Journal of Cardiothoracic-Renal Research (2006) 1, 135—139

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Angiotensin II-mediated apoptosis on human vascular smooth muscle cells

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Received 20 May 2006; accepted 20 May 2006

KEYWORDS
Angiotensin II;
Apoptosis;
Vascular smooth muscle cells;
AT-1 receptor;
AT-2 receptor

Summary While previous studies demonstrated that angiotensin II is a potent vasoconstrictor and mitogenic factor, the effect of angiotensin II on apoptosis in vascular smooth muscle cells remain controversial. Therefore, the current study was designed to investigate the action of angiotensin II on apoptosis in human vascular smooth muscle cells. Human saphenous vein was obtained from coronary artery bypass surgery ($n=6$) and was minced and incubated in the special tissue culture system in the absence or presence of angiotensin II (10^{-7} M) for 24 h. These studies were repeated with co-incubation of losartan (AT-1 receptor antagonist, 10^{-6} M) or PD-123319 (AT-2 receptor antagonist, 10^{-6} M). To detect the in situ DNA fragmentation, TUNEL staining was performed. TUNEL staining demonstrated that angiotensin II increased apoptosis in human vascular smooth muscle cells. This action of angiotensin II was enhanced by losartan and attenuated by PD-123319. Furthermore, co-incubation with both losartan and PD-123319 significantly reduced apoptosis levels. In conclusion, these data demonstrated that angiotensin II has potent apoptotic effect in human vascular smooth muscle cells through both AT-1 and AT-2 receptors. Furthermore, angiotensin II through AT-2 receptor has more potent apoptotic action in human vascular smooth muscle cells. This study indicated that angiotensin II plays an important role in the processes of apoptosis via angiotensin II receptors in human vascular smooth muscle cells.

Introduction

Apoptosis of vascular smooth muscle cells has been described in disease such as atherosclerosis, and restenosis after angioplasty and bypass grafting, as well as develop-

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doi:10.1016/j.jccr.2006.05.005

ment and physiological remodeling of vessels [1—4]. It has been believed that vascular structure is determined in large part by a balance between cell growth and cell death by apoptosis. However, the factors that regulate this balance between vascular smooth muscle cells death and survival remains to be defined.

Angiotensin II importantly contributes to the pathobiology of atherosclerosis and vascular disease not only via its role in hypertension but also via its direct effects on vascular smooth muscle cells. Angiotensin II has previously been shown to promote the growth of vascular smooth muscle cells via activation of AT-1 receptor [5—8]. More recent studies have demonstrated that the angiotensin II through AT-1 receptor or AT-2 receptor mediates apoptosis in vascular smooth muscle cells [9,10]. Angiotensin II represents a bifunctional growth factor by simultaneously stimulating proliferative and apoptotic pathways.

Based upon these previous studies, we hypothesize that angiotensin II may produce apoptosis in human vascular smooth muscle cells of saphenous vein via angiotensin II receptors. Therefore, the present study was designed to investigate the pathophysiological roles of angiotensin II-mediate apoptosis in human vascular smooth muscle cells.

Methods

Human subjects and venous tissue incubation

Saphenous veins of six patients were obtained intraoperatively during the coronary artery bypass surgery. This study was performed with the approval of the Institutional Review Board of the University of Maryland School of Medicine. The mean age (±S.E.M.) of the patients was 66 ± 2 years (range, 55—77). These patients include five men and one woman. After venous tissue excision, the samples were immediately placed in oxygenated, nominally Ca^{2+}-free Tyrode solution for transport to the laboratory. The tissues were chopped with scissors into cubic chunks (0.5 mm^3) in nominally Ca^{2+}-free Tyrode solution (36 °C). The Tyrode solution contained (mmol/L): NaCl 126.0, KCl 5.4, $MgCl_2$ 1.0, NaH_2PO_4 0.33, glucose 10.0, and HEPES 10.0, pH 7.4. The tissues then were placed in the special tissue culture system with 5% serum and culture medium (Clontech Laboratories, Inc., San Diego, CA) for 24 h. For negative control, we also investigated the TUNEL staining in fresh human saphenous vein tissue without incubation. Venous samples from each patient were divided into seven study groups: (1) control (vehicle group); (2) angiotensin II alone (10^{-7} M); (3) angiotensin II with losartan (10^{-6} M, AT-1 receptor antagonist); (4) angiotensin II with PD-123319 (10^{-6} M, AT-2 receptor antagonist); (5) angiotensin II with both losartan and PD-123319; (6) losartan alone; (7) PD-123319 alone. After incubation, the venous tissues were immediately fixed with 10% buffered formalin for further studies.

The venous tissue viability was determined by following methods: (1) staining with anti-alpha-1 anti-trypsin antibody, anti-kappa light chain antibody and anti-lambda light chain antibody; (2) plasma membrane marker ouabain-sensitive Na^+, K^+-ATPase activities; (3) RNA quality, judging from 28S/18S ratio of rRNA and GAPDH mRNA; (4) tetrazolium derivative reduction (MTT) assay; (5) electro microscopy; (6) TUNEL staining and DNA gel electrophoresis. Our previous studies demonstrated that all of these evaluations were no significant difference between fresh tissue and tissue after 24-h incubation in our special tissue culture system. These evidences established that our tissue culture system is reliable with no significant influence on the results of our experiments.

In situ identification of nuclear DNA fragmentation

To detect the DNA fragmentation in situ, nick-end labeling was performed with the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). The procedure is based upon the method described previously [1—4,9,10] with minor modifications. Briefly, human venous tissue was fixed in 10% formalin and embedded in paraffin. Serial sections were prepared at a thickness of 5 μm. Tissue sections were deparaffinized and treated with 3% hydrogen peroxide. The slides were incubated with 2% H_2O_2 for 7 min to inactivate the endogenous peroxidase and covered with 0.3 μg/μL terminal deoxynucleotidyl transferase (TDT, Boehringer Mannheim) and 0.04 nmol/μL biotinylated dUTP (Boehringer Mannheim) in TDT buffer containing 30 mmol/L cobalt chloride for 90 min at 37 °C. The reaction was terminated with buffer containing 30 mmol/L sodium citrate and 300 mmol/L NaCl. The slides were covered with 5% normal goat serum and applied with horseradish peroxidase-conjugated streptavidin (Nichirel). Peroxidase was visualized using the chromogen 3,3′-diaminobenzidine and H_2O_2. Counterstaining was performed with hematoxylin. For negative control to DNA fragmentation labeling, the serial sections were stained without terminal ozynucleotidyl transferase. An average of 1000 nuclei from random fields was analyzed for each data point. The percentage of apoptotic cells was determined by means of an apoptotic index. The apoptotic index (percentage of apoptotic nuclei) was calculated as (apoptotic nuclei/total nuclei) × 100%. Apoptotic index of 0.5 or less was considered to indicate the absence of apoptosis. Sample identities were concealed during scoring, and at least three samples were scored per group. The nuclei without DNA fragmentation stained blue as a result of counterstaining with hematoxylin. The nuclei with DNA fragmentation stained brown color, and nuclei without DNA fragmentation had clear blue nuclear regions.

Statistics

Results of the quantitative studies are expressed as means ± S.E.M. Statistical significance ($p < 0.05$) in comparisons between two measurements and among groups was determined by the two-tailed Student's *t*-test and by analysis of variance with the Bonferroni method, respectively.

Results

TUNEL staining demonstrated that angiotensin II increased apoptosis in human vascular smooth muscle cells. The apoptotic index in angiotensin II group (Fig. 1, TUNEL staining score: 27.6 ± 4.1%) was significantly increased compared