Apoxin I, a Novel Apoptosis-inducing Factor with L-Amino Acid Oxidase Activity Purified from Western Diamondback Rattlesnake Venom*

(Received for publication, August 13, 1996, and in revised form, January 21, 1997)

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From the ‡Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan, and the Institute for Research Resources Bank (Tokyo, Japan) and maintained in RPMI 1640 medium (Nissui) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were subcultured at the rate of 0.2 ml/min. The elution profile was monitored at 280 nm, and SDS-polyacrylamide gel electrophoresis analysis was carried out as described previously (16), and proteins were stained using a two-dimensional silver stain kit (Daiichi, Tokyo, Japan).

Cell Lines and Cell Culture—Human umbilical vein endothelial cells obtained from InvitroCYTE (Seattle, WA) were cultured and maintained in endothelial cell growth medium with low serum growth supplement according to the manufacturer's instructions. The cells were cultured in collagen-coated dishes (Iwaki Glass, Tokyo, Japan). Human promyelocytic leukemia HL-60, human ovarian carcinoma A2780, and mouse endothelial KN-3 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and maintained in RPMI 1640 medium (Nissui) supplemented with 10% heat-inactivated fetal bovine serum and 100 mg/ml kanamycin in a humidified atmosphere of 5% CO2 and 95% air. All experiments were performed using cells during the exponential growth phase.

Nuclear Morphological Changes—Cells (0.5 × 10⁶) were treated with 10 μg/ml purified apoxin I for 18 h, except for HL-60 cells, which were treated for 4 h, in the growth medium and then harvested. After cells were washed in phosphate-buffered saline and fixed in methanol/acetate (3:1) for 10 min, cells were stained with 20 μl of 1 μg/ml 4,6-diamidino-2-phenylindole (Sigma); one drop of fixed cells was placed on a glass slide. Nuclear morphology was observed by a Nikon UFX-HA fluorescent microscope as described previously (17).

DNA Fragmentation Assay—After treatment with apoxin I, HL-60 cells (0.5 × 10⁶) were suspended in 20 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5 mg/ml proteinase K (Sigma). After incubation at 50 °C for 1 h, 1 μl of 0.5 μg/ml RNase A solution was added, and the suspension was incubated for an additional hour. The sample was
mixed with 10 μl of the preheated (70 °C) solution containing 10 mM EDTA (pH 8.0), 1% (w/v) low melting point agarose (Sigma), 0.25% bromphenol blue, and 40% sucrose. DNA was analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide, and then photographed on a UV transilluminator (18).

**Flow Cytometric Analysis**—HL-60 cells (0.5 × 10⁶) were pretreated with or without 160 μM Z-VAD, 200 μM Z-Asp, or antioxidants for 1 h, and then cells were treated with 5 μg/ml apoxin I or 20 μM H₂O₂ for 4 h. Cells were harvested, fixed in 70% ethanol, treated with RNase A (1 mg/ml in 0.1 M phosphate buffer, pH 7.0), and stained with propidium iodide solution (50 μg/ml in 0.1% sodium citrate and 0.1% Nonidet P-40). Cells were analyzed using a Becton Dickinson (Braintree, MA) FACScan flow cytometer (18).

**Amino Acid Sequence**—The purified apoxin I was cloned onto a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) and applied to Applied Biosystems 477A and Beckman LF3400D peptide sequencers. Data were analyzed by the sequencers automatically, and we confirmed the data by checking the chromatograms.

**Measurement of LAO Activity**—The reaction mixtures (1 ml) containing the 1 μg/ml or 2 μg/ml purified apoxin I, 50 μg/ml horseradish peroxidase (5 milliunits/ml), 10 μM O-dianisidine, and 10 μM 1-leucine or 1-leucine in 100 mM Tris-HCl (pH 8.5) were incubated at 25 °C, and the initial rate was measured as the increase in absorbance at 436 nm (19) with a Beckman DU 640 spectrophotometer.

**Subcellular Distribution of Apoxin I**—Purified apoxin I was labeled with fluorescein 5'-isothiocyanate as described previously (20). HL-60 cells were treated with the fluorescein 5'-isothiocyanate-labeled apoxin I (5 μg/ml) for 4 h at 37 °C, and observed by fluorescent microscopy.

**RESULTS AND DISCUSSION**

Crude venom from rattlesnake was first subjected to a Sephadex G-100 gel filtration column followed by a Rotofor isoelectric focusing apparatus; major apoptosis-inducing activity was detected in fractions of pI 6.0–6.5. The active fractions were collected and applied to fast protein liquid chromatography equipped with a Superdex 200 HR 10/30 column directly connected to a TSK 3000 PWXL column. The activity was eluted as a single peak with a molecular mass of approximately 100 kDa (Fig. 1a). The purification yielded 1.48% of the crude venom proteins. According to the LAO activity (described below), the purified protein was concentrated 56-fold from the crude venom, and the recovery of the LAO activity was 83%. The purified protein, named apoxin I, showed a homogenous single band of 55 kDa in SDS-polyacrylamide gel electrophoresis (Fig. 1b).

**FIG. 1. Purification of apoxin I from western diamondback rattlesnake venom.** a, chromatogram of the final purification step of apoxin I by Superdex 200 HR 10/30 connected to a TSK 3000 PWXL gel filtration column. The **shaded peak** contains apoptosis-inducing activity. b, SDS-polyacrylamide gel electrophoresis analysis of the purified apoxin I, showing a purified 55-kDa single band.

**FIG. 2. Nuclear morphological changes in apoxin I-treated cells.** Human umbilical vein endothelial cells (a and b), HL-60 cells (c and d), A2780 cells (e), and KN-3 cells (f) were treated without (a and c) or with 10 μg/ml apoxin I (b and d–f) for 18 h (b, e, and f) or 4 h (d), and the nuclei were stained with 4,6-diamidino-2-phenylindole as described under “Experimental Procedures.”

**FIG. 3. Oligonucleosomal DNA fragmentation in apoxin I-treated cells.** HL-60 (0.5 × 10⁶) cells were treated with indicated concentrations of apoxin I for 4 h (a) or with 5 μg/ml apoxin I for the indicated times (b). After harvest of the cells, nuclear DNA fragmentation was analyzed by 2% agarose gel electrophoresis.

**FIG. 4. Amino Acid Sequence.** Amino Acid Sequence—The purified apoxin I was cloned onto a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) and applied to Applied Biosystems 477A and Beckman LF3400D peptide sequencers. Data were analyzed by the sequencers automatically, and we confirmed the data by checking the chromatograms.
containing DNA at less than G1 (subdiploid cells). The data represent oxidized L-leucine in a dose- and time-dependent manner, whereas it did not oxidize D-leucine (not shown). These results indicate that apoxin I induced apoptosis in such cell lines.

We next analyzed the N-terminal amino acid sequence of the purified apoxin I. Nineteen of 20 amino acids were identified in the N-terminal amino acid sequence of apoxin I from the western diamondback rattlesnake (C. atrox), L-amino acid oxidase from the Malayan pit viper (C. rhodostoma), and from king cobra (O. hannah). X, undetermined sequence most probably due to decomposition of cysteine during reactions. Underlined characters, identical amino acids with apoxin I.

Apoxin I: \text{AHRNFL-EEXFRETDYEEFL-}

M. pit viper LAO: \text{ADDNFLAE-FQENDYEEFL-}

King cobra LAO: \text{HVIN-L-EESFQPEYXNH-}

inhibited the nuclear condensation caused by 10 μg/ml Apoxin I (not shown). These results indicate that apoxin I induced apoptosis in such cell lines.

Treatment of apoxin I with heat (70°C, 10 min) or repeated freezing and thawing abolished both the LAO- and apoptosis-inducing activity (not shown), suggesting the involvement of the LAO activity in apoxin I-induced apoptosis. The oxidation by LAO of L-amino acid under oxygenated conditions results in the production of the corresponding α-keto acid, NH3, and H2O2 (21, 22). Therefore, we tested the effects of scavengers of reactive oxygen species on the apoxin I-induced apoptosis. As shown in Fig. 7, catalase completely inhibited apoptosis induced by apoxin I and H2O2, indicating that H2O2 mediates apoxin I-induced apoptosis. We prepared fluorescein 5′-isothiocyanate-labeled apoxin I to investigate subcellular localization of apoxin I by fluorescent microscopy. Our preliminary results showed that apoxin I accumulated in the cellular plasma membrane. Further study is now in progress. According to these results, we tested antioxidants for the inhibition of the apoxin I-induced apoptosis. A membrane antioxidant, trolox (23), but not a water-soluble antioxidant, L-ascorbate (24), inhibited the apoxin I-induced apoptosis (Fig. 7). These results suggest that apoptosis is triggered by membrane oxidation by H2O2 produced by apoxin I accumulating in the cellular plasma membrane.

Venoms of several snake species contain large amounts of LAO, but its biological effects are largely unknown. It was reported that LAO from king cobra (O. hannah) venom induced platelet aggregation (22). The LAO-induced platelet aggregation is inhibited by catalase, suggesting that H2O2 also mediates LAO-induced platelet aggregation (22). We do not know whether all the LAOs from different snake species show similar biological effects; however, it is likely they do, because H2O2 mediated the biological effects of LAO. These observations, together with our present study, suggest that treatment with
antioxidants might protect tissues affected by snake bite from various types of harmful snake venom.

Acknowledgments—We are grateful to Drs. A. Tomida, N. Fujita, and T. Mashima for helpful discussions and encouragement. We are also grateful to Dr. S. Tomioka (Tokyo University) for helpful suggestions and technical advice. We thank Drs. S. Kataoka and H. Kawai (Kirin Brewery) for providing Z-Asp and Z-VAD. We thank Dr. S. Sawai (Beckman) for analysis of the N-terminal peptide sequence of apoxin I. We thank Dr. Robert H. Morton (Janssen-Kyowa) for checking the manuscript.

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J. Biol. Chem. 1997, 272:9539-9542.
doi: 10.1074/jbc.272.14.9539

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