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Induction of Apoptotic Cell Death in Human Endothelial Cells Treated with Snake Venom: Implication of Intracellular Reactive Oxygen Species and Protective Effects of Glutathione and Superoxide Dismutases[1]

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Human vascular endothelial cells play a pivotal role in atherosclerotic changes but are resistant to apoptotic inducers such as Fas ligand and it has been difficult to induce apoptosis. We developed an experimental model for the apoptosis in the endothelial cells by using snake venom treatment. Snake venom was found to generate intracellular reactive oxygen species (ROS) in the endothelial cells, which leads to apoptosis as judged by electron microscopy as well as by DNA cleavage. Buthionine sulfoximine (BSO) and diethyldithiocarbamate (DDC) accelerated the apoptosis, indicating intracellular glutathione and superoxide levels play a critical role. Pretreatment with tumor necrosis factor (TNF) or phorbol ester (TPA), which increases the Mn-SOD level, prevented the apoptosis. These data suggest that intracellular ROS enhances apoptosis whereas several anti-oxidants are protective in human endothelial cells. The induction of apoptosis by ROS of endothelial cells may be related to initiation of atherosclerotic changes.

Key words: glutathione, reactive oxygen species, snake venom, superoxide dismutase, vascular endothelial cells.

The apoptosis of various cells requires inducers including free radicals (*1-3*). Endothelial cells or vascular smooth muscle cells are generally considered to be resistant to apoptosis but recent studies suggested that such a change in these cells is related to atherosclerotic changes (*4*). Therefore, in order to know the mechanism of apoptosis, it is very important to develop the experimental model for apoptosis in endothelial cells. Endothelial cells act as a barrier between blood and tissues, and thus are exposed to free radicals. However, the significance of free radicals in the apoptosis of these cells is not clear. In cell cultures the depletion of such survival components as fetal calf serum and various growth factors has been associated with the development of apoptosis of endothelial cells (*5*). TNF was used in some experiments (*6*), but endothelial cells are essentially resistant to this protein and Mn-SOD in endothelial cells is dramatically induced by TNF (*7*). So, there was no suitable fatal factor which induces apoptosis in endothelial cells. Recently, several factors, including snake venom, alkyllysophospholipids and vanadate, were reported to induce apoptosis of endothelial cells (*8-10*). However, in these experiments serum or growth factors in the culture medium prevented apoptosis. In our studies, we found that snake venom generated ROS, and induced the apoptosis of endothelial cells in the presence of serum and growth factors, and the apoptosis was confirmed by electron microscopy as well as by DNA cleavage. Using this material to generate intracellular ROS, we have investigated the roles of free radicals and anti-oxidants in the development of apoptosis of endothelial cells without the influence of survival factor depletion.

MATERIALS AND METHODS

Materials—The venom of *Crotalus viridis oreganus* (V-7500) and BSO were products of Sigma. DDC was purchased from Wako Pure Chemical, and recombinant TNF-α was obtained from Ube Industries. γ-Glutamyl-cysteine ethylester was a gift from Teijin Co., Ltd. Other reagents were of the highest grade available.

Endothelial Cells—Human endothelial cells were isolated from human umbilical veins as previously described (*7*). These cells were grown at 37°C in 100-mm Petri dishes or 96-well flat bottom microplates with MCDB131 medium containing fetal calf serum (10%), recombinant human basic fibroblast growth factor (10 ng/ml), hydrocortisone (1

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Abbreviations: SOD, superoxide dismutase; ROS, reactive oxygen species; TNF, tumor necrosis factor-α; GSH, glutathione; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DDC, diethyldithiocarbamate; BSO, buthionine sulfoximine; TE, Tris-EDTA buffer; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; GC-E, γ-glutamyl-cysteine ester (ethylester).

mg/ml), penicillin G (100 u/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml), under a humid atmosphere containing 5% CO_2. Cultures of subconfluent cells were treated with TNFα, BSO, or DDC.

Cell Viability Assay—The viability of cells in 96-well culture plates was determined 24 h after the addition of snake venom by the methylene blue method (*11*). Twenty microliters of a 25% glutaraldehyde solution was added to each well followed by incubation for 15 min to fix the surviving cells. After washing with PBS, the fixed cells were stained for 15 min with 100 ml of a 0.05% methylene blue solution. After washing 3 to 4 times with PBS, 200 ml of 0.33 N HCl was added to each well to extract the methylene blue, whose level was determined as the absorbance at 665 nm.

Measurement of SOD and GSH—The Mn-SOD levels of endothelial cells were measured using an ELISA kit, which was developed in our laboratory using a specific monoclonal antibody (*12*). GSH levels were measured by the modified Tietze method (*13*).

Scanning Electron Microscopy—After incubation with snake venom, endothelial cells were fixed with 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4) and then postfixed with 1% OsO_4 in the same buffer. The specimen was dehydrated with a graded ethanol series, freeze-dried using *t*-butyl alcohol, and then coated with platinum and carbon.

Determination of DNA Cleavage—Following treatment with snake venom, DNA cleavage of cells was determined by agarose gel electrophoresis as previously described (*14*). Briefly, endothelial cells were collected 4 h after the addition of snake venom. Non-attached cells were collected by aspiration and the remaining attached cells were removed with a Rubber Policeman. Both cell collections were washed twice with PBS and concentrated by centrifugation. They were then incubated with lysis buffer containing 100 mg/ml proteinase K, and the nuclear lysates were extracted with phenol and phenol-chloroform-isoamyl alcohol. The DNA was precipitated with ethanol, pelleted by centrifugation, and dried. After being dissolved in TE buffer containing 20 mg/liter of RNase A and extraction with phenol-chloroform-isoamyl alcohol, the samples were analyzed by electrophoresis on 1.5% agarose gels. The bands were visualized by staining.

Staining of Nuclear DNA—Endothelial cells treated with snake venom were fixed for 30 min at room temperature with 3% paraformaldehyde in PBS, washed with PBS, and treated for 4 min with 1% Triton X-100/PBS, and then washed with PBS. The fixed and treated cells were incubated for 60 min at room temperature with 0.5 mg/ml of 4′,6-diamino-2-phenylindole hydrochloride in PBS, washed with PBS, and then mounted. Cells were inspected under Olympus microscope in the fluorescence mode. Cells which contained highly condensed chromatin and irregular DNA inclusions were defined as being apoptotic (*15*).

Measurement of Intracellular ROS by Flow Cytometry—Levels were assessed using an oxidation-sensitive fluorescent probe DCFH-DA, as previously described (*16*). In the presence of a variety of intracellular ROS, which are thought to be mainly peroxides, DCFH is oxidized to 2′,7′-dichlorofluorescein, a highly fluorescent compound. Endothelial cells which had been treated with snake venom were

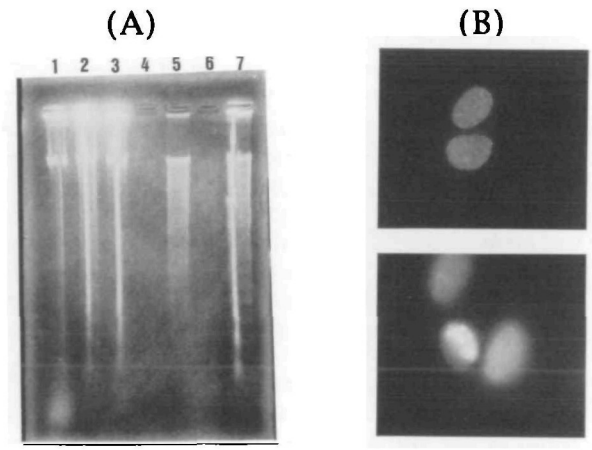


Fig. 1. **DNA cleavage and fragmentation of chromatin in endothelial cells treated with snake venom.** (A) Snake venom concentrations used per ml.: Lane 1, 0 μg; 2, 1 μg; 3, 2 μg; 4, 10 μg (attached); 5, 10 μg (non-attached); 6, 20 μg (attached); 7, 20 μg (non-attached). (B) Upper, control; lower, snake venom 5 μg/ml, 12 h after treatment.

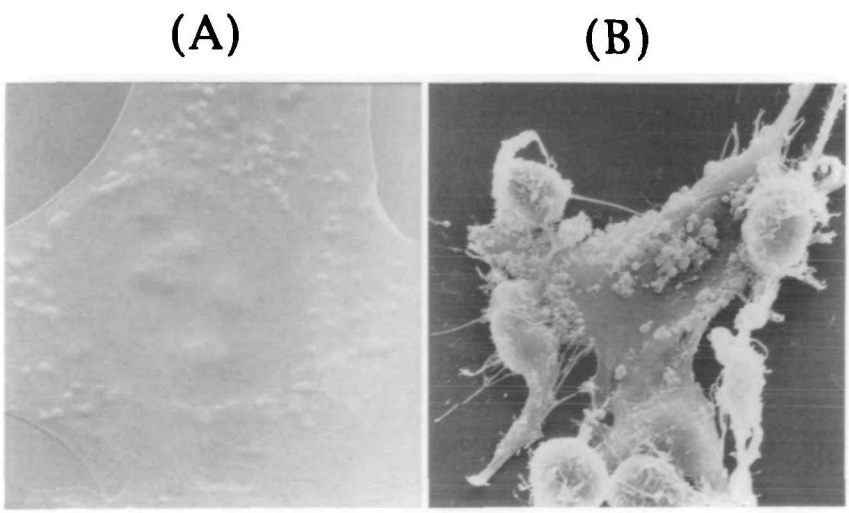


Fig. 2. **Results of scanning electron microscopy of endothelial cells after treatment with snake venom.** Untreated cells show smooth and flat cell surfaces (A), while treated cells have many spherical bodies and/or protuberances (B). A, control; B, snake venom 10 μg/ml.

incubated with 5 mM DCHA-DA. Their cellular fluorescence intensities were measured using a FACScan (Becton-Dickinson).

RESULTS

Detection of Apoptosis of Endothelial Cells—Lane 7 in Fig. 1A shows the DNA cleavage in endothelial cells which had been treated with snake venom. Figure 1B also shows fragmentation of nuclear chromatin of cells treated with snake venom.

On scanning electron microscopic examination untreated endothelial cells showed smooth and flattened surfaces (Fig. 2A). After 24 h treatment with snake venom, the surfaces became irregular and bumpy with many protuberances and spherical bodies (Fig. 2B). Cells with such surfaces are considered to be apoptotic.

Flow Cytometric Analysis of Intracellular ROS—The production of ROS by endothelial cells treated with snake venom was evaluated by flow cytometry (Fig. 3). Cells treated for 4 h showed markedly increased ROS contents, which returned to normal levels after 24 h.

Effects of Pretreatment with BSO and DDC on the Apoptosis of Endothelial Cells—Figure 4A shows the effect of BSO pretreatment (24 h) on the viability of endothelial cells following treatment with snake venom. Pretreatment with BSO lowered the glutathione level and decreased cell viability (Fig. 4B). The sensitivity of endothelial cells to snake venom decreased as the glutathione level decreased.

Treatment with DDC, an inhibitor of Cu,Zn-SOD, resulted in a dose-dependent decrease in cell viability (Fig. 5).

Effects of TNF and TPA Pretreatments on the Apoptosis of Endothelial Cells—In our previous studies, we found TNF and/or TPA induces Mn-SOD in endothelial cells, as judged by immuno-electron microscopies as well as by ELISA (7). Pretreatments with them markedly increased the viability of cells which had been treated with snake venom (Fig. 6). The increase in the Mn-SOD level is in keeping with the enhanced cell viability.

Effect of γ-Glutamyl-Cysteine Ester—The γ-glutamyl-cysteine ester is able to cross the cell membrane and cleaved by esterase to form γ-glutamyl-cysteine. γ-Glutamyl-cysteine is the substrate of glutathione synthase and is easily converted to glutathione. BSO plus γ-glutamyl-cys-

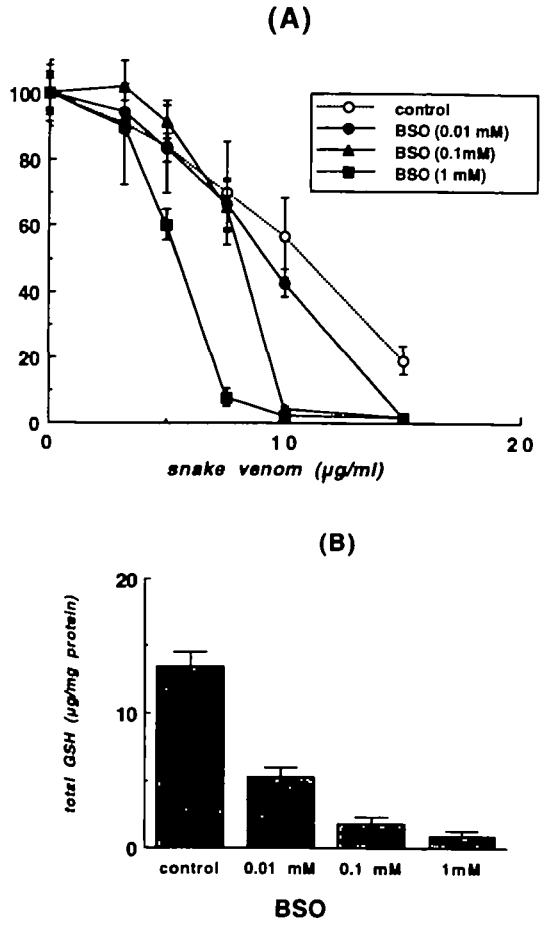


Fig. 4. **Effect of pretreatment with BSO on the viability of endothelial cells.** (A) Dose-dependent effect of pretreatment with BSO on cell viability ($n=8$, mean±SD). (B) Glutathione levels in endothelial cells pretreated with BSO.

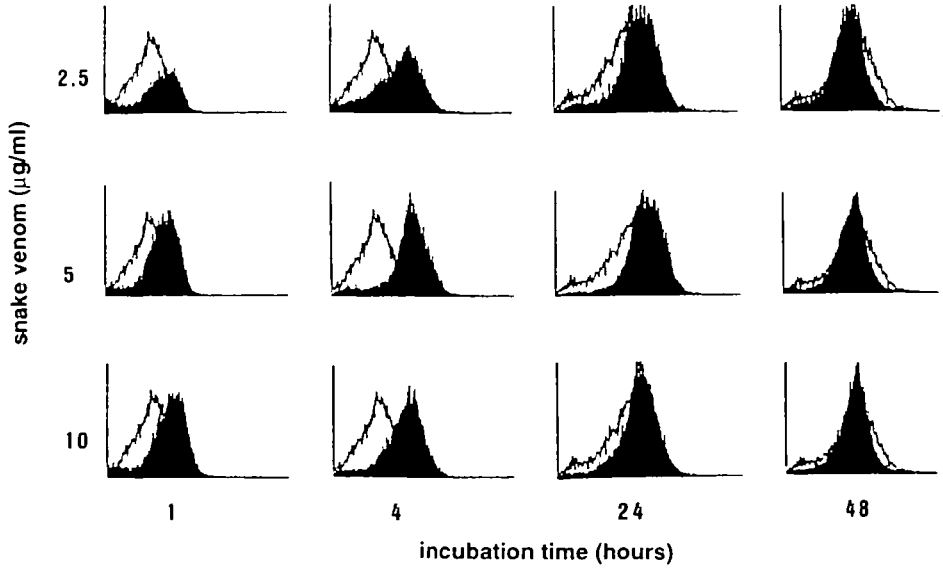


Fig. 3. **Intracellular reactive oxygen species determination by flow cytometry.** White, control cells; black, cells treated with snake venom. Vertical axes, relative frequency; horizontal axes, log fluorescence.

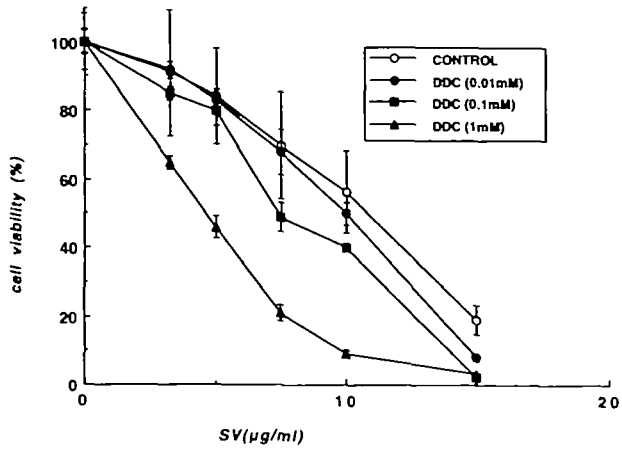


Fig. 5. **Effect of pretreatment with DDC on the viability of endothelial cells.** Dose-dependent effect of pretreatment with DDC on cell viability ($n=8$, mean$\pm$SD).

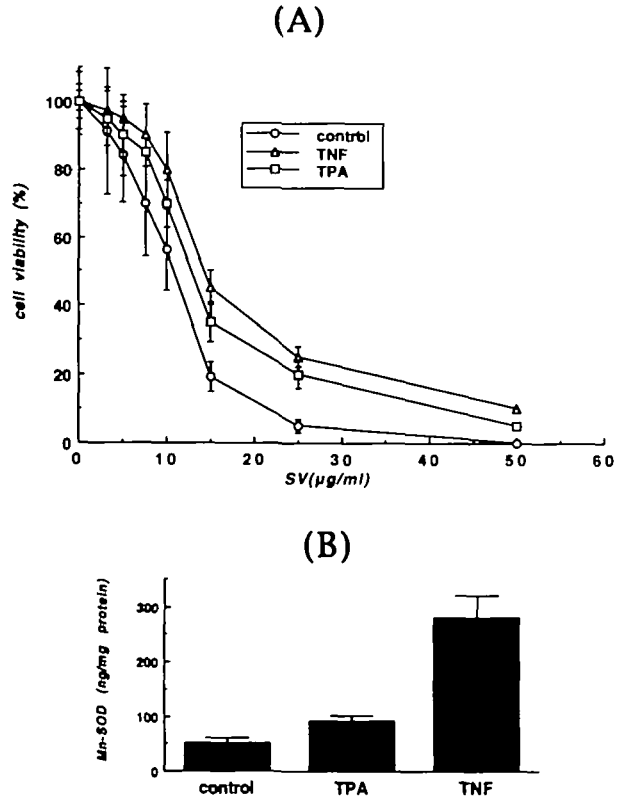
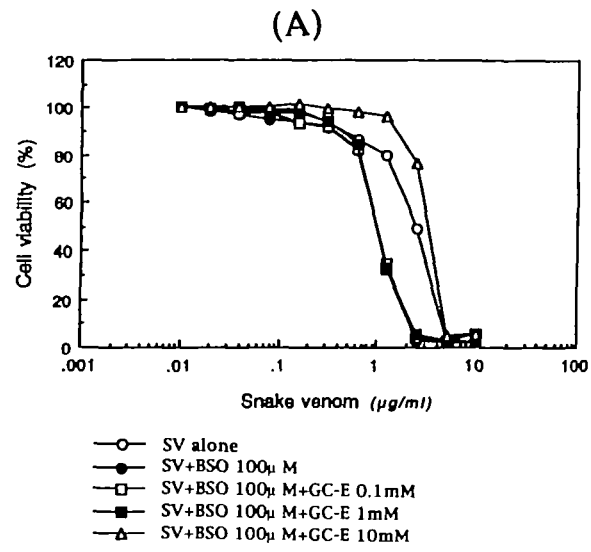


Fig. 6. **Effects of pretreatment with TNF and TPA on the apoptosis of endothelial cells.** (A) Effect of TNF (10 ng/ml) and TPA (100 ng/ml) on cell viability ($n=8$, mean$\pm$SD). (B) Mn-SOD content of endothelial cells.

teine ester selectively increased the glutathione level in endothelial cells, and the addition of it led to complete recovery of the cytotoxicity of snake venom, which was augmented by BSO (Fig. 7). The glutathione levels of BSO plus γ-glutamyl-cysteine ester (10 mM) was 24 ± 7.2 μg/mg protein, which was higher than control.



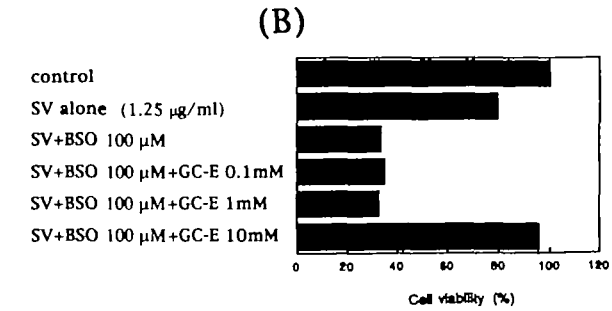


Fig. 7. **Effect of GC-E on the apoptosis of endothelial cells.** (A) Cell viability results ($n=8$, mean$\pm$SD). (B) Cell viability at 1.25 μg/ml snake venom.

DISCUSSION

Even though studies on apoptosis of various cells have been extensive, the mechanism by which it occurs in endothelial cells remains controversial. Such cells are resistant to apoptotic inducers such as Fas ligand (*17*), and it has also been difficult to induce apoptosis without depletion of survival factors such as basic fibroblast growth factors or fetal calf serum. The participation of free radicals in the induction of apoptosis of endothelial cells has received little attention. However, no direct evidence of the role of free radicals or anti-oxidants was presented. The previous inability to generate ROS has made it difficult to investigate the roles of free radicals and anti-oxidants in apoptosis. In the present study we have demonstrated that snake venom generates intracellular ROS in endothelial cells, and induces apoptosis under normal culture conditions with fetal calf serum and basic fibroblast growth factors, and have also shown that anti-oxidants may play important roles in the development of such conditions.

BSO inhibits γ-glutamyl-cysteine synthetase, which is a rate-limiting enzyme in GSH synthesis and decreases the GSH levels in endothelial cells. The decrease in the GSH level correlated with cell survival, and the addition of γ-glutamyl-cysteine ester, which elevates the GSH level

resulted in a recovery of cell viability. This is thus direct evidence that GSH is a protective agent against the development of apoptosis of endothelial cells. DDC, a metal chelator which reduces the activity of Cu,Zn-SOD in various cells (*18*), also decreased cell viability. Anti-oxidants, such as glutathione and Cu,Zn-SOD, may play protective roles against apoptosis. Pretreatment of cells with TNF and TPA also increased cell viability. It has already been reported that TNF and/or TPA induce(s) Mn-SOD in endothelial cells dramatically without a change in the level of Cu,Zn-SOD. It has also been reported that TNF, lipopolysaccharide, or endotoxin pretreatment protects cells or organs from ischemia-repurfusion injury through the induction of Mn-SOD (*19, 20*). In the present study, it appeared that the increase in Mn-SOD induced by TNF or TPA may protect endothelial cells from ROS generated by snake venom.

Apoptosis may be initiated through a variety of pathways. Free radical or ROS generation would be one of them. Endothelial cells appear to be much more resistant to apoptosis than others. While many blood cells undergo apoptosis, endothelial types in blood vessels are relatively stable. These cells possess abundant anti-oxidants. For example, extracellular SOD exists on endothelial cells, and the mitochondria of endothelial cells also contain a larger amounts of Mn-SOD than myocytes. It has recently been reported that the addition of SOD to the culture medium did not protect endothelial cells from apoptosis induced by endotoxin (*21*). This supports the finding in our study that intracellular but not extracellular anti-oxidants are important in inhibiting the development of apoptosis of endothelial cells. However, when an imbalance of anti-oxidants in endothelial cells occurs in various pathological conditions along with increased levels of intracellular ROS, apoptotic changes of endothelial cells may likely be initiated. This may lead to atherosclerotic changes or other organ disturbances.

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