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INDUCTION OF APOPTOSIS BY HEMORRHAGIC SNAKE VENOM IN VASCULAR ENDOTHELIAL CELLS

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Vascular degeneration appears to play crucial roles in producing many vascular mulfunctions (1-3). In order to identify specific inducers of programmed death in vascular endothelial cells (VEC), examinations were made of the effects of substances that are known to affect the vascular system by using VEC in culture (4,5). We found that hemorrhagic snake venoms induced apoptotic cell death or programmed cell death of VEC. By contrast, neurotoxic snake venoms did not induce programmed cell death but caused necrosis at much higher doses of the venoms. No effect of hemorrhagic venom was observed with many types of cultured cells other than VEC. Thus, hemorrhagic snake venom appears to be a useful tool for studies of the molecular mechanisms of vascular apoptosis. The results also suggest a possible mechanism of action of hemorrhagic snake venom on the vascular system.

Apoptotic cell death is one of the characteristics of multicellular organisms that requires controlled differentiation, morphogenesis, etc (6). Recently, several specific factors that induce apoptosis in the immune system (e.g., self-antigen (7), HIV (8)) or in the nervous system (e.g., glutamate (9)) have been identified. Such studies have helped not only in the understanding of mechanisms relevant to the malfunction of these systems but have also suggested possible new therapeutic modalities (10). In the vascular system, mechanisms associated with malfunctions, such as arteriosclerosis (1) and hemorrhagic necrosis in tumors (2) have been studied in some detail. Apoptosis in vitro of VEC has provided another system for studies of vascular malfunction. Since VEC, like lymphocytes and neurons, undergo apoptosis upon removal of fibroblast growth factor (FGF) (4,5), we postulated that vascular homeostasis might be controlled by a mechanism that was mediated by a specific apoptosis-inducing and, hence, lethal factor. As a candidate for an

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agent that might mimic the effect of such factors, hemorrhagic snake venom was examined for its effect on VEC because such venom is known to induce degeneration of the vascular system by an as yet unknown mechanism.

MATERIALS AND METHODS

Reagents. MCDB-104 was purchased from Kyokuto Pharmaceutical Industry, Tokyo, Japan. MEM was purchased from Nissui Pharmaceutical Co. Tokyo, Japan. Fetal bovine serum (FBS) was purchased from GIBCO, Grand Island, NY, U. S. A. Snake venoms were obtained commercially (See legends to figures). All other chemicals were of ultra pure grade.

Cell cultures. VEC from human umbilical vein (4) were cultured on collagen-coated plastic dishes in a culture medium, MCDB-104, supplemented with 10% FBS and 70 ng/ml FGF (supplemented with 100 μg/ml heparin) at 37°C in 5% carbondioxide and 95% air. BFAS-63C (bovine smooth muscle cells) and HPAEC (human pulmonary artery endothelial cells) were cultured in a medium consisting of MCD-104 supplemented with 10% FBS. RSMC-3 (rat smooth muscle cells) and HF (human embryonic lung fibroblasts) were cultured in a medium consisting of MEM supplemented with 10% FBS.

Viability assay. Cells were grown until culture reached sub-confluent state, then the medium was replaced with MCDB-104 medium after washing once with the same medium. The cells were kept in an incubator with or without venom. The number of trypsinized cells was counted by a Coulter counter after 24h or 30 hr. Cells detached from the dish was washed off before the treatment with trypsin.

DNA analysis.DNA was extracted and analyzed as described previously (4). DNA was stained with ethydium bromide to obtain profiles.

RESULTS AND DISCUSSION

When a small amount of rattlesnake venom was added to a sub-confluent (11) culture of VEC, cells started to die within 6 h after the addition of venom. After 24 h, quite few cells remained intact. Microscopic observation of the process of cell death revealed that the cells exhibited morphological changes characteristic of apoptotic cell death, namely, the fragmentation of cells. The process of cell death induced by the venom could be inhibited by the addition with venom of FGF or cycloheximide an inhibitor of protein synthesis (Fig. 1). These observations were also evidence of venom-induced apoptotic cell death. By contrast, addition of cobra venom, a neurotoxic venom, did not induce cell death unless a relatively large amount of venom was added. Furthermore, the cell death induced by cobra venom was not inhibited by the addition of cycloheximide or FGF, observations that suggest that cell death was necrotic in this case. The results indicated that snake venoms have differential effects on cell death in VEC. We purchased hemorrhagic venoms from the Southern European sand viper, Japanese mamushi, puff adder, habu, and rattlesnake; and we also purchased neurotoxic venoms from the banded krait, sea snake, and Thailand cobra. All of the hemorrhagic venoms induced cell death which could be

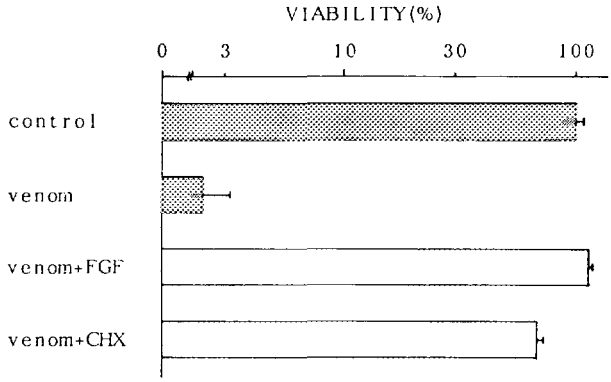

Fig. 1. Viability of VEC in the presence of hemorrhagic snake venom. A sub-confluent culture (about 10^5 cells/cm^2, 10-20 PDL) of VEC cells from human umbilical vein (4) was stimulated with 2 μg/ml rattlesnake (Crotalus atrox) venom (Sigma Co., St Louis MO, USA), the same amount of venom plus 70 ng/ml FGF, or the same amount of venom plus 1 μg/ml cycloheximide. The number of living cells after 24 h of such stimulation was counted with a Coulter counter. Averages of results from three experiments are shown. (PDL=population doubling level, age related).

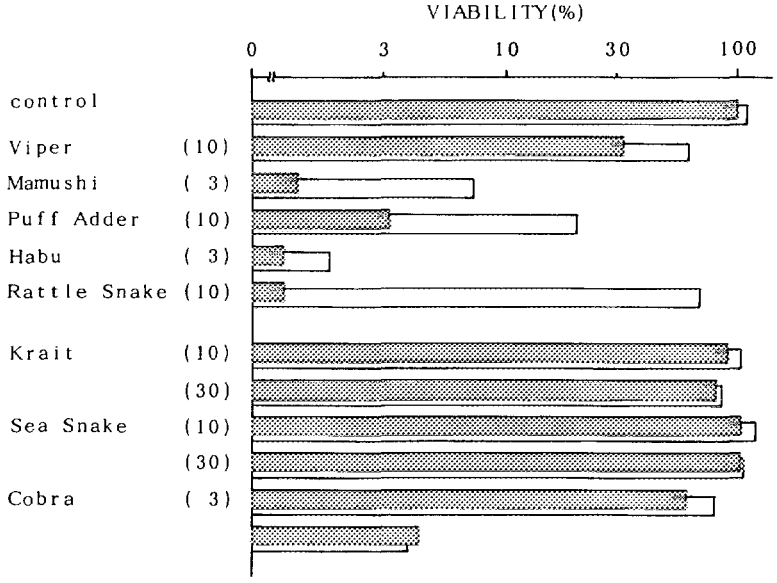

Fig. 2. Viability of VEC in the presence of various snake venoms. The experimental conditions were the same as those described in the legend to Fig. 1. and the number of living cells was counted after 30 h of stimulation. Venoms tested were as follows: viper, Viper ammodytes (Sigma); mamushi, Agkistrodon halys blomhottii (Sigma); paff adder, Bitis arietans (Sigma); habu, Trimeresurus flavoviridis (Wako Pure Chemicals, Osaka, Japan); rattlesnake; bungarus, Bungarus fasciatus (Sigma); sea snake, Enhydrina schistosa (Sigma); cobra, Naja naja kaouthia (Wako).
The shaded bars represent viabilities in the presence of venom and open bars represent viabilities in the presence of venom plus 1 μg/ml cycloheximide. Representative results are shown.

prevented by cycloheximide, while in no case did the neurotoxic venoms induce cell death that could be inhibited by cycloheximide (Fig. 2).

It appears that, in VEC, hemorrhagic venoms induce apoptotic cell death and neurotoxic venoms induce necrotic cell death, as judged by the morphological changes and the effects of cycloheximide. In order to confirm this point, we collected dead cells and extracted their DNA. Fractionation of the DNA by electophoresis revealed the characteristic fragmentation of DNA in cells killed by hemorrhagic venom but not in cells killed by neurotoxic venom (Fig. 3). Thus, we were able to conclude that hemorrhagic snake venoms and not neurotoxic venoms induce apoptosis in VEC.

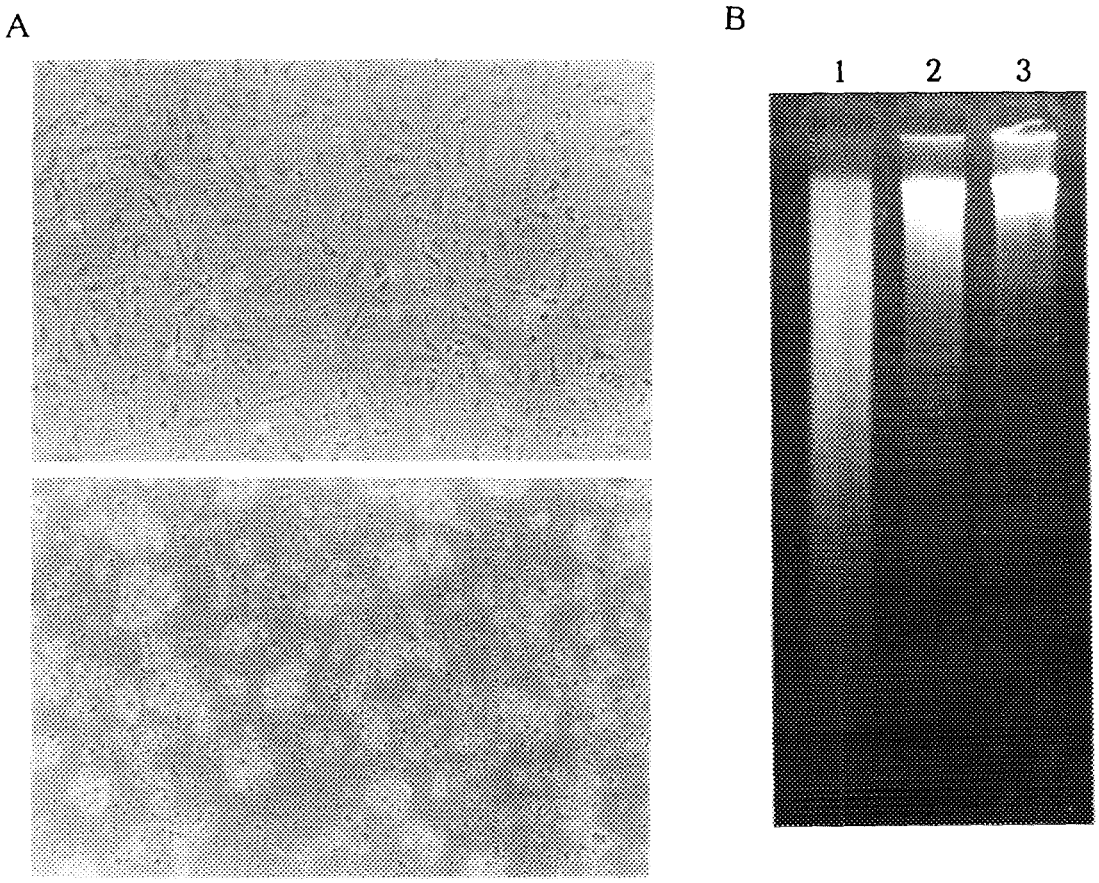

Fig. 3. Venom-dependent induction of apoptotic cell death or necrotic cell death. A. Morphological differences between VEC stimulated with hemorrhagic venom and those stimulated with neurotoxic venom. The upper panel shows cells treated with 10 μg/ml cobra venom; the lower panel shows cells treated with 10 μg/ml rattlesnake venom. B. Size distribution of DNA obtained from dead cells after 12 h of treatment. DNA was extracted from the treated cells and analyzed by electrophoresis. Profiles are shown of DNA from dead cells that had been treated with cobra venom (lane 1), rattlesnake venom (lane 2), and habu venom (lane 3).

Next, we asked whether the apoptotic response was a unique feature of vascular cells. The effects of hemorrhagic venom on bovine smooth muscle cells, rat smooth muscle cells, human embryonic lung fibroblast cells, and human pulmonary artery endothelial cells were examined. Hemorrhagic venom induced apoptotic cell death in artery endothelial cells but not in any other cells (Fig. 4). At high concentrations of hemorrhagic venom, cells other than VEC were subject to necrotic cell death and such cell death was not inhibited by cycloheximide.

The study described here shows that a primary effect of hemorrhagic venom on vascular cells is the triggering of the mechanism responsible for apoptosis. The data obtained so far suggest that this type of response to such venom may be a unique property of VEC. The results agree to the in vivo observations of the effect of hemorrahgic venom that showed the venom acted on vascular system to produce a pin-hole instead to alter the permeability of the system (12). Snake venom is a complex mixture of many

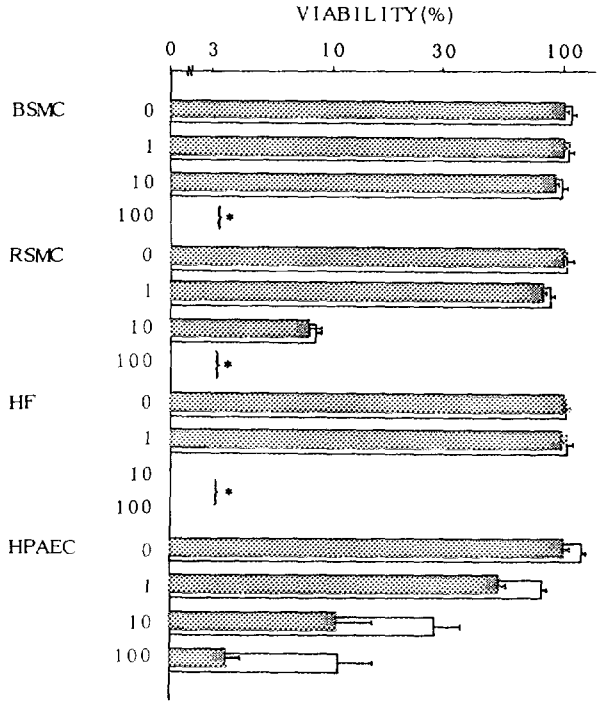

Fig. 4. Effects of hemorrhagic venom on the viability of several types of mammalian cell. The experimental conditions were the same as described in the legend to Fig. 1. The cells tested were BSMC, BFAS-63C (bovine smooth muscle cells, 28 PDL); RSMC, RSMC-3 (rat smooth muscle cells, 12 PDL); TIG-7, human embryonic lung fibroblasts (30 PDL); P4-EC, human pulmonary artery endothelial cells (5 PDL). Shaded bars, in the presence of venom alone; open bars, in the presence of venom plus 1 μg/ml cycloheximide. The asterisks indicate that the value is less than 3%. The data are the averages from three independent experiments.

biologically active polypeptides (13) and, therefore, identification of the substance(s) that is responsible for the apparently specific induction of apoptotic cell death is our next goal. Several polypeptides have been identified as participants in the initiation of the hemorrhagic response. The targets of these polypeptides are thought to be platelets, blood plasma, basement membrane, etc. However, their capacities for induction of apoptotic cell death in vascular endothelial cells are unknown. The results reported here should help us to understand the molecular mechanism of the hemorrhagic action of snake venoms and of homeostasis of vascular systems.

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