well in 96-well plates and were cultured in the presence of 20 % NRS. Cultures were harvested every 24 h on 1 to 7 d after growth arrest for determination of radioactivity.

Determination of the uptake of [^{3}H]thymidine For proliferation experiments, VSMC were seeded as previously described at a density of 1×10^6 cells/well in 96-well plates (160 μL/well) with the standard 20 % NRS containing medium. Cells were then preincubated for 48 h with 0.4 % NRS (vol/vol) containing medium to become quiescent before the addition of Lig or specimen serum. Growth stimulation was started with 20 % rabbit serum of each specimen (40 μL/well) or 20 % NRS with Lig of various concentrations, each in triplicate wells. Incubated at 37 ℃ in humidified atmosphere of 95 % air + 5 % CO_2 for 96 h, the serum-containing medium was removed and the cells were washed twice with serum-free DMEM and incubated under serum-free condition for 24 h. Then [^{3}H]thymidine (74 kBq/well) was added. The culture was terminated 8 h later by washing cells twice with ice-cold phosphate buffered saline (PBS). The cells were then trypsinized and retrieved onto filter paper disc with a cell harvester and measured by an LS-3801 liquid scintillation counter (Beckman).

Statistics Results were expressed as $\bar{x} \pm s$ and compared with t-test.

RESULTS

A growth curve on VSMC seeded at densities of 2×10^9 cells·L^{-1} showed typical lag and log phases of growth with a maximal doubling time of 48 h during log phase growth (Fig 1A). With this protocol, by d 2 cells growing under control conditions reached a stationary phase of growth. The effect of different densities of seeding cells on [^{3}H] thymidine incorporation. VSMC were seeded at densities of $5 \times 10^5 - 1 \times 10^6$ cells/well, [^{3}H]thymidine incorporation increased progressively. It suggested that a density of 1×10^6 cells/well was optimum choice for seeding VSMC in culture (Fig 1B). VSMC increased rapidly during the 96 – 120 h incubation period (Fig 1C). With this protocol, the appropriate period for incubation was 96 h.

Direct addition of Lig at dose of 40 – 4000 μg/well had inhibitory effect on thymidine uptake in VSMC. Lig inhibited VSMC proliferation in a dose-dependent manner (Fig 2).

Thymidine uptake in VSMC incubated in the presence of rabbit serum, Lig group (45 ± 8) Bq/well was lower than control group (64 ± 11) Bq/well ($P < 0.01$). Also, the group after received Lig was significantly lower than before (54 ± 8) Bq/well ($P < 0.05$). In addition, [^{3}H]thymidine uptake in VSMC of control group after arterial injury was higher than before (54 ± 9) Bq/well ($P < 0.05$).

In animals in which the arterial was injured, Lig treatment caused an increase in lumen diameter and a decrease in intimal thickness. Intimal thickness significantly increased and lumen diameter decreased at dilated sites than at normal adjacent wall in both Lig group and control group, except lumen diameter in Lig group ($t = 1.348$, $P = 0.208$) (Tab 1).

DISCUSSION

It is generally accepted that VSMC respond to the

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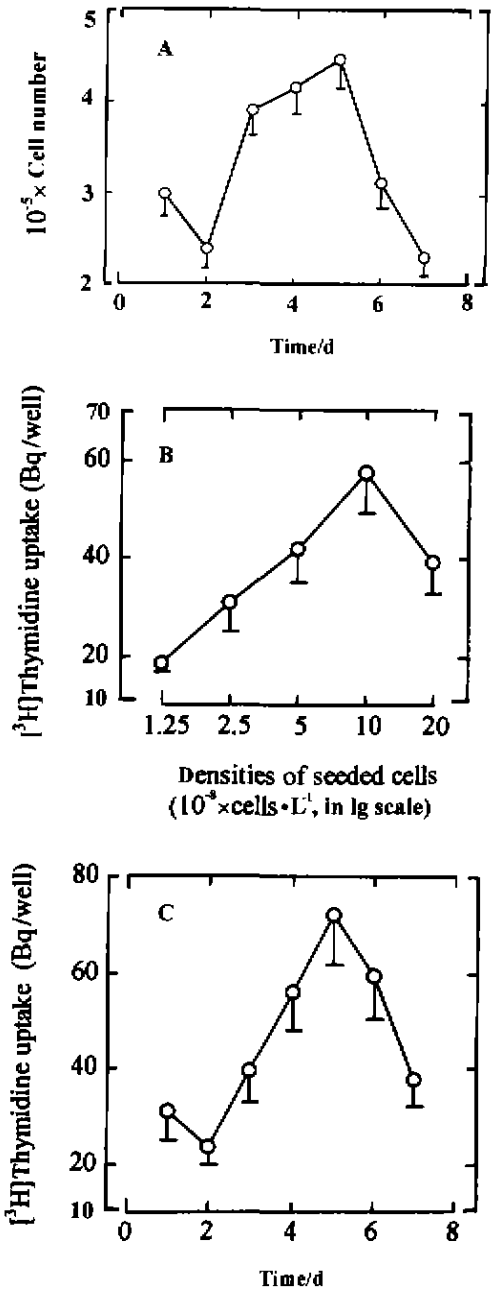

Fig 1. Growth behavior of aortic smooth muscle cells from New Zealand white rabbit. (A) Aortic smooth muscle cells seeded at density of 2×10^9 cells · L^{-1}. The cell counts were taken on day 1 to 7. (B) VSMC were seeded separately at different densities and cultured. Each point is the $\bar{x} \pm s$ of triplicate cultures. (C) VSMC were seeded at a density of 1×10^6 cells/well.

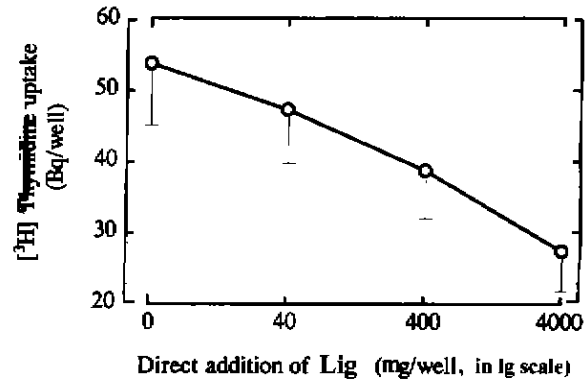

Fig 2. Direct addition of Lig (mg/well) on proliferation of VSMC. Aortic VSMC from New Zealand white rabbit were incubated for 96 h as described in Methods. $\bar{x} \pm s$ of triplicate determinations of tritiated thymidine uptake.

Tab 1. Effects of Lig on intimal thickness and lumen diameter after balloon catheter dilation in aortic wall. The mean values from three consecutive transverse sections were used. Lumen diameter values were calculated according to cross-sectional areas of intima. $n = 10$ segments. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* normal adjacent site.

Groups	Arterial injury site	Normal adjacent site
	Intimal thickness/μm	
Control group	77 ± 23	41 ± 13^f
Drug group	56 ± 16^{be}	38 ± 12
	Lumen diameter/μm	
Control group	877 ± 118	1033 ± 175^b
Drug group	1023 ± 157de	1128 ± 193

injury stimulus by migrating out of the media and proliferating to form a thickened intima[1,2]. In the present study, we used balloon catheter dilation as model system to investigate the mechanism of stenosis. Balloon injury markedly increased primary cultured aortic VSMC [^{3}H]thymidine incorporation, which usually taken to represent the synthesis of DNA. It indicated that balloon induced proliferation of VSMC in balloon-injured sites. The progress of proliferation and migration of VSMC towards the intima and secretion of mitogenes such as platelet-derived growth factor (PDGF), leading to the formation of a neointima[9,10]. This finding is consistent with our morphometric analysis. Our previous report has also demonstrated that PDGF receptor mRNA expression of aortic endothelium augmented after arterial injury[8]. The

enhanced uptake of VSMC [^{3}H]thymidine stimulated by balloon procedure was markedly inhibited by Lig. In this study, Lig has been found to inhibit intima hyperplasia. Evidence Lig may hold-back VSMC from static phase (G_1) to DNA synthestic phase (S)[7]. The results indicated that Lig could inhibit significantly the transcription of procollagen genes alpha 1-Ⅰ and alpha 1-Ⅲ[11]. It has also been reported that Lig causes increase of intracellular cAMP in both platelets and coronary artery[5], However, the mechanism of the inhibitory effect of Lig involved in proliferation of VSMC in the injured artery is unknown.

To our knowledge, no study has explored the role of Lig in inhibition of VSMC proliferation in balloon-induced animal model. Although there are distinct differences between coronary artery and aortic artery, VSMC is the most prominent cell type in the arterial wall. Furthermore, the present study on cultured VSMC was performed using rabbit serum obtained from those animals pretreated with the Lig. This new pharmacological testing method[12] has been claimed to make the result be similar to that obtained in an *in vivo* experiment. Therefore, the present investigation is helpful for further study on VSMC after balloon injury *in vivo*. However, the animal model system is designed only for the study of the inhibitory effect on stenosis, but not on restenosis. The effects of Lig on entirely mimic restenosis await further demonstration. Moreover many drugs have proved with antiproliferative effects in the rat and rabbit models after balloon angioplasty, but clinical trials with these agents have failed to show any benefit in reducing restenosis[13, 14]. It is necessary to open up new potential drugs in prevention of restenosis.

Taken together, our results indicated that Lig showed an obvious anti-proliferation effect of VSMC after balloon injury. Nevertheless, the mechanism and the role of Lig underlying the prevention effects on restenosis after PTCA warrant further investigation.

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川芎嗪对家兔主动脉损伤平滑肌细胞增殖的抑制作用[1]

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关键词 血管平滑肌; 培养的细胞; 胸主动脉; 气囊扩张术; 川芎嗪; 细胞分裂

目的: 探讨川芎嗪对主动脉球囊损伤平滑肌细胞增殖的抑制作用. **方法**: 20 只新西兰大白兔行主动脉球囊术. 以川芎嗪(40 mg·kg^{-1}·d^{-1}, iv)共 21 天(术前 7 天, 术后 14 天)处理的兔血清, 或直接加入培养基, 检测对原代培养的主动脉平滑肌细胞[^{3}H]胸腺嘧啶核苷酸掺入的影响. 并对血管壁组织切片显微测量内膜厚度和管径. **结果**: 球囊扩张处内膜厚度(77 ± 23) μm 和管腔狭窄(877 ± 118) μm 均较邻近正常处显著增加[分别为, (41 ± 13) μm, $P < 0.01$; (1033 ± 175) μm, $P < 0.05$], 而川芎嗪显著降低损伤后内膜的增厚(56 ± 16) μm, ($P < 0.05$)和管腔的变窄(1023 ± 157) μm, ($P < 0.05$). 用川芎嗪治疗后血清培养主动脉平滑肌细胞, 显示对[^{3}H]胸腺嘧啶核苷酸掺入明显抑制, 直接加川芎嗪入培养基对掺入抑制呈剂量依赖[(40 - 4000) μg/well]. **结论**: 川芎嗪对家兔主动脉球囊损伤平滑肌细胞增殖有显著抑制.

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