DNA Synthesis and Apoptosis in Smooth Muscle Cells From a Model of Genetic Hypertension

Alison M. Devlin, James S. Clark, John L. Reid, Anna F. Dominiczak

Abstract—The present study was designed to assess vascular smooth muscle cell (VSMC) proliferation and apoptosis in primary cultured VSMCs prepared from the aortic tunica media of adult (4 to 5 months old) age- and gender-matched groups of stroke-prone spontaneously hypertensive rats (SHRSP) and the normotensive reference strain, Wistar-Kyoto (WKY) rats. In the present study, VSMC proliferation was assessed with measurement of DNA synthesis in response to stimulation of G1/G0 arrested VSMCs with 10% serum, whereas apoptosis was measured in response to serum deprivation. Apoptosis in aortic VSMCs was assessed in vitro with the technique of Annexin V binding in combination with propidium iodide exclusion with bivariate flow cytometric analysis. The percentage of necrotic VSMCs in the cell populations was assessed simultaneously. The light-scattering properties of the cells were assessed to provide further information on cell shrinkage and chromatin condensation. Results of the present study have shown enhanced DNA synthesis in VSMCs from SHRSP (n=10; 5.2±0.9 cpm×10^3/mg protein) compared with WKY (n=12; 2.4±0.7 cpm×10^3 /mg protein; P<0.05, 95% CI, −5271 to −296). In addition, the results of the present study have demonstrated the role of serum in the survival of VSMCs in vitro, because SHRSP VSMCs underwent significantly more apoptosis in response to insult by serum deprivation (n=13; 10.21±1.8%) than WKY VSMCs (n=7; 3.44±1.4%; P<0.01, 95% CI, −11.5 to −2.0). Thus, it appears that both proliferation and apoptosis are enhanced in synthetic phenotype aortic medial VSMCs from the SHRSP in vitro. (Hypertension. 2000;36:110-115.)

Key Words: rats, inbred stroke-prone SHR ■ muscle, smooth, vascular ■ cells ■ apoptosis ■ annexin V

Apoptosis, or programmed cell death, is a well-documented phenomenon in many cellular systems. It plays a key role in tissue and organ development and has been identified in the physiological hypertrophy and remodeling of the vasculature that is found in hypertension, atherosclerosis, and restenosis after angioplasty. It has been postulated that apoptosis occurs as a consequence of a defective cell cycle, and it is known that damage to the cell cycle is an efficient trigger of apoptosis. The cell cycle regulatory molecules and signaling mechanisms involved in vascular smooth muscle cell (VSMC) proliferation and apoptosis are being elucidated in vitro, but the majority of these studies were not conducted with VSMCs from well-characterized models of genetic hypertension.

Previous in vitro studies have identified several factors that can modulate, in parallel or in opposition, VSMC proliferation and apoptosis. For example, DNA replication is inhibited and apoptosis is increased via the cAMP or nitric oxide pathway. In contrast, platelet-derived growth factor or insulin-like growth factor-I inhibits apoptosis and promotes DNA replication. In addition, overexpression of the transcription factor c-myc or its adenoviral functional homolog, E1A, increases both proliferation and apoptosis in VSMCs. The effect of growth factors and cytokines on cell fate is also extremely tissue specific, as is illustrated in a recent study in which transforming growth factor-β was found to potentiate endothelial cell apoptosis yet inhibit VSMC apoptosis. In the context of hypertension, apoptosis has been shown to be enhanced in target organs of spontaneously hypertensive rats (SHR) in vivo, and it is also known that VSMCs from the SHR exhibit abnormal growth in vitro that is manifest as an accelerated entry into S phase of the cell cycle as well as a hyperresponsiveness to growth factors. The current status of the regulation of vascular cell apoptosis both in vivo and in vitro, including the gene products and the signaling pathways involved, was comprehensively reviewed by Orlov et al.

One of the earliest features of apoptotic cells is the translocation of the negatively charged phospholipid species phosphatidylserine (PS) to the outer surface of the cell membrane. This feature was first reported to occur in cells of the immune system and was later demonstrated to unequivocally occur in apoptotic VSMCs. Furthermore, previous studies have shown that VSMCs undergo marked shrinkage and a reduced cell volume due to loss of intracellular water when undergoing death by apoptosis. The differential staining of VSMCs with Annexin V protein and propidium iodide through dual color “bivariate” flow cytometry may be used to quantitatively assess apoptosis and necrosis in vitro.

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Because the regulation of apoptosis in VSMCs derived from models of genetic hypertension remains poorly characterized, the aim of the present study was to assess VSMC proliferation as measured with \[^{3}H\]thymidine incorporation into newly synthesized DNA and apoptosis, as measured with Annexin V binding, in vitro in primary cultured aortic VSMCs isolated from adult age- and gender-matched stroke-prone SHR (SHRSP) and Wistar-Kyoto (WKY) animals. This present work examines further the phenotypic differences in growth and susceptibility to apoptosis in VSMCs isolated from hypertensive compared with normotensive arteries with the use of one of the best characterized models of genetic hypertension, the SHRSP.12,13

Methods

Experimental Animals

Adult parental SHRSP and WKY animals were obtained from colonies established in the Department of Medicine and Therapeutics, University of Glasgow, which have been maintained since 1991 by brother×sister mating as previously described.14 A total of 8 adult (4 to 5 months old) parental SHRSP (4 male, 4 female) and 6 adult (4 to 5 months old) parental WKY (3 male, 3 female) animals were used for the preparation of primary aortic medial VSMCs. Blood pressure measurements were made with tail-cuff plethysmography in conscious, restrained rats as previously described.12,13 Animals were sacrificed with halothane overdose, and tissue was removed as described later. These studies were approved by the Home Office according to regulations regarding experiments with animals in the United Kingdom. These regulations meet all the requirements of the American Physiological Society.

TABLE 1. Data on Blood Pressure, Age, and Gender of Rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>SHRSP\text{\textsubscript{Gla}}</th>
<th>WKY\text{\textsubscript{Gla}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>170.1±5.2\textsuperscript{*}</td>
<td>128.7±1.5</td>
</tr>
<tr>
<td>Age, d</td>
<td>150±6</td>
<td>146±11</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>4/4</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

\*P<0.0001 compared with WKY with Student's unpaired t test.

Preparation of Primary Aortic VSMCs From SHRSP and WKY

Animals were killed, and the full length of the thoracic aorta was carefully dissected. Primary VSMCs were isolated from the tunica media of thoracic aortas from SHRSP and WKY through a stepwise enzymatic digestion procedure as described previously.12 VSMCs, which were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (vol/vol) fetal bovine serum, 10% (vol/vol) equine serum, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL), had the usual growth characteristics and at confluence exhibited the typical “hill-and-valley” pattern and other VSMC characteristics as reported previously.12

Determination of VSMC DNA Synthesis In Vitro

VSMCs were used at passages 5 to 8 for all proliferation experiments. VSMCs were plated onto 24-well plates at the range of 3 to 4×10^4 cells/mL, 1 mL/well, in complete DMEM growth medium. VSMCs were allowed to grow until they reached subconfluency (80% to 90% confluent), after which the growth medium was replaced with serum-free medium, to synchronize the VSMCs at G0/G1 phase of the cell cycle. After a 24-hour serum-deprivation period, VSMCs were treated with DMEM that contained 10% (vol/vol) fetal bovine serum, 10% (vol/vol) equine serum, 1-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL), the usual growth characteristics and at confluence exhibited the typical “hill-and-valley” pattern and other VSMC characteristics as reported previously.12

TABLE 2. Growth of VSMCs in the Presence of 10% Serum as Determined with \[^{3}H\]Thymidine Incorporation Into Newly Synthesized DNA

<table>
<thead>
<tr>
<th>Strain</th>
<th>[^{3}H]Thymidine Incorporation, cpm×10^3/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKYGla</td>
<td>2.4±0.7</td>
</tr>
<tr>
<td>SHRSP\text{\textsubscript{Gla}}</td>
<td>5.2±0.9\textsuperscript{*}</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Cells were used at passages 5 to 8 for all experiments (n=10 to 12 separate experiments).

\*P<0.05 compared with WKY with Student’s unpaired t test.
Determinaton of VSMC Apoptosis In Vitro

VSMCs were used at passages 6 to 10 for all apoptosis experiments. VSMCs were plated onto 12-well plates at a density of $4.5 \times 10^4$ cells/mL, 3 mL/well, in complete DMEM growth medium and allowed to grow for a period of 24 to 48 hours. DMEM was then removed, and wells were washed once with serum-free medium; then, VSMCs were treated with DMEM containing 10% serum or serum-free medium for 24 hours to induce death by apoptosis. VSMCs were then harvested and pooled with their respective supernatant, so as to include any detached apoptotic VSMCs. VSMCs were pelleted by centrifugation at 2000 rpm for 5 minutes in a cold (4°C) PBS. Samples were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl$_2$) at a concentration of $1 \times 10^6$ cells/mL, to which was added sequentially 10 μL of 10 μg/mL fluorescein isothiocyanate–conjugated Annexin V protein (FITC Annexin V) followed by 10 μL of propidium iodide (50 μg/mL). There were 3 to 6 repeats for each experimental treatment conducted, and additional wells of VSMCs were harvested simultaneously for a series of controls to validate the correct electronic compensation of the fluorochromes.

After the addition of the fluorochromes and a 15-minute incubation period at 20°C to 25°C (room temperature) in the dark, binding was stopped by the addition of 400 μL of propidium iodide negative, apoptotic cells are Annexin V positive and propidium iodide positive (R2). Figure 1A shows a representative flow cytometric dot-plot for the binding of FITC-Annexin V to VSMCs grown in the presence of 10% serum in which the apoptotic population (R1) equals 7.1% and the necrotic population (R2) equals 3.8%. Figure 1B shows a representative flow cytometric dot-plot for VSMCs after 24-hour serum deprivation in which the apoptotic population (R1) is increased to 13.4% and the necrotic population (R2) is increased to 9.6%.

Statistical Analysis

The results are presented as mean ± SEM. Statistical analysis was performed with Student’s unpaired t test. A value of $P<0.05$ was considered statistically significant.

Materials

DMEM, fetal bovine serum, equine serum, penicillin/streptomycin, and L-glutamine were from Gibco Life Technologies Ltd. Annexin V (FITC) protein and other standard chemicals were purchased from Sigma Chemical Company Ltd. Annexin V (FITC) protein was from R&D Systems Europe Ltd. [methyl-3H]Thymidine (20 Ci/mmol) was from NEN/DuPont.

Results

A total of 8 separate preparations of primary VSMCs were established from 8 separate adult SHRSP animals, and 6 separate preparations of primary VSMCs were prepared from 6 separate adult WKY animals. Both groups of animals were age and gender matched, and the mean systemic blood pressures (SBPs) are given in Table 1. As expected, there was a highly significant difference in mean SBP between WKY (n=6; 128.7±1.5 mm Hg) and SHRSP (n=6; 170.1±5.2 mm Hg; P<0.0001, 95% CI, 28.9 to 54.0).

[3H]Thymidine Incorporation Into DNA in VSMCs From SHRSP and WKY

For [3H]thymidine incorporation experiments, aortic VSMCs from 5 (3 female, 2 male) SHRSP (SBP 163.2±2.9 mm Hg) and 4 (2 female, 2 male) WKY (SBP 129.7±1.5 mm Hg; P<0.001; 95% CI, 24.9 to 42.0) animals were used. Synchronized WKY and SHRSP VSMCs in vitro presented different responses in [3H]thymidine incorporation into new DNA in response to stimulation with 10% serum, which is the usual serum concentration used in cell growth studies. VSMCs from SHRSP (n=10; 5.2±0.9 cpm×10^3/mg protein) incorporated significantly more [3H]thymidine into newly synthesized DNA than did control VSMCs from WKY (n=12;
Apoptosis in VSMCs from SHRSP and WKY In Vitro

For in vitro apoptosis experiments with Annexin V (FITC) binding, primary aortic VSMCs from 5 (3 female, 2 male) SHRSP (SBP 172.2±8.3 mm Hg) and 4 (2 female, 2 male) WKY (SBP 127.75±1.7 mm Hg; P<0.01; 95% CI, 20.8 to 68.1) animals were used. A representative flow cytometric histogram for the binding of Annexin V (FITC) to VSMCs maintained in the presence of 10% serum or after treatment with serum deprivation for 24 hours is presented in Figure 2. The profile shows a shift in the FL-1 (FITC) Annexin V parameter, which corresponds to increased binding to PS residues exposed on the surface of VSMCs undergoing apoptosis in vitro.

In response to serum deprivation, VSMCs from SHRSP underwent significantly more death by apoptosis as assessed with Annexin V binding compared with VSMCs from age- and gender-matched WKY animals (WKY: n=7, 3.44±1.4%; SHRSP: n=13, 10.21±1.8%; P<0.01; 95% CI, −11.5 to −2.0) (Figure 3). VSMCs from SHRSP also underwent more death by necrosis in response to serum deprivation compared with VSMCs from WKY (WKY: n=9, 3.02±0.6%; SHRSP: n=10, 5.16±0.83%; P=0.054; 95% CI, −4.32 to 0.05).

Figure 4 displays representative forward light scatter (FSC), which is related to cell volume, versus side light scatter (SSC), which is related to granularity, cell frequency dot-plots for VSMCs in the presence of 10% serum (Figure 4A) compared with cells treated by serum deprivation for 24 hours (Figure 4B). A decrease in FSC and an increase in SSC is evident and corresponds to a decrease in VSMC size and an increase in cell granularity or density, which in turn corresponds to cell shrinkage and chromatin condensation. Therefore, the mean FSC parameter confirms VSMC volume decrease or shrinkage in response to serum deprivation treatment. In VSMCs from WKY (n=7), there is a reduction in mean FSC (cell volume) from 396.9±31 to 377.6±16 arbitrary units, whereas in VSMCs from SHRSP (n=15), there is a significant reduction in mean FSC from 384±17 to 337.9±9.9 arbitrary units (P<0.05; 95% CI, −86.7 to −5). However, the mean decrease in FSC in VSMCs from SHRSP compared with WKY in response to treatment with serum deprivation was nonsignificant. The results for cell volume in VSMCs from SHRSP and WKY before and after the induction of apoptosis are also summarized in Figure 4.

Discussion

This is the first study in which apoptosis has been assessed in conjunction with proliferation in primary cultured aortic VSMCs from SHRSP compared with WKY. Consistent with previous reports in the SHR model,2,8 we have documented enhanced growth that is accompanied by enhanced susceptibility to apoptosis in VSMCs in vitro from the SHRSP compared with the WKY. The results of the present in vitro study provide a further line of evidence to suggest that...
dysregulation of VSMC growth and apoptosis in hypertension is not merely secondary to increased blood pressure. Apoptosis was originally identified and reported on the basis of the unique cellular pathology and morphological changes that occur as revealed by electron microscopy; a technique that to this day remains a definitive means of identifying apoptotic cells. However, as far as we are aware, this is one of the first reports of the suitable application of Annexin V/propidium iodide staining to quantify VSMC apoptosis in vitro, although other workers have previously applied the technique to quantify the incidence of B cell and endothelial cell apoptosis in vitro. In our previous in vivo pharmacological intervention studies in the SHRSP, we have shown that levels of angiotensin II (Ang II) contribute to the development of VSMC polyploidy and hypertrophy in the blood vessel wall via the Ang II type 1 (AT1) receptor. In addition, previous studies in the SHR model have identified apoptosis as the cellular mechanism whereby vascular structure is normalized in response to AT1 receptor antagonism in vivo. Because polyploidy occurs when cells double their DNA content but fail to complete mitosis, it follows that the polyploid VSMCs in the blood vessel wall may represent a highly differentiated VSMC phenotype that is possibly more susceptible to death by apoptosis in vivo, although this has yet to be proved. However, the present study was conducted with primary aortic VSMCs in vitro, and it is well known that VSMCs’ dedifferentiate in vitro and adopt a fetal pattern of gene expression consistent with the “synthetic” phenotype. We have shown here that the abnormal proliferation of VSMCs from the model of genetic hypertension, the SHRSP, is associated with increased susceptibility to death by apoptosis once the serum mitogens and survival factors are completely withdrawn. A natural interpretation of this observation is that the mechanisms that control the cell cycle and apoptosis in VSMCs from the SHRSP are very closely related. Indeed, a recent report has documented a default molecule, which is expressed in the G2 M phase, that is responsible for the control of cell cycle progression or death by apoptosis. In this study, workers identified a protein that is an inhibitor of apoptosis known as survivin that may also preserve genetic fidelity, including the control of ploidy during cell division, although the study was not conducted in vascular cells. The present study with the SHRSP model provides further insight to confirm that VSMC growth and apoptosis dysregulation occurs in genetic hypertension. However, it is pertinent to note that the primary VSMCs used in the present study were all prepared in an identical fashion from tightly age- and gender-matched animal groups because previous workers have reported that cultured VSMCs exhibit developmentally regulated growth phenotypes that mimic the in vivo pattern. For this reason, the phenotypic differences observed here in the rates of VSMC proliferation and apoptosis in the SHRSP relate to adult animals in which hypertension is well established. However, in blood vessels of adult rodent models of hypertension, such as the SHR in vivo, the VSMC phenotype is highly differentiated, and recent studies with Ang II type 2 (AT2) receptor knockout mouse strains have documented the important role of the AT2 receptor subtype in the mediation of vasculogenesis and, more recently, in the mediation of VSMC differentiation. Because this receptor subtype has recently been shown to mediate VSMC apoptosis in vitro and has been previously shown to have different levels of expression in VSMCs from models of hypertension compared with respective controls, it is also possible that the differences in growth and apoptosis in VSMCs isolated from the SHRSP compared with the WKY, as reported in the present study, may be related to altered AT1/AT2 receptor expression levels or function. Such cellular mechanisms are presently hypothetical because they were not assessed in this study. However, these hypotheses that remain to be fully tested in models of genetic hypertension in vivo and in vitro will be of major interest in future studies.

Acknowledgments

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References


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