BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 134–139 (1996)
ARTICLE NO. 0996

Identification of the Snake Venom Substance That Induces Apoptosis

Sung-Min Suhr and Doo-Sik Kim[1]

Department of Biochemistry, College of Science, and Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

Received May 21, 1996

Hemorrhagic snake venom induces apoptosis of vascular endothelial cells [S. Araki, T. Ishida, T. Yamamoto, K. Kaji, and H. Hayashi (1993) *Biochem. Biophys. Res. Comm.* 190, 148–153]. We have identified that a cytotoxic substance of Korean snake venom which is responsible for the apoptosis is L-amino acid oxidase (LAO). The purified enzyme is a homodimeric glycoprotein of 110,000 and is capable of generating H_2O_2 by catalyzing oxidation of L-amino acid. In the presence of the enzyme, cultured L1210 cell nuclei were splitted and showed the characteristic ladder-like pattern of DNA fragmentation. The enzyme binds directly to the cell surface, thereby increasing local concentration of H_2O_2. However, experimental evidence suggests that the LAO-induced apoptotic mechanism is distinguished from the one caused by exogenous H_2O_2.

Apoptosis is a morphologically and biochemically distinct form of programmed cell death that plays a major role during development(1-3), as a major mechanism for the precise regulation of cell numbers(4), and as a defense mechanism to remove unwanted and potentially dangerous cells that have been infected by viruses and tumor cells(5). Apoptosis can be initiated by pathological and physiological stimuli such as ionized radiation, glucocorticoids, hyperthermia, oxidants, free radicals, growth factor withdrawal, and triggering of cytokine receptors(6-7). Cells undergoing apoptotic cell death reveal a characteristic morphological and biochemical changes including membrane blebbing, nuclear and cytoplasmic condensation, and DNA fragmentation: large fragments of 300-750kb are generated first, followed by fragments of 50-70kb, and finally (but not always) shows the familiar 'nucleosomal ladder' (8). Previous report by Araki *et al.* has demonstrated that the apoptotic cell death is induced when hemorrhagic snake venom was added to a subconfluent culture of vascular endothelial cells(VEC), while many other types of cultured cells are unaffected(9). Snake venom is a complex mixture of many physiologically active proteins and peptides including fibrinolytic protease(10), thrombin-like enzyme(11), and phospholipase A_2 as myotoxin(12). In this report, we demonstrate the purification and identification of a substance, L-amino acid oxidase, from the venom of Korean snake (*Agkistrodon halys*) that is responsible for induction of apoptosis in several cultured cell lines.

MATERIALS AND METHODS

Cell culture and treatments. L1210 (mouse lymphocytic leukemia), MOLT-4 (human T-cell leukemia), RPMI1788 (human hematopoietic cell), HL60 (human promyelocytic cell), and HeLa(human epitheloid carcinoma)cell lines were used. Splenocyte was isolated from mouse spleen. Cells were cultured in RPMI 1640 media supplemented with 5% FBS, 10mM Hepes, 100 IU/ml penicillin and streptomycin, and kept in a controlled atmosphere (5% CO_2 incubator at 37°C). Cells(2×10^5/ml) were treated with apoptotic sample on a 96 well microtiter plate for 3 to 24hr. Cell viability was measured by a MTT (3-(4,5-dimethylthiazol-2yl)-2.5-diphenyltetrazollium bromide) assay (13). The resulting formazan product was solubilized in 0.5N HCl, isopropanol and measured spectrophotometrically at 570nm.

[1] To whom correspondence should be addressed. Fax: 82-2-362-9897.

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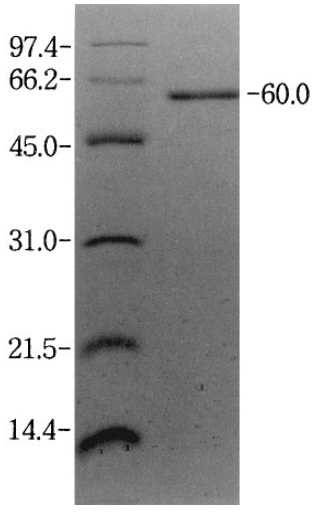

FIG. 1. SDS polyacrylamide gel electrophoresis of snake venom L-amino acid oxidase. Purified L-amino acid oxidase from the venom of *Agkistrodon halys* was subjected to electrophoresis in 15% polyacrylamide gel in the presence of SDS.

Analysis of DNA. A total of 2×10^5 cells were lysed in a hypotonic lysing buffer containing 10mM Tris, 1mM EDTA(pH 7.5), and 0.2% Triton X-100. Nucleic acids were extracted by phenol, ethanol precipitated, and incubated in 10μg/ml RNase A for 60min at 37°C. The purified DNA was loaded on a 1% agarose gel in TAE buffer, stained with 1μg/ml ethydium bromide(14). For quantitative analysis of fragmented DNA, cells (2×10^5/ml) were pulsed with 0.5 μCi of [^{3}H]thymidine/well for 8hr. Cells were harvested after 24hr incubation. The pellet was lysed with hypotonic lysing buffer and the lysates were centrifuged at 13,000×G for 10min to separate fragmented chromatin. The radioactivities in the cell pellet and in the supernatant were quantified by scintillation counting (4).

Protein purification. Apoptotic protein was fractionated by a series of native PAGE electro-elution, Sephacryl S-200 gel filtration, and Heparin affinity chromatography. Molcular weight was determined by SDS/PAGE, native pore gradient PAGE and gel filtration. L-amino acid oxidase assay was coupled with horseradish peroxidase and guaiacol(15). Carbohydrate staining of the protein was carried out as described by Hawkes(16).

Electron microscopy. Cells were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1M cacodylate buffer/0.1M NaCl/0.2%$CaCl_2$ and then prefixed in 1% OsO_4. Cells were stained with uranylacetate and leadcitrate, and embedded for transmission EM(17).

Fluorescence microscopy. Conjugation of cytotoxic protein with fluorescein isothiocyanate (FITC) was performed by the addition of FITC stock solution (1mg/ml in dimethyl sulfoxide) to the concentrated protein solution (0.5mg/ml in 0.1M sodium carbonate pH9.5) under continuous stirring(18). The unbound dye from the conjugate was separated by washing twice in PBS using Centricon-30. Cells were treated with FITC-conjugated protein for time course incubation, collected by centrifugation and observed by fluorescence microscope.

RESULTS AND DISCUSSION

The snake venom proteins (9.6mg) of *Agkistrodon halys* were initially resolved by native PAGE. Separated protein bands were then extracted from the gel slices in phosphate buffered saline (PBS) for 24hr. Fractions that have cytotoxic activity to L1210 cells were combined, concentrated, and further purified by Sephacryl S-200 gel filtration and Heparin affinity chromatography. The purified cytotoxic activity migrated as a single glycoprotein band of 60,000 on SDS/PAGE (Fig. 1). The functional molecular size was estimated to be 110,000 both by native pore-gradient PAGE and by gel filtration suggesting homodimeric structure of the cytotoxic protein. Since Ueda *et al.*(19) demonstrated purification of snake venom L-amino acid oxidase(LAO) that catalyzes H_2O_2 production, the enzyme activity was examined for the purified protein by a coupled assay system containing peroxidase

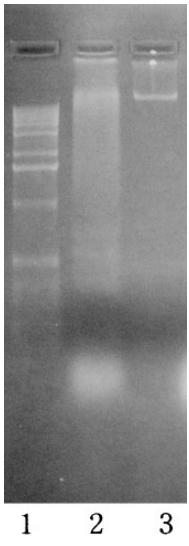

FIG. 2. Agarose gel electrophoresis of DNA isolated from L1210 cells. Cells were incubated with or without LAO for 24hr. Lane 1, molecular weight marker; lane 2, LAO treated; lane 3, untreated.

and guaiacol (15). Result of the enzymatic assay in the cell culture media clearly showed that the cytotoxic protein is LAO.

In order to study the mechanism related to the LAO-induced cell death, we have analyzed the chromosomal DNA of the L1210 cells. Agarose gel electrophoresis of the DNA isolated from the LAO-treated L1210 cells demonstrated a distinct ladder-like fragmentation pattern as shown in Fig. 2. A more quantitative analysis for the DNA fragmentation of L1210 cells pre-incorporated with [^{3}H]thymidine in 24hr incubation revealed 80.6% fragmentation of the total DNA. Although it is generally assumed that the endonucleolytic DNA fragmentation is an important criterion during apoptosis(20), a further indication associated with the apoptotic cell death of the L1210 cells was obtained by transmission electron microscopy. When the cells were exposed to the LAO in culture media, the nuclear division into several pieces, decreased cell volume and increased vacuolization were visualized (Fig. 3). Damage of the plasma membrane was assessed by measuring extracellular lactate dehydrogenase (LDH) activity. Comparing to the total cell lysate, the LAO-treated cells released 18.3% of LDH activity in 9hr incubation, but 71% of the activity in 24hrs. Hydrogen peroxide has been reported to induce apoptosis at lower concentration of below 2.5mM, but to cause necrosis at higher level of above 7.5mM(21). Our experimental observations are in agreement with the above, that is, snake venom LAO induced apoptosis in 9hr incubation, but caused necrosis in 24hr due to generated H_2O_2 by the enzyme reaction. These results also confirm that apoptotic cells might degrade and undergo secondary necrosis *in vitro* when phagocytic cells are absent(22). In order to examine whether the LAO-induced cytotoxic effect solely depends on H_2O_2, the L1210 cells were cultured with the LAO and catalase to get rid of the generated H_2O_2 in culture media(17,23). Nevertheless, the cell viability was not fully recovered but about 70%(Fig. 4). This result indicates that the LAO-induced apoptosis is not fully explained by the effect of H_2O_2 generated by the enzyme reaction in culture media.

When the cells were incubated for 5hr with the LAO labeled with FITC(18), fluorescence microscopic observation revealed the chromophores attached to the cell surface of L1210 but not to the HeLa cells (Fig. 5). Therefore it is hypothesized that the local concentration of H_2O_2

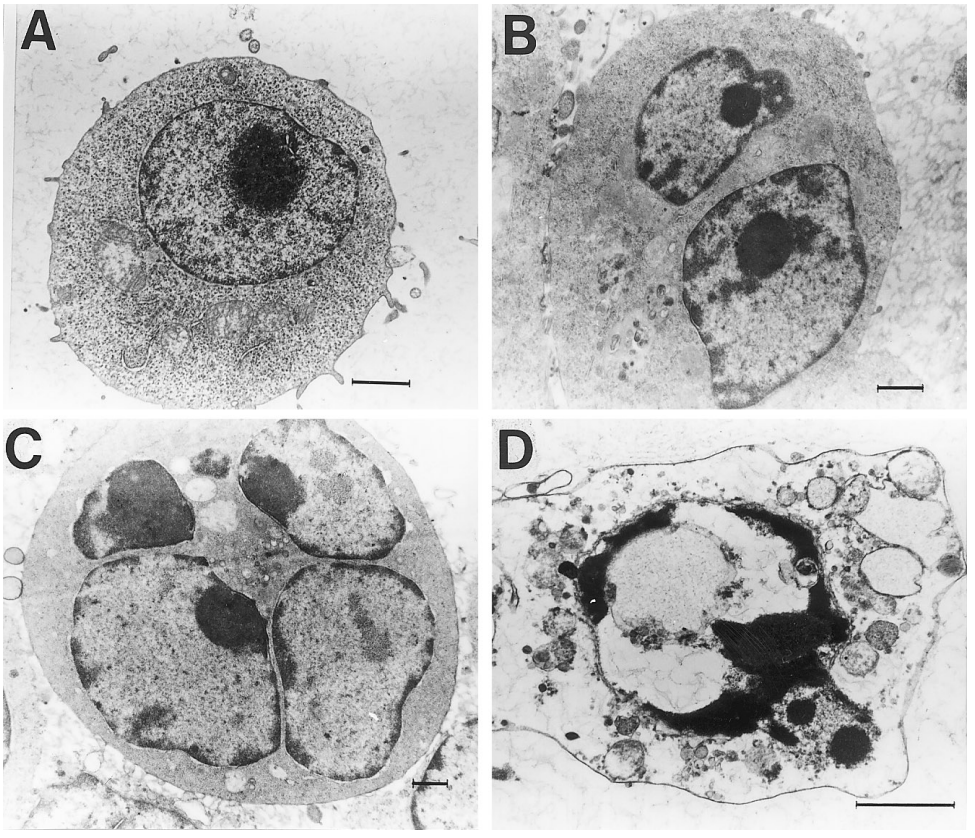

FIG. 3. Transmission electron micrographs of normal and apoptotic L1210 cells. (A) Control L1210 cells; (B, C) L1210 cells treated with L-aminoacid oxidase for 9hr; (D) cells examined 24hr after treatment. Bar, 1μm.

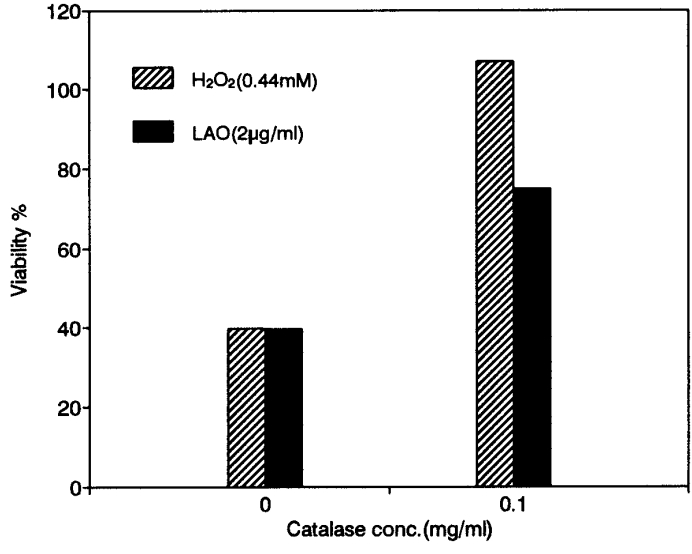

FIG. 4. Protection effect of catalase against cytotoxicity. L1210 cells were incubated with exogenous H_2O_2 or L-amino acid oxidase for 24hr.

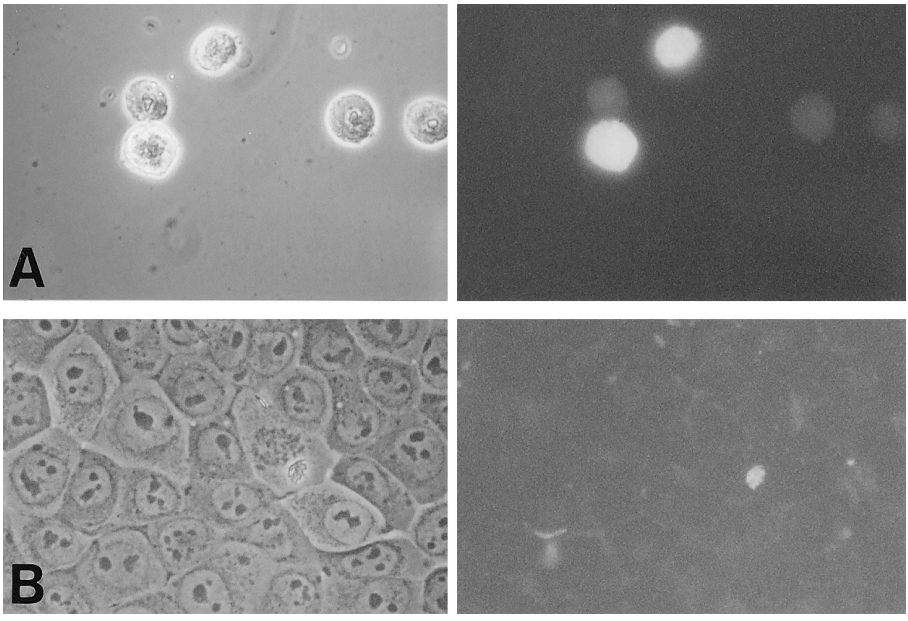

FIG. 5. Differential binding of L-amino acid oxidase to the cell surface. L1210 cells (A) or HeLa cells (B) were treated with FITC-labeled L-amino acid oxidase for 5 hr followed by fluorescence microscopic observation.

near the plasma membrane is greatly increased by the bound LAO. Cytotoxic specificity of the snake venom LAO was tested with several cell lines including L1210, MOLT-4, RPMI 1788, HL-60, mouse splenocyte and HeLa cell line. As shown in Table 1, the different susceptibilities may be explained by the degree of LAO binding to the cell surface as evidenced in Fig. 5. In conclusion, it is clear that the LAO is a responsible element of snake venom-induced apoptotic cell death, and the extent of the effect is variable depending on cell lines. Hydroxyl radicals are the most oxygen free radical species, capable of inducing oxidative damage to macromolecules including DNA, protein, and lipid membranes(22). The effects of excess production of hydrogen peroxide, such as DNA strand breaks and membrane blebbing, match the hallmark features of apoptosis. Further investgations will be focused on searching for which intracellular components are correlated to the apoptotic event induced by the snake venom LAO.

TABLE 1
Cytotoxic Effect of L-Amino Acid Oxidase on Different Cell Lines

Cell type	% of viability
L1210	8.9
MOLT-4	23.9
HL 60	43.1
RPMI 1788	40.7
Splenocyte	65.8
HeLa	93.1

Note. L-amino acid oxidase (2μg/ml) was incubated with each cell line (2×10^5 cells/ml).

ACKNOWLEDGMENT

This study was supported in part by a research grant from Bioproducts Research Center of Yonsei University (Project No. 95-K3-03 01 01-01-3).

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