

SHORT COMMUNICATION

Involvement of gene expressions in apoptosis of vascular endothelial cells induced by rattlesnake venom

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ABSTRACT

Formation of apoptotic bodies is a typical character of apoptotic cell death, but how the processes are controlled is not known. In this study, we compared two apoptosis inducing systems in vascular endothelial cells (VEC). We found that the formation of apoptotic bodies during apoptosis induced by rattlesnake venom, which is an unique and specific apoptosis inducer to vascular endothelial cells, was much faster than that induced by deprivation of survival factors (aFGF and serum). When we blocked the synthesis of mRNAs in cells treated with rattlesnake venom by DRB (5, 6-dichloro-1- β -D-ribofuranosylbenzimidazole), an inhibitor of transcription, the formation of apoptotic bodies was dramatically inhibited. We examined the expression of P⁵³ gene and found that its expression was much higher in apoptosis induced by rattlesnake venom than that in apoptosis induced by deprivation of aFGF and serum. Our results suggest that gene expression is important and P⁵³ gene may play a major role in inducing the formation of apoptotic bodies in VEC.

Key words: *Apoptosis, rattlesnake venom, gene expressions, P⁵³ gene, endothelial cells.*

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INTRODUCTION

Apoptosis is a pathway of cell death characterized by a series of specific morphological changes. The great majority of programmed cell death from insects to mammals exhibit striking consistent morphological features, typical for apoptosis, suggesting that a molecular machinery for cell death has been conserved in the animal kingdom. This machinery which is still largely uncharacterized, executes a series of changes in the cells resulting in the condensation of the nucleus, fragmentation of DNA and plasma membrane changes like the formation of apoptotic bodies (membrane bound vesicles)[1-5]. A recent report showed that cell death and the induction of the formation of apoptotic body might be regulated by distinct pathways in hepatocytes[6]. In our laboratory, apoptosis of vascular endothelial cells has been studied, and we found that formation of apoptotic bodies during apoptosis triggered by rattlesnake venom is much faster than that in apoptosis induced by deprivation of FGF and serum[7],[8]. However, the regulation process of the formation of apoptotic bodies is unknown till now. In this study, we examined gene expressions and the possible role of P⁵³ gene in the formation of apoptotic bodies in human vascular endothelial cells induced by rattlesnake venom.

MATERIAL AND METHODS

Reagents

MCDB-104 medium was purchased from Kyokuto Pharmaceutical Industry, Tokyo, Japan. Fetal bovine serum (FBS) was purchased from Wako Pure Chemical Industry, Osaka, Japan. aFGF was extracted from bovine brains by the method of Lobb and Fett[9]. Rattlesnake venom was obtained commercially from Sigma Co., St. Louis, MO, USA. A Quick Prep mRNA Purification Kit was purchased from Pharmacia P-L Biochemicals Inc. Tokyo, Japan. Human P53 cDNA (2.16 kb) was obtained from Japanese Cancer Research Resources Bank (JCRB), Tokyo, Japan. A cDNA probe for the human gene for beta-actin was purchased from Wako Pure Chemical Industry, Osaka, Japan. A Random Primer DNA Labeling Kit was obtained commercially from Takara Industry, Otu, Japan. DRB (5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole) was purchased from Funakoshi Inc., Tokyo, Japan. All other reagents were of ultra-pure grade.

Cell culture and induction of apoptosis

Human umbilical vein endothelial cells (HUVEC) were obtained by the method of Jaffe[10]. The cells were cultured on gelatin-coated plastic dish in MCDB-104 medium supplemented with 10% FBS and 70 ng/ml aFGF (plus 100 μg/ml heparin) at 37°C in 5 % CO₂ and 95% air. In all of our experiments, we used cells with a population doubling level of 10 to 20. When the cells were grown until they became confluence, the medium was replaced by aFGF- and FBS- free MCDB-104 medium after washing once with the same medium. The cells were kept in aFGF- and FBS-free MCDB-104 medium with or without venom. The number of trypsinized cells was counted by a Coulter counter after deprivation of survival factors or being treated with rattlesnake venom for 0 h, 2 h, 4 h, 6 h, or 8 h. But the detached cells were washed off before trypsin digestion. The cells which were unstained by erythrosin B (0.5 %, Sigma) and kept attached to dishes after washing were regarded as living cells. Apoptosis (%) was calculated by the following equation: apoptosis (%) = 100 x (total number of cells-number of viable cells)/total number of cells.

Analysis of DNA fragmentation

2×10^6 cells were incubated in a digestion buffer that contained 200 $\mu\text{g/ml}$ proteinase K at 50 °C for 5 h. The cellular DNA was extracted once with phenol and once with a mixture of phenol, chloroform and 3-methyl-1-butanol (25:24:1, v/v). After digestion with RNase (final concentration, 600 $\mu\text{g/ml}$) at 37 °C for 30 min, the sample was subjected to electrophoresis on a 2% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The gel was then stained with ethidium bromide and photographed on a UV transilluminator.

Nuclear fragmentation assay

This experiment was performed according to the published method[11].

Extraction of mRNA and northern blot analysis

Control cells and cells harvested after 3 h and 6 h of treatment with rattlesnake venom were washed twice in PBS and their respective mRNA was extracted and purified with a Quick Prep mRNA Purification Kit. Nucleolytic activity in the snake venom did not interfere with the preparation of mRNA, as judged by the yield of mRNA and its ability to hybridize with b-actin cDNA. Purified mRNA (2 μg) was subjected to electrophoresis in a 1 % agarose-formaldehyde gel for 1 h at 60 V and then for 2 h at 120 V in $1 \times$ MOPS buffer. mRNAs were transferred to a Gene Screen Plus Nylon membrane. The membrane was prehybridized for 4 h at 42 °C in 50 % (v/v) formamide, 10 % sodium dextrin sulfate (Mr 500,000), $5 \times$ SSPE, 10 % (w/v) SDS, $5 \times$ Denhardt's solution, and 100 mg/L denatured salmon sperm DNA. Then the membrane was allowed to hybridize with a probe for P⁵³ that had been labeled with [³²P]-dCTP by the random priming method (Random Primer Labeling Kit protocol) at 42 °C for 24 h in the hybridization buffer, which was the same as the prehybridization buffer. The membrane was then washed twice at room temperature in $2 \times$ SSPE for 15 min then twice for 30 min. in $2 \times$ SSPE and 2 % SDS at 65 °C, and final two times in $0.1 \times$ SSPE at room temperature for 15 min. Washed membranes were exposed to x-ray film at -80 °C. For normalization of signals, membranes were reprobbed with a cDNA probe for b-actin mRNA that had been labeled with [³²P]-dCTP after stripping off the previously used DNA probe by boiling the membrane for 20 min. in $0.1 \times$ SSC and 1% SDS solution. (The compositions of buffers were as follows: TE buffer, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA; Tris-acetate buffer, 40 mM Tris-acetate, pH 8.0, 1 mM EDTA; PBS, 137 mM NaCl, 2.68 mM KCl, 8.07 mM Na₂HPO₄, 1.47 mM KH₂PO₄; MOPS buffer, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.4; SSPE, 10 mM Na₂PO₄, pH 7.4, 150 mM NaCl, 1 mM EDTA; $5 \times$ Denhardt's solution, 0.1 % (w/v) Ficoll, 0.1 % PVP, 0.1 % BSA and SSC, 150 mM NaCl, 15 mM sodium citrate).

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of apoptosis in VEC

When confluent cultures of VEC were deprived of aFGF and serum, some cells started to round up and finally detached from the dish[7]. Several membrane vesicles, named as apoptotic bodies, were shed from the detached cells. The process of the formation of apoptotic bodies proceeded with a very low speed during apoptosis triggered by the deprivation of aFGF and serum. By contrast, when rattlesnake venom (from 2 to 10 $\mu\text{g/ml}$) was added to the cultures of VEC, cells rapidly detached from the dish[8], and simultaneously, many apoptotic bodies were formed. After 8 h, about 70 % of cells became detached from the dish, accompanied with many apoptotic bodies (Fig 1).

The cells that had been deprived of survival factors (α FGF and serum) and that treated

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Fig 1.

The progress of apoptosis of VEC in two apoptosis inducing systems. Apoptosis (%) were calculated as described in Materials and Methods. Black triangle, cells cultured in MCDB-104 medium deprived of aFGF and serum; black circle, cells similarly cultured in the presence of rattlesnake venom. The concentration of rattlesnake venom was 6 mg/ml. Apoptosis (%) of control VEC was zero. Means and SD (bars) from five independent experiments.

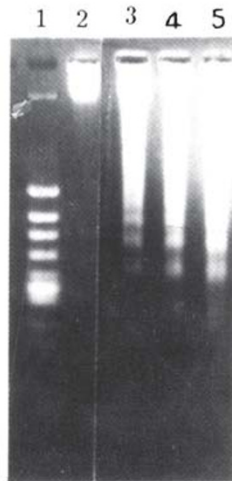
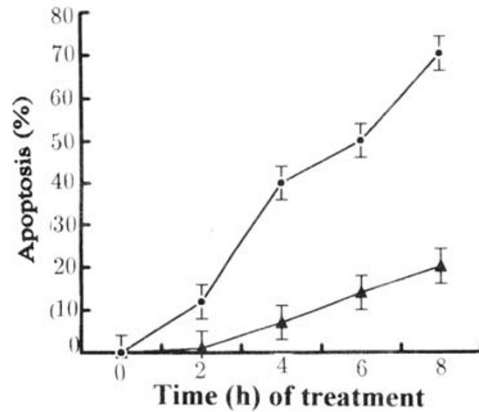


Fig 2.

DNA fragmentation assay of apoptosis of VEC as treated in Fig 1.

Lane 1, DNA markers (fragments of ϕ x-174 DNA, 79-1,057 bp);

Lane 2, DNA extracted from control cells;

Lane 3, DNA extracted from cells deprived of aFGF and serum for 6 h;

Lane 4, and lane 5, DNA extracted from cells treated with 6 μ g/ml rattlesnake venom for 3 h and 6 h, respectively.

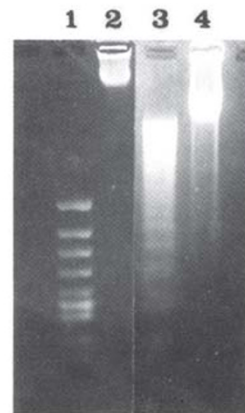


Fig 3.

DRB inhibition of DNA fragmentation in VEC induced by rattlesnake venom.

Lane 1, DNA markers (fragments of ϕ x-174 DNA, 79-1,057 bp); lane 2, DNA extracted from control cells;

lane 3, DNA of cells treated with 10 μ g/ml rattlesnake venom for 24 h;

lane 4, DNA of cells treated with 25 μ M DRB and 10 μ g/ml rattlesnake venom for 24 h.

with rattlesnake venom for 6 h were collected and the DNAs extracted respectively. Electrophoretic fractionation of DNA on an agarose gel revealed its fragmentation which is characteristic of apoptotic cell death (Fig 2).

Involvement of gene expression in formation of apoptotic bodies in VEC

It is interesting to know why the formation of apoptotic bodies in apoptosis induced by

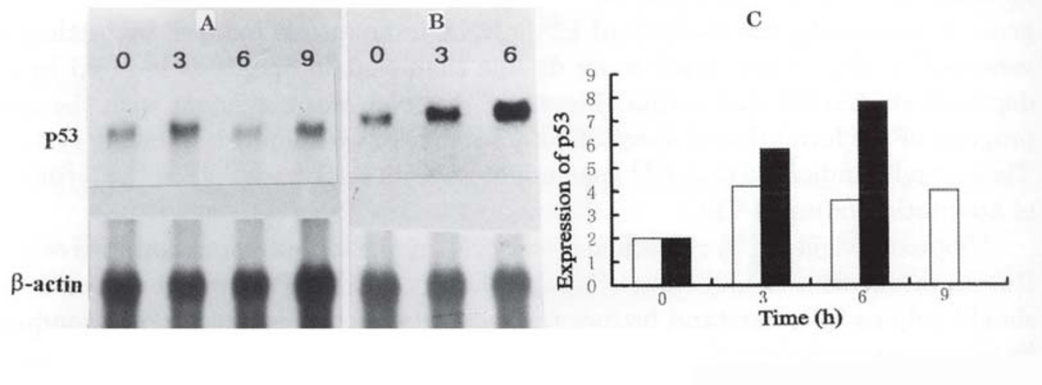


Fig 4.

Northern blot analysis and the relative level of the expression of P^{53} gene in the course of apoptosis of VEC in the two different inducing systems.

A: Cells deprived of aFGF and serum for 0 h (lane 0), 3 h (lane 3), 6 h (lane 6) and 9 h (lane 9) respectively. mRNA (2 μ g) in each lane was probed with [32 P]-dCTP by the random priming method.

B: Cells deprived of aFGF and serum were treated with 10 μ g/ml rattlesnake venom for 0 h (lane 0), 3 h (lane 3) and 6 h (lane 6) respectively. mRNA (2 mg) of each lane was probed by the same method as in A.

C: The relative level of expression of P^{53} gene under the same condition as in A (open bars) and B (filled bars). The relative levels of gene expression were determined by densitometry of autoradiograms.

The result was a representative one from three independent reproducible experiments.

rattlesnake venom was so much faster than that in apoptosis induced by deprivation of aFGF and serum. The mRNA synthesis of VEC in the presence of rattlesnake venom was thus blocked by using a specific inhibitor of transcription, DRB. About 100 % of cells treated with 10 μ g/ml rattlesnake venom for 24 h became fragmented into apoptotic bodies, but, almost all cells treated with both 25 μ M DRB and 10 μ g/ml rattlesnake venom for the same period remained round. These data suggested that gene expression was necessary for the formation of apoptotic bodies in VEC apoptosis.

When the nuclear fragmentation and DNA fragmentation were examined for the cells treated only with rattlesnake venom and those treated with both DRB and rattlesnake venom, the death of the former cells was clearly due to apoptosis, but in case of latter cells, DRB drastically inhibited the nuclear fragmentation and DNA fragmentation induced by rattlesnake venom (Fig 3).

The possible role of P^{53} gene expression in the formation of apoptotic bodies in VEC

To confirm the involvement of gene expression in the formation of apoptotic bodies, the

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change of P⁵³ mRNA expression was followed in these two apoptosis inducing systems of VEC, with the results showing the increased expression of P⁵³ gene. Interestingly, the increase of P⁵³ mRNA in apoptosis induced by rattlesnake venom (Fig 4B, C) was much more drastic than that in apoptosis induced by the deprivation of α FGF and serum (Fig 4A, C)), which was consistent with the rapid progress of the formation of apoptotic bodies in the two apoptosis inducing systems. These results indicated that P⁵³ gene might have an important role in the formation of apoptotic bodies in VEC.

Apoptosis triggered by rattlesnake venom is unique to vascular endothelial cells[8]. But its mechanism of induction of apoptosis is not clear. The results reported here should help us to understand further the molecular mechanism of apoptosis induced by rattlesnake venom.

The formation of apoptotic bodies is a typical character of apoptotic cell death, however, how the processes are controlled is not known. In a recent report, P⁵³ gene-deficient hepatocytes were found to be not able to form apoptotic bodies despite that IFN-g induced cell death in normal hepatocytes, which implied that P⁵³ gene may play a major role in the induction of apoptotic body formation[6]. Our results reported here are in line with this notion.

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