In vitro inhibition of proliferation of vascular smooth muscle cells by serum of rats treated with Dahuang Zhechong pill

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Abstract

Dahuang Zhechong pill (DHZCP) is a famous and classical Chinese herbal prescription, which is clinically used to treat hepatic, gynecological and cardiovascular diseases in China. The aim of this study was to observe the effects of the serum of rats treated with DHZCP on the proliferation of cultured rat vascular smooth muscle cells (VSMCs) stimulated by platelet-derived growth factor (PDGF), oxidized low density lipoprotein (ox-LDL) and hyperlipidemic serum (HLS), and on DNA, protein and collagen syntheses of VSMCs induced by PDGF in vitro. VSMCs proliferation was assayed by measuring the cell viability with MTT method, and syntheses of DNA, protein and collagen were evaluated by detecting [3H]-thymidine, [3H]-leucine and [3H]-proline incorporations, respectively. The results showed that PDGF, ox-LDL and HLS stimulated the proliferation of rat VSMCs in vitro. The serum of rats treated with DHZCP significantly inhibited the proliferation of rat VSMCs induced by the above stimulants and the incorporations of [3H]-thymidine, [3H]-leucine and [3H]-proline into rat VSMCs induced by PDGF in comparison with the model control group (P < 0.01). The data suggest that DHZCP is able to obviously inhibit VSMCs proliferation via interfering with syntheses of DNA and protein, and to decrease production of extracellular matrix by VSMCs through antagonizing collagen synthesis.

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1. Introduction

Dahuang Zhechong pill (DHZCP), a famous classical Chinese prescription from Traditional Chinese Medicine classic “Synopsis of Prescriptions of the Golden Chamber”, consists of 12 traditional Chinese drugs: Eupolyphaga sinensis Walker. (Polyphagidae), Rheum officinale Baill. (Polygonaceae), Scutellaria baicalensis Georgi. (Labiatae), Glycyrrhiza uralensis Fisch. (Leguminosae), Prunus persica Batsch. (Rosaceae), Prunus armeniaca L. (Rosaceae), Paeonia lactiflora Pall. (Ranunculaceae), Rehmannia glutinosa Libosch. (Scrophulariaceae), Toxicodendron vernicifluum F.A. Barkl. (Anacardiaceae), Tabanus bivittatus Mats. (Tabanidae), Hirudo nipponica Whitman. (Hirudinidae), Holotrichia diomphalia Bates. (Scarabaeidae) (Yan and Chen, 2000). It is reported that DHZCP produces the functions of invigorating blood and removing blood stasis, mollifying stomach and strengthening weakness and is mainly used to treat hepatic diseases in clinic practice and occasionally used for atherosclerotic therapy with good clinical effect in recent years.

Vascular smooth muscle cells (VSMCs) are one of the major constituents of blood vessel wall and very important for maintenance of vascular structure and function. Proliferation and migration of VSMCs play a key role in formation and progression of atherosclerosis and in development of restenosis (Ross, 1993; Schwartz et al., 1995). It is reported that platelet-derived growth factor (PDGF), oxidized low density lipoprotein (ox-LDL), hyperlipidemic serum (HLS) and many other factors stimulate proliferation of VSMCs (Witzum and Steinberg, 1991; Hughes et al., 1996). Our previous studies suggest that DHZCP is a potentially inhibitory agent on the experimental atherosclerosis for its anti-oxidative activity in vivo (Li et al., 2006). But, so far in vitro effect of DHZCP on VSMCs proliferation involved in formation of atherosclerosis is not defined. In this paper, it was of interest to determine whether DHZCP inhibited the proliferation of VSMCs induced by different patho-
logical stimulants and the primary mechanisms of action in vitro. Serum Pharmacology was first denominated by Tashino in 1984 (Tashino, 1988). Briefly, drug or compound of drugs are orally given to animals and blood is collected to separate serum after the definite time. Then, the serum containing drug(s) is used for in vitro experimental study of effect of the drug or the compound of drugs. This method is more fit to in vitro study of traditional Chinese drugs so as to avoid an interference with result from impurities that the crude drugs contain. Therefore, the method of Serum Pharmacology was used for the above purpose of research.

2. Materials and methods

2.1. Materials

DHZCP was purchased from Xi’an C.P. Pharmaceutical Co. Ltd. (PR China). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco RBL (Grand Island, NY, USA). HEPES, propidium iodide (PI), trypsin, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) and monoclonal antibody against smooth muscle actin were from Sigma Chemical Co. (USA). PDGF-BB was produced by RnD (USA). [3H]-thymidine, [3H]-leucine and [3H]-proline were from Beijing Atom High Tech Co. Ltd. (PR China).

ox-LDL was prepared according to the following method. LDL isolated from fresh plasma of health adult with density gradient centrifugation was oxygenized by CuCl2 (Chapman et al., 1981). Degree and content of oxidation were determined by the method of Lowry (Diessner and Knore, 1968) and the method of thiobarbituric acid (Yamazaki, 1967). HLS was from 3-month-old male Sprague–Dawley (SD) rats which were fed an atherogenic diet (21% (w/w) fat, 0.5% (w/w) cholesterol, 19.5% (w/w) casein) for 4 weeks and contained 87.07 mmol/l total cholesterol, 42.39 mmol/l triglyceride and 15.13 mmol/l high density lipoprotein.

2.2. Preparation of serum containing the tested drugs

Male SD rats weighing 200–250 g were purchased from the Experimental Animal Center of Xi’an Jiaotong University School of Medicine, Xi’an, PR China. Animals were housed in a room with temperature of 21–25°C, relative humidity of 50–60% and a 12 h light/12 h dark cycle (lights on at 08:00 a.m.). They had free access to food and water. All experimental procedures carried out in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Xi’an Jiaotong University. Ten rats were randomly grouped to normal serum group, positive control group and three doses of DHZCP-treated groups. Rats in normal serum group were treated with oral saline for 14 days. DHZCP at doses of 4.3, 8.5 and 17 g/kg was intraorally administrated to rats in the drug-treated groups for 14 days. Salvia miltiorrhiza Bge. (Labiatae) (SMB), which is demonstrated to inhibit proliferation of VSMCs (Meng et al., 1995), was given to rats in positive control group at 4.8 g/kg in the same way. At 2 h after the last administration, blood was aseptically obtained from the abdominal aorta of rat. The tubes containing blood were allowed to stand for about 0.5 h at 25°C before the serum was acquired by centrifugation at 2000 rpm for 20 min. Following two times of filtration with 0.22 μm cellulose acetate membrane, the serum was bottled, calculated in 56°C water for 30 min and stored at −20°C for use.

2.3. Cell culture

VSMCs were prepared from thoracic aorta of 2–3-month-old male SD rats weighing 100–150 g using the explant method (Skalli, 1986). Briefly, the aorta was freed of connective tissue and adherent fat, the endothelial cell layer of intima was removed with mechanical method, and the aortic artery was cut into about 3 mm cubes. Then, they were placed in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37°C until VSMCs exhibited a typical “hill and valley” growth pattern. Finally, identity of VSMCs was confirmed by the morphological examination and staining for α-actin. At confluency, the cells were trypsinized, counted and seeded. The confluent cells at passages 3–6 were used for the study.

2.4. Experimental procedures

The cell suspension solution in concentration of 1 × 105 ml−1 was inoculated into 96-well culture plate with 0.1 ml each well. After the cell incubation in 5% CO2 culture tank for 24 h under the conditions of 37°C and 80% relative humidity, the upper clear liquid was removed. Then, the experiment was carried out as the following procedures. The cells were divided into six groups. DMEM containing 10% normal serum was added into culture system in the normal control group, DMEM containing both 10% normal serum and stimulants (PDGF-BB, ox-LDL, HLS) into culture system in the model control group and DMEM containing 10% SMB-treated serum and the above-mentioned stimulants into culture system in the positive control group. In DHZCP-treated groups, DMEM containing 10% different doses of DHZCP-treated serum and the stimulants was added into culture system. At the same time, six parallel samples were kept in each group.

2.5. Investigation of proliferation of VSMCs stimulated by PDGF, ox-LDL and HLS

VSMCs were grown in 96-well plate for 24 h and starved in serum-free medium for 24 h. After the cell incubation with serum containing different treatments for 24 h, 50 ng/ml PDGF-BB (or 10 μg/ml ox-LDL or 100 μg/ml HLS) was added into the culture system for further 24 h incubation. Then, the cell viability was assayed by MTT method (Mosmann, 1983). Briefly, a volume of 200 μl of 0.5 mg/ml MTT in DMEM medium was added to each well and incubated with the cells for 4 h. Then, 150 μl of DMSO was added to the culture system to dissolve formazan
2.6. Observation of DNA and protein syntheses of VSMCs

DNA and protein syntheses were evaluated by measuring \([^{3}H]\)-thymidine and \([^{3}H]\)-leucine incorporations, respectively (Pakala et al., 1997). The cells were counted and seeded into a 96-well plate in complete medium at a density of 5000 cells/100 μl per well. The cells were allowed to adhere for 24 h at 37 °C, 5% CO2 and then incubated in the basal medium for another 24 h for synchronization of cell cycle. After the cell incubation with serum containing different treatments for 24 h, 50 ng/ml PDGF-BB was added into the culture system for further 14 h incubation. Then, the cells were supplemented with \([^{3}H]\)-thymidine or \([^{3}H]\)-leucine at 37 kBq/ml for an additional 10 h incubation. At end of the incubation, the medium was aspirated, and the cells were washed three times with PBS, removed with trypsin–EDTA and collected on filter plate using a cell harvester. The cells on the filter plate were added to 0.5 ml of scintillation fluid, and incorporation of \([^{3}H]\)-thymidine into DNA and incorporation of \([^{3}H]\)-leucine into protein were determined by a liquid scintillation counter (Model LS6500, Beckman, Germany). The incorporation was expressed as average cpm of \([^{3}H]\)-thymidine or \([^{3}H]\)-leucine per culture condition.

2.7. Assessment of collagen syntheses of VSMCs

Collagen synthesis of VSMCs was assayed by measuring \([^{3}H]\)-proline incorporation. The incubation and treatment of the cells were the same as Section 2.6. After 50 ng/ml PDGF-BB was added into the culture system for 14 h incubation, the cells were supplemented with \([^{3}H]\)-proline at 37 kBq/ml for an additional 10 h incubation. At end of the incubation, the medium was aspirated, and then the cells were washed three times with PBS, removed with trypsin–EDTA and collected on filter plate using a cell harvester. The cells on the filter plate were added to 0.5 ml of scintillation fluid, and incorporation of \([^{3}H]\)-proline into collagen was determined by a liquid scintillation counter. The incorporation was expressed as average cpm of \([^{3}H]\)-proline per culture condition.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>PDGF OD value</th>
<th>Inhibition rate (%)</th>
<th>OD value</th>
<th>Inhibition rate (%)</th>
<th>OD value</th>
<th>Inhibition rate (%)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>0.464 ± 0.038**</td>
<td>0.476 ± 0.069**</td>
<td></td>
<td></td>
<td>0.520 ± 0.072**</td>
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<tr>
<td>Model</td>
<td>0.965 ± 0.086</td>
<td>0.798 ± 0.043</td>
<td></td>
<td></td>
<td>0.894 ± 0.079</td>
<td></td>
</tr>
<tr>
<td>SMB</td>
<td>0.799 ± 0.069**</td>
<td>0.625 ± 0.012**</td>
<td>17.2</td>
<td>0.631 ± 0.050**</td>
<td>29.4</td>
<td></td>
</tr>
<tr>
<td>DHZCP (4.3 g/kg)</td>
<td>0.620 ± 0.058**</td>
<td>0.502 ± 0.002**</td>
<td>35.8</td>
<td>0.576 ± 0.064**</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>DHZCP (8.5 g/kg)</td>
<td>0.756 ± 0.095**</td>
<td>0.688 ± 0.064**</td>
<td>21.7</td>
<td>0.741 ± 0.060**</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td>DHZCP (17 g/kg)</td>
<td>0.908 ± 0.108</td>
<td>0.754 ± 0.076</td>
<td>6.9</td>
<td>0.836 ± 0.046</td>
<td>6.5</td>
<td></td>
</tr>
</tbody>
</table>

VSMCs were precultured in DMEM containing 10% different treated serum. The normal serum from rats treated with saline was used for the normal and model control groups. The serum from rats treated with Salvia miltiorrhiza Bge. (Labiatae) was used for SMB group as a positive control. The serum from rats treated with different doses of DHZCP was used for DHZCP groups. After 24 h, 50 ng/ml PDGF-BB or 10 μg/ml ox-LDL or 100 μg/ml HLS was added into the culture system for further 24 h incubation except the normal control group. Then, cell viability was assayed by MTT method. The data were presented as mean ± S.D. of six separate experiments. **P<0.01 vs. model control group.
versus model control). It is demonstrated that the serum of rats treated with DHZCP potently inhibits proliferation of rat VSMCs induced by the pathological factors such as PDGF, ox-LDL and HLS in vitro.

3.2. Effect of the serum of rats treated with DHZCP on DNA and protein syntheses of VSMCs induced by PDGF

Effect of the serum from rats treated with DHZCP on DNA and protein syntheses of rat VSMCs induced by PDGF was presented in Figs. 1 and 2, respectively. Compared with the normal control group, PDGF used in the model control group increased $[^{3}H]$-thymidine incorporation into the cells by 83.6% and $[^{3}H]$-leucine incorporation into the cells by 98.3% ($P < 0.01$). However, the serum from rats treated with 8.5 and 17 g/kg of DHZCP obviously inhibited PDGF-induced $[^{3}H]$-thymidine and $[^{3}H]$-leucine incorporations into the cells ($P < 0.01$ versus model control), which the maximal inhibitions achieved in the serum from rats treated with 17 g/kg of DHZCP were 43.9% for $[^{3}H]$-thymidine incorporation and 34.3% for $[^{3}H]$-leucine incorporation. The result shows that the serum from rats treated with DHZCP effectively inhibits DNA and protein syntheses of rat VSMCs induced by PDGF.

3.3. Effect of the serum of rats treated with DHZCP on collagen synthesis of VSMCs induced by PDGF

Effect of the serum from rats treated with DHZCP on collagen synthesis of VSMCs induced by PDGF was illustrated in Fig. 3. Compared with the normal control group, PDGF added in the model control group increased $[^{3}H]$-proline incorporation into the cells by 85.0% ($P < 0.01$). But, the serum from rats treated with 8.5 and 17 g/kg of DHZCP significantly decreased $[^{3}H]$-proline incorporation into the cells ($P < 0.01$ versus model control). The result suggests that the serum from rats treated with DHZCP is also able to inhibit collagen synthesis of rat VSMCs induced by PDGF.

4. Discussion

In methods of traditional pharmacology used for study of effect of traditional Chinese drugs in vitro, crude drug or compound of crude drugs is directly added into culture system of cells or tissues (Chen et al., 2003; Nishida and Satoh, 2003). Among the process, pH, osmotic pressure, impurities, physical and chemical characters of crude drugs in traditional Chinese drugs are bound to result in the changes of physiological conditions of in vitro reaction system so as to affect validity of experimental results (Meng et al., 1999). Moreover, compositions of traditional Chinese drugs from plant, animal and mineral are complex and many of the compositions do not work on the body until they undergo a series of biotransformation after absorption from gastrointestinal tract. Serum Pharmacology, which is put forward by Tashino (Tashino, 1988), gets over interferes of physical and chemical characters of crude drugs on experiment result so that in vitro experiment conditions are similar to the environment in which drugs work in vivo. So, Serum Pharmacology has extensively been used to study effects and mechanisms of traditional Chinese drugs in vitro.

Proliferation of VSMCs is a potential pathological progression in cardiovascular diseases such as hypertension, atherosclerosis and restenosis. Consequently, inhibition of VSMCs proliferation represents an important therapeutic strategy for treatment of these diseases (Ross, 1993; Schwartz et al., 1995). It is well known that the growth factors such as PDGF, epidermal growth factor (EGF) and angiotensin II are able to stimulate VSMCs proliferation (Newby and George, 1993). Among several growth factors stimulating VSMCs proliferation, PDGF plays a critical role in development of restenosis and atherosclerosis (Ferns et al., 1991). PDGF, as a VSMCs
mitogen in vitro, is able to mediate phenotypic modulation of VSMCs migration and proliferation in vivo. ox-LDL has also been shown to directly stimulate proliferation of VSMCs in several species as revealed by [3H]-thymidine incorporation to DNA (Sachinidis et al., 1997). The mitogenic effect of ox-LDL may be indirectly mediated through induction of PDGF production in macrophages, VSMCs (Stiko-Rahm et al., 1992) and other cells of the vessel wall (Boulanger et al., 1992). Meanwhile, HLS also induces proliferation of VSMCs via enhancing accumulation of cAMP (Kishi et al., 1985). Therefore, three different stimuli from PDGF, ox-LDL and HLS were used for different models of VSMCs proliferation in the study. Our results showed that the serum of rats receiving treatment of DHZCP significantly inhibited PDGF, ox-LDL and HLS stimulated VSMCs proliferation, indicating that DHZCP may be an effective inhibitor of VSMCs proliferation.

Proliferation of VSMCs is mainly based on syntheses of DNA and protein. In order to elucidate basis of inhibition of VSMCs proliferation by DHZCP, effect of the serum of rats receiving treatment of DHZCP on syntheses of DNA and protein of VSMCs was evaluated. The result demonstrated that DHZCP produced an inhibition of syntheses of DNA and protein of VSMCs.

Extracellular matrix (ECM) in vascular wall synthesized and secreted by VSMCs also plays a pivotal role in the formation of atherosclerotic plaque. ECM includes many non-cell components, among which collagen is a main protein molecule. Our result showed that the serum of rats receiving treatment of DHZCP inhibited synthesis of collagen by VSMCs.

Together considering our previous in vivo research (Li et al., 2006) and the present result, DHZCP has the ability to inhibit proliferation of VSMCs and synthesis of ECM collagen, which may be one of its pharmacological bases for prevention and treatment of atherosclerosis. However, we still do not know which crude drug or composition in the classic prescription mainly contributes to the effects. More experiments will be done to clarify effective components and action mechanisms of inhibition proliferation of VSMCs by DHZCP.

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References