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Purification of a Vascular Apoptosis-Inducing Factor from Hemorrhagic Snake Venom

S. Masuda, S. Araki, T. Yamamoto, K. Kaji,* and H. Hayashi

*Sugashima Marine Biological Laboratory, School of Science, Nagoya University, Toba, Mie 517, Japan; and *Department of Life Health Science, Shizuoka University, Shizuoka, Shizuoka 422, Japan*

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Hemorrhagic snake venom induces apoptosis in vascular endothelial cells specifically. We report here the purification of an apoptosis-inducing factor from the venom of the rattlesnake *Crotalus atrox*. The purified factor was a homodimeric protein with a molecular mass of 110 kD and an isoelectric point of 8.5. When exposed to the factor, vascular endothelial cells in culture died, with the characteristic features of apoptosis, such as fragmentation of cells and cleavage of DNA into fragments that yielded a characteristic ladder pattern upon electrophoresis. The activity seemed to be specific to endothelial cells. This factor may prove to be a useful tool for studies of the molecular mechanisms of vascular apoptosis. © 1997 Academic Press

Vascular endothelial cells (VEC) are important in wound healing, in the progression of cancer, in the homeostasis of the circulatory system and in embryogenesis as a consequence of their capacity for angiogenesis (1,2). Vascular degradation is of equal importance in such phenomena. For example, degeneration of tumor vessels induces tumor regression (3), and degradation of vessels during the healing of a wound is important for the prevention of a hypertrophic scar (4). Furthermore, many vascular degenerative disease are known, such as atherosclerosis and diabetic angiopathy, as well as hemorrhagic diseases or conditions caused by viruses, bacteria, snake venoms and autoantigens (5).

We have been investigating cell death in VEC because it seems possible that cell death might play an active role in the control of vascular degeneration (8,9). We showed previously that crude hemorrhagic snake venoms are effective for the induction of apoptosis in VEC. The activity is specific to VEC (10), and neurotoxic snake venoms lack such activity. In this study, we purified a vascular apoptosis-inducing factor from the hemorrhagic venom of the rattlesnake *Crotarus atrox*.

MATERIALS AND METHODS

Reagents. MCDB-107 medium for cell culture was purchased from Kyokuto Pharmaceutical Industry (Tokyo, Japan). Fibroblast growth factor (FGF) was extracted from bovine brains by the method of Lobb and Fett (6). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY, U.S.A.). Crude snake venom and crystalline bovine serum albumin were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Cycloheximide (CHX) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of ultrapure grade.

Cell culture. Human umbilical vein endothelial cells were obtained by the method of Jaffe *et al.* (7). The cells were cultured on collagen-coated plastic dishes in MCDB-107 culture medium that had been supplemented with 10% FBS, 70 ng/ml FGF, and 100 μg/ml heparin at 37 °C in an atmosphere of 5% carbon dioxide and 95% air. The cells were stained immunochemically by treatment with antibodies raised in rabbit against human factor VIII to confirm that they were endothelial cells. RSMC-3 (rat smooth muscle cells), TIG-7 (human fibroblasts) and ECV-304 (transformed VEC) were cultured in a medium that consisted of MEM supplemented with 10% FBS.

Assay of viability. VEC were grown until cultures had reached confluence or sub-confluence. Then the medium was replaced with the basal medium (without FGF and FBS) after cells had been washed once with phosphate-buffered saline without magnesium or calcium (PBS^-). The cells in test media were kept in an incubator with or without the fraction to be tested. In order to determine numbers of living cells, which adhered to the surface of the plastic dishes, trypsinized cells were counted in a Coulter counter (Coulter Electronic, Inc., Hialeah, FL, U.S.A.) after an appropriate time interval. Cells that had disintegrated and detached from dishes were washed away with PBS^- before treatment with trypsin. The cells that remained attached to dishes after washing were not stained with erythrosin B and were regarded as living cells.

Purification of the protein. Crude snake venom from *Crotalus atrox* was fractionated by column chromatography on hydroxyapatite and isoelectric focusing in a polyacrylamide gel. The activity of fractions was assayed by monitoring the viability of cultured VEC and fractions with apoptotic activity were identified. Protein concentrations were measured by the BCA assay (Pierce Chemical Co. Rockford, IL, U.S.A.) on microtiter plates with bovine serum albumin as the standard.

Gel electrophoresis. Cells were plated on dishes of 100 mm in diameter. After treatment with test medium, DNA was extracted from cells and loaded immediately onto a 2.0% agarose gel for conventional electrophoresis, as described previously (8).

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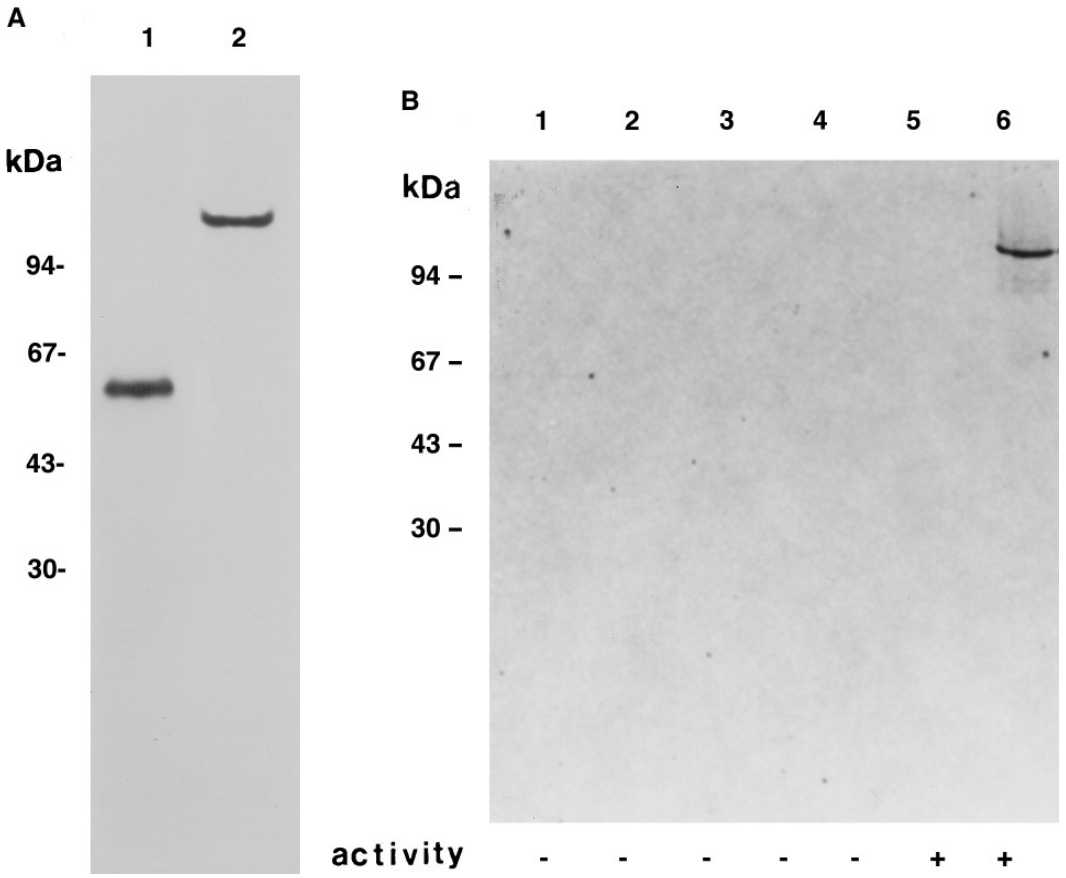

FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified protein. A) Purified VAP from *Crotalus atrox* was subjected to electrophoresis in an SDS-polyacrylamide gel (12.5% polyacrylamide). Lane 1, VAP after reduction by 2-mercaptoethanol, lane 2, native VAP. Mobilities of marker proteins are also shown. B) Separation of the purified protein by chromatography on a column of phenyl-Sepharose. 20 μg of the purified protein, in a buffer of 10 mM Tris-HCl (pH 7.0)-1 mM $CaCl_2$ supplemented with 1 M ammonium sulfate, was applied onto a column of phenyl-Sepharose (5 mm i.d. $\times$ 15 mm). Adsorbed protein was eluted by stepwise decrease of the concentration of ammonium sulfate, and then by increase of the concentration of Triton-X100 in the same buffer. The activity of each fraction and profile of protein were investigated. Lanes 1, 2, 3, and 4 are the fractions eluted by 1 M, 0.1 M, 0.01 M and 0 M ammonium sulfate, respectively. Lanes 5 and 6 are the fractions eluted by 0.1% and 1% Triton-X100, respectively.

Assay of the degradation of phospholipids and of proteolytic activity. Phospholipid degradation was assayed by two-dimensional thin-layer chromatography. Cultures of VEC were labeled with [^{32}P]H_3PO_4 for 1 day. Cells were harvested and phospholipids were extracted by the method of Bligh and Dyer (18). Phospholipids were separated by two-dimensional thin-layer chromatography with a mixture of chloroform, methanol and 20% aqueous ammonia (30:50:10, v/v) in the first dimension and with a mixture of chloroform, methanol, acetic acid and water (135:60:18:3, v/v) in the second dimension. Phospholipids were detected by autoradiography. Proteolytic activity was assayed by the degradation of denatured bovine serum albumin as the substrate.

RESULTS

Crude snake venom from *Crotalus atrox* was fractionated by column chromatography on hydroxyapatite. The column (0.8 cm i.d. $\times$ 3 cm) was equilibrated to 10 mM sodium phosphate buffer (pH7.0) and the solution of the venom (10 mg in 1 ml of the same buffer) was loaded onto the column. The adsorbed protein were eluted successively with 1) 10 mM phosphate buffer, 2) 0.1 M phosphate buffer, 3) 0.5 M phosphate buffer. Fractions that induced apoptosis in vascular endothelial cells were eluted from the column by 0.1 M sodium phosphate buffer. The active fractions were combined and concentrated by ultrafiltration with 30,000 NMWL membrane (Millipore) and then fractionated further by isoelectric focusing (column size 4 mm i.d. $\times$ 11.5 cm). The activity migrated with a pI of 8.5. After these two purification steps, the active fraction apparently contained only a single protein, as indicated by SDS-PAGE. The purified protein was confirmed to be the active factor as follows. First, the activity required the presence of Ca^{2+} ions, and the mobility of the purified protein during IEF shifted in the presence of 1 mM $CaCl_2$. Second, the activity and the protein coincided after chromatography on a column of phenyl-Sepha-

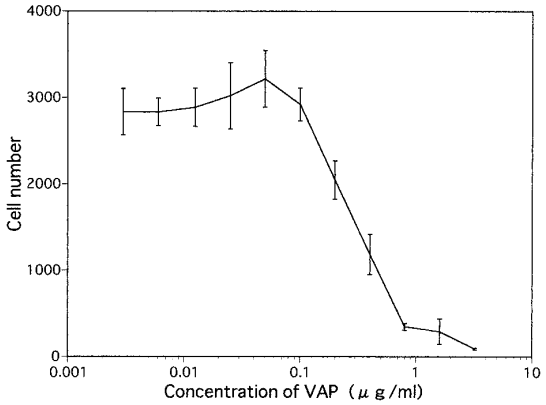

FIG. 2. Dose-response curve for purified VAP. The complete medium was replaced by medium that had been supplemented with VAP at the indicated concentration and the number of viable cells in each sample was determined after 24 h. Each number was normalized to that of the control sample which was taken as 100%. The data are averages (+S.E.) of results from four samples of cells in each case.

rose. Third, the activity was sensitive to reducing agents such as mercaptoethanol and glutathione. The purified protein migrated as a single band of a protein of 60 kD during SDS-PAGE (Fig. 1A, 1B). In the absence of a reducing agent it migrated as a protein of 110 kD, a result that suggested that the active protein was a homodimer. We named the purified protein vascular apoptosis-inducing protein (VAP). The amount of VAP in the crude venom was estimated to be about 0.5% (w/w). The half-lethal dose of VAP against VEC in culture was 0.3 μg/ml (Fig. 2). This value was probably an underestimate since substantial inactivation of VAP was apparent during the purification.

VAP induced extensive cell death in VEC within 12 hours at a concentration of 1 μg/ml (Fig. 3). In previous studies on cell death *in vitro*, we identified the features that are typical of apoptotic cell death in VEC (8–10). namely, cell shrinkage and the formation of blebs on the cell surface that finally results in generation of apoptotic bodies. These morphological changes can be observed under the light microscope. When VEC were treated with VAP, morphological changes that were typical of apoptosis were observed. Thus, it appeared that the VAP-induced cell death was a type of apoptosis. In contrast to the crude venom, VAP did not trigger the detachment of cells nor did it induce ne-

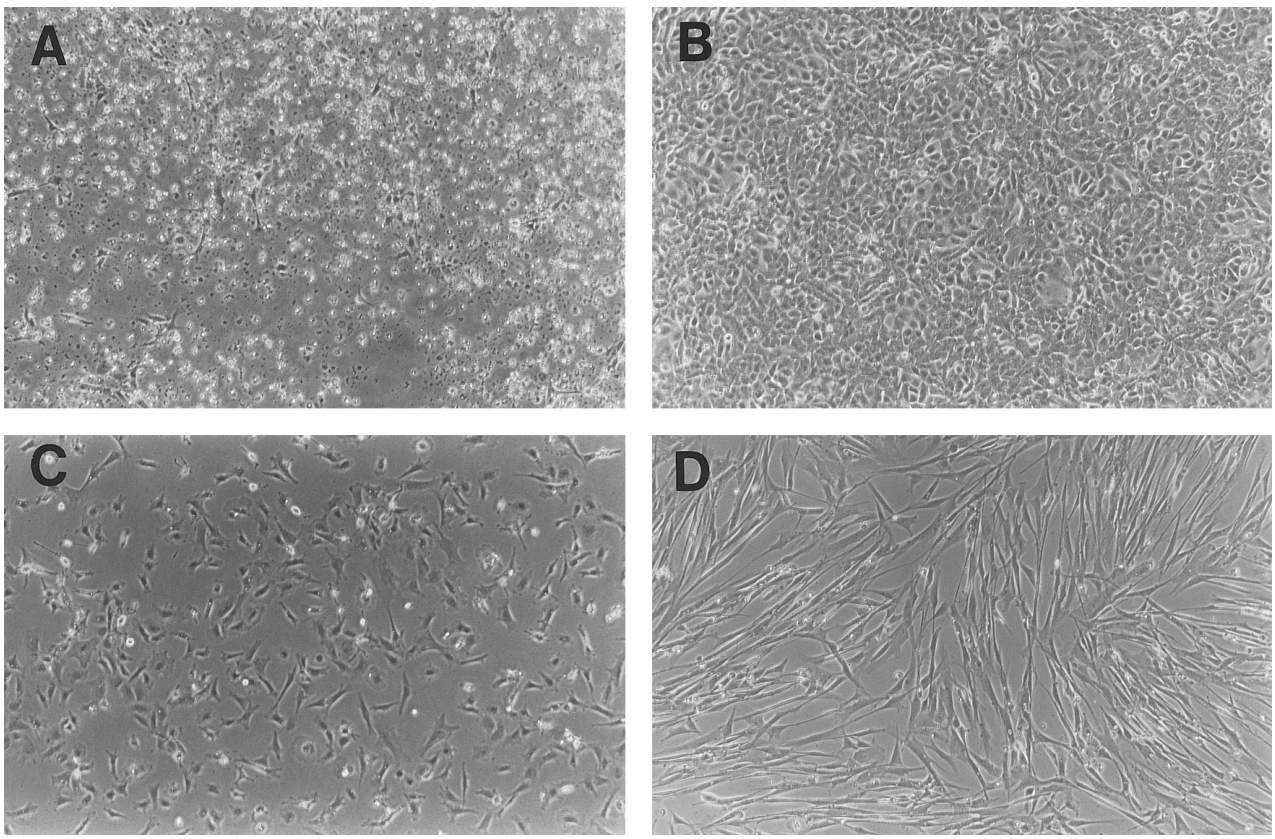

FIG. 3. Induction of cell death by VAP. Cultures of various cells were stimulated with VAP at 1 μg/ml for 12 h. Death of cells in the presence of VAP was observed under the light microscope. The complete medium was replaced with FBS-free medium that contained VAP to examine the effect of VAP on A, VEC from human umbilical vein; B, ECV-304 cells (transformed VEC); C, RSMC-3 (rat smooth muscle cells); and D, TIG-7 (human fibroblasts). (Original magnification $\times$100).

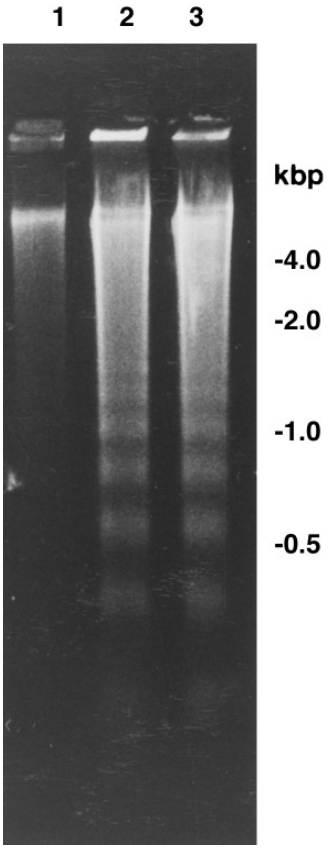

FIG. 4. Agarose gel electrophoresis showing fragmentation of DNA after treatment of VEC with VAP for 12 h. DNA was extracted from control cells and from cells treated with either crude venom or purified VAP as follows: lane 1, control VEC after 12 h; lane 2, VEC after treatment with 10 μg/ml crude venom for 12 h; lane 3, VEC after treatment with VAP at the concentration of 1 μg/ml for 12 h.

crotic cell death. VAP did not induce apoptosis in cells other than VEC, such as smooth muscle cells and fibroblasts (Fig. 3). In addition, it did not induce apoptosis in ECV-304, a line of transformed VEC.

The definitive biochemical, as distinct from morphological, hallmark of apoptosis in many types of cell is the cleavage of DNA into fragments with lengths that are multiples of the distance between nucleosomes (2). We extracted DNA from VEC with or without prior exposure to VAP and analyzed the DNA by agarose gel electrophoresis. After treatment of VEC with VAP for 12 h, fragmentation of DNA was clearly detectable (Fig. 4).

We examined VAP to determine whether it might have some enzymatic activity since snake venoms include many toxic enzymes, such as proteases and phospholipases. The factor had no detectable proteolytic activity against bovine serum albumin (data not shown) nor did it have any phospholipase activity against cellular phospholipids.

DISCUSSION

Toxins that cause hemorrhage or cytolysis are widely distributed in the venoms of hemorrhagic snakes such as those in the families *Crotalidae* and *Viperidae*. Some of these toxins have been identified as hemorrhagic metalloproteinases and myotoxins, for example. A number of hemorrhagic proteins have been isolated and characterized as metalloproteinases, with molecular masses that differ from enzyme to enzyme. The proteinases have been shown to have no direct cytotoxic effects on endothelial cells (15), although it has been reported that these enzymes caused direct damage to cells at high concentrations of the order of 0.1 μM. However, such direct activity was not reversed by FGF (16). The activity of VAP as well as that of the crude venom can be attenuated by FGF (10), an indication that the effect of VAP is different from that of a protease. Furthermore, the proteinases identified to date in the venom of *Crotalus atrox* have different molecular masses and isoelectric points from those of VAP.

Myotoxins, such as myonecrotic phospholipase A2, are cytotoxic to endothelial cells (14,15) and they increase vascular permeability (17). However, they do not always have high-level phospholipase activity. The myotoxins in rattlesnake venom are reported to be small polypeptides of 13-18 kD. They induce necrosis, and their cytotoxic effect on endothelial cells is not especially strong (14).

The activity of VAP is expressed rather slowly as compared with that of the myotoxins. Therefore, it might not be associated with the rapid hemorrhagic process that occurs within only a few minutes. However, endothelial damage might be a critical step in the later development of widespread hemorrhage as toxins are delivered to tissues distant from the original site of the snakebite.

Each component in hemorrhagic venom functions effectively against its own target: platelets are targets for disintegrin; basement membrane are targets for hemorrhagic proteinases; and fibrinogen is the target for snake fibrinogenase (19). Similarly, VAP might play an important role, acting on vascular endothelial cells to induce hemorrhage in concert with toxins that are specific for other targets.

It is of interest that the preferred target of VAP is vascular endothelial cells. The mechanism responsible for this specificity is unknown, but VAP may prove to be a useful tool for studies of vascular apoptosis.

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