J Tokyo Wom Med Univ
71 (9・10) 669~678 (2001)

The Cytoprotective Effect of Protease Inhibitor on Programmed Cell Death of Endothelial Cell

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(Received Jan. 19, 2001)

Failure of the vascular endothelial cell is first step of the crisis of multiple organ dysfunction syndrome (MODS), and cell injury by oxidative stress is deeply concerned in this mechanism. For the purpose of this study is clarifying the mechanism of cytoprotective effect for cell injury of protease inhibitor by assuming that the apoptosis relation protein is concerned in this cell injury. Hydrogen peroxide was given in the vascular endothelial cell, and the expression of Bax and Bcl-2 were measured using monoclonal antibody in flow cytometer. And, the stress was similarly given in the independent culture group and protease inhibitor cocultivation group, and the change of the expression of Bcl-2 and Bax were measured. The expression of Bcl-2 of the stimulus group showed the decrease in comparison with the control population, and the Bax/Bcl-2 ratio showed the tendency in the stress which increased concentration-dependent. In addition, Bax and Bcl-2 expression were compared in the protease inhibitor cocultivation and independent culture group, in the cocultivation group, the decrease in the expression of Bcl-2 was suppressed in comparison with the independent culture group, and the increase tendency of the Bax/Bcl-2 ratio was also suppressed. And the form of DNA was kept in the measurement by the PI dyeing. The result indicate in which Bcl-2 family who is the apoptosis relation protein from them in the injury of the vascular endothelial cell was concerned, and the protease inhibitor caught that the cytoprotective effect may be shown by the action of normalizing the change of Bax/Bcl-2 ratio by the oxidative stimulation as a phenomenon.

Introduction

There are a number of aggressive insult situations that suddenly develop in patients who are brought in an emergency, including those of external causes, such as injuries, burns, hemorrhage, intoxication, and infections; and those of intrinsic nature, such as dehydration, pain, and tumors. Various test procedures, as well as surgery and chemotherapy act as added stresses to human bodies. Experimental studies of these aggressive insults have undoubtedly contributed to the progress in clinical emergency care, improve the safety of extensive surgical procedures, and ultimately improved the outcome of treatment for those patients with poor risks. Dramatic progress has recently been shown in the results of

studies on aggressive insults. In the field of emergency medicine, there reported a great number of studies on the significance of a host invasion and multiple organ dysfunction syndrome (MODS)[1)2)], which indicated rapid progress in clarifying the defense and control mechanism of the host against aggressive insults.

A patient subjected to an overwhelming aggressive insult often succumbs to multiple organ failure that develops as a consequence of this aggressive insult, rather than as a direct result of the aggressive insult itself. The prognosis of a patient with multiple organ failure is extremely poor and the condition is usually refractory to any treatments.

Cytokines are significantly involved in the development of multiple organ failure. It has been reported that under a stress condition, the stimulation by mediators, such as cytokines produces excessive quantities of active oxygen as a result of changes in the blood flow in tissues and organs, which in turn, triggers tissue damage[3)]. It has also been reported that the activated oxygen induced stress protein in cells[4)], acted as a factor to cause cell proliferation, and induced and promoted apoptosis[5)]. It is known that many of the aforementioned mediators have direct or indirect effects on the function of vascular endothelial cells. These cells not only play an important role in the host under stress: but also are injured by the action of activated oxygen, which initiates the process by which major organs start malfunctioning.

The mechanism for the development of organ failure following stress can be described as follows: when stimulated, the immune responsive cells secrete chemical mediators, such as cytokines, and the aggressive cells that receive the cytokine signals generate aggression factors, such as free radicals and proteases, through the intracellular transduction system[6)]. Ultimately, cellular functions become deranged, tissues are destroyed, and organ failure ensues[6)]. In addition, through the uptake of oxygen by the host, various types of activated oxygen are produced; molecules, such as activated oxygen species and free radicals, seek electrons to form pairs and exist in highly unstable and reactive states. Under a stress condition, changes in the blood flow in organs and stimulation by mediators such as cytokines result in excessive generation of activated oxygen. This condition also enhances the generation of activated neutrophils, mediators of inflammation, and IL-8, a neutrophil migration factor, from vascular endothelial cells; and it causes adhesion of neutrophils to vascular endothelial cells[7)]. This represents one possible mechanism through which tissue damage evolves. Activated oxygen also aids in the transformation of xanthine dehydrogenase to xanthine oxidase. The excess quantity of activated oxygen that has been produced inactivates enzymes, such as α-1 antiprotease and it becomes impossible to suppress elastase activity.

The present study is intended to examine the signal transduction mechanism that may trigger *in vivo* responses to stresses through some artificial experiments in vitro, with a special emphasis on injuries to the vascular endothelial cells. It was presumed that apoptosis participates in the mechanism through which vascular endothelial cells are injured under the stress. By investigating the possibility that Bcl-2 associated X protein (Bax) and the B cell lymphomal leukemia-2 (Bcl-2) system, apoptosis-related proteins, are involved in this mechanism, the process in which vascular endothelial cells are damaged under the stress may be elucidated. Some new discoveries in the present study were also made on the cell protective actions of protease inhibitors against this damaging mechanism, the details of which are reported below. In prior studies on aggressive insult, reports have been made in each spe-

cific area without any correlationship. The techniques employed in the present study are based on a new approach and the application of a systematic methodology, to obtain basic and scientific information on sequential changes resulting from an aggressive insult.

Materials and Methods

Materials

Human umbilical vein endothelial cells (HUVEC; Bio Whitaker Inc, Walkersville, MD, USA) were used as the material. The cells were placed in a CO_2 incubator that was adjusted to a temperature of 37 ℃ and CO_2 concentration of 5.0 %. A culture medium was used for HUVECs (EGM-2; containing 20 ml/L FBS, 4 ml/L hFGF-B, 1 ml/L VEGF, 1 ml/L R3-IGF-1, 1 ml/L heparin, 1 ml/L hEGF, GA-1000, and antibiotics; Clonetics Co, San Diego, CA, USA). HUVECs of a single strain were serially cultured for the material.

Experimental protocol

HUVEC that had been cultured singly under the conditions given above and HUVEC that were raised with a purified human urinary trypsin inhibitor (UTI, by Mochida Pharmaceutical Co, Tokyo, Japan) used as a protease inhibitor at a final concentration of 1 mM to 1 nM (1μM = 200 U/ml: computed with the relative activity of a pure product as 3000 U/mg and apparent molecular weight of the solution as 66000) were employed. For a stress donor, hydrogen peroxide (H_2O_2: 0.25 mM to 1 mM) was allowed to act on both HUVECs for 24 hours after primary culture for 4 or 5 days of second or third generation and the expression of apoptosis-related proteins that were induced by oxidative stress on both HUVECs was analyzed by using a digital flow cytometer (Epics XL-MCL System II: Beckman Coulter Co, Tokyo). The frequencies of expression were described as the percentage of the number of HUVECs with positive expression to the total number of HUVECs.

Analysis of Bax and Bcl-2 expression

Apoptosis-related proteins of vascular endothelial cells that have been subjected to oxidative stress were allowed to react immunochemically with the FITC-labelled anti-Bax monoclonal antibody (CellProbe: Beckman Coulter Co, Tokyo) and FITC-labelled anti-Bcl-2 monoclonal antibody (CellProbe: Beckman Coulter Co, Tokyo). The induction of their expression was determined by using a digital flow.

DNA fragmentation

A propidium iodide solution (PI solution: 50μg/ml propidium iodide, 0.1 M sodium citrate, 0.1 M triton X-100) was added to 1×10^6 of vascular endothelial cells that had been subjected to oxidative stress. The cells were incubated at 4 ℃ in darkness. After 30 minutes, DNA fragmentation was determined by using a digital flow cytometer.

For analytical software, named Flow Centre System (Expo 2; Beckman Coulter Co, Tokyo) was used.

Results

When the singly cultured vascular endothelial cells were exposed to a stress donor (H_2O_2: 1 mM to 1 nM) for 24 hours and the frequency of expression of Bax, an apoptosis-enhancing protein, was determined by using a flow cytometer, it was confirmed that the Bax expression frequency of the stressed cells had significantly increased in comparison with the control without stress (Fig. 1). Similarly, the frequencies of the expression of Bcl-2, another apoptosis-inhibiting protein, were compared between the cells exposed to the stress donor and the non-stressed control cell; this time it was confirmed that, when compared to the control, the frequency of expression was significantly reduced in those cells that had been exposed to the stress donor (Fig. 2).

When these changes were examined in relation to the concentration of the stress donor, Bax

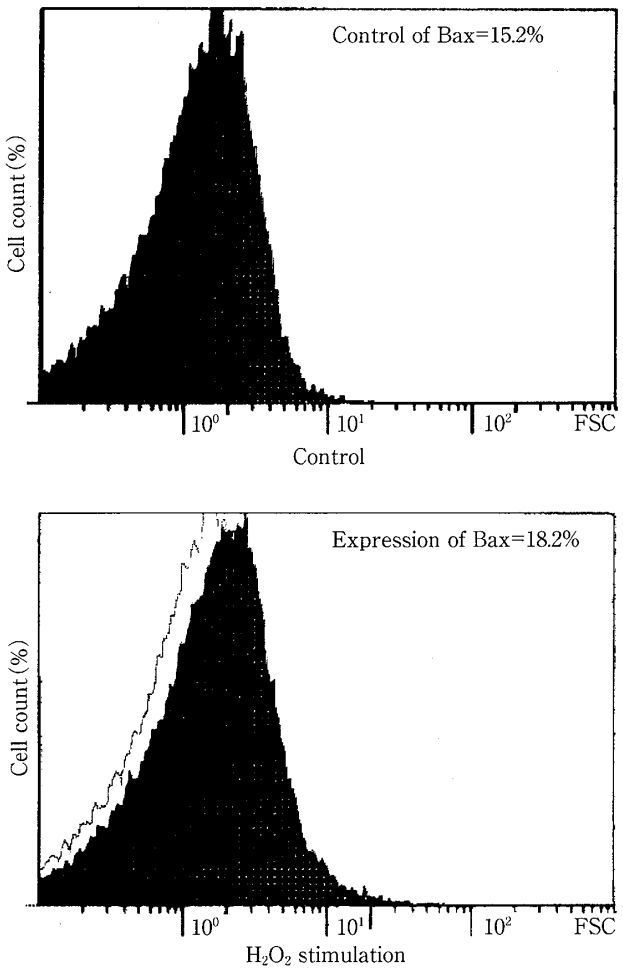

Fig. 1 Bax antigen expression
Expression of Bax in the H_2O_2-stimulated HUVECs (H_2O_2 : 0.5 mM), in comparison with the control: A slight increase is noted after H_2O_2 stimulation (indicated by using a monoclonal antibody).

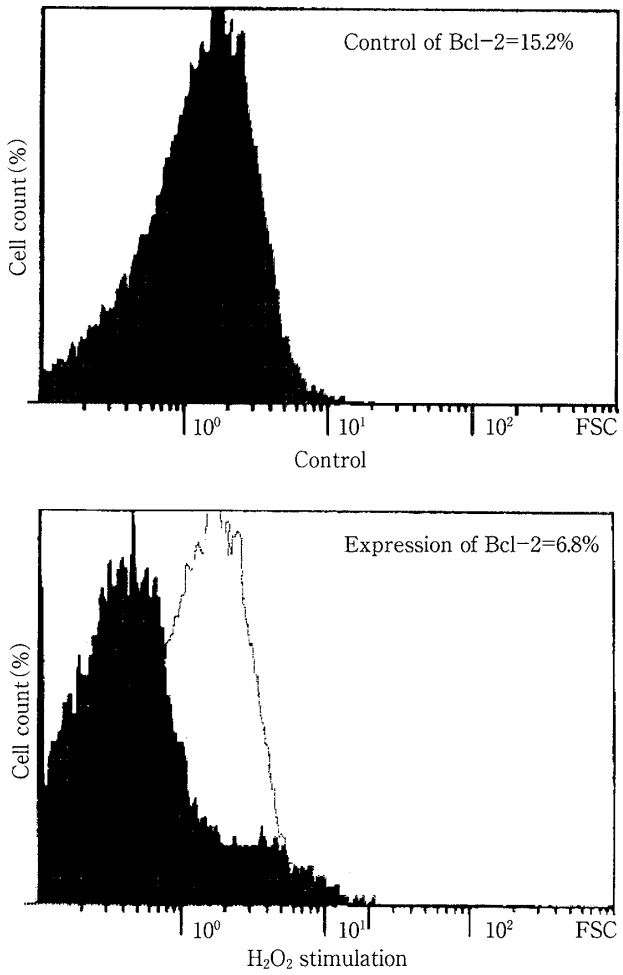

Fig. 2 Bcl-2 antigen expression
The frequency of Bcl-2 expression in cultured HUVECs after H_2O_2-stimulate (H_2O_2 : 0.5 mM) in cnotrol: A decline is evident in the former.

showed no prominent changes with a rise in the concentration of the stress donor, while the frequency of Bcl-2 expression showed a tendency to be reduced, depending on the concentration of the stress donor (Fig. 3). When the ratio of the expression frequencies, Bax/Bcl-2, was computed, it was found that the ratio increased dependent on the concentration of the stress donor (Fig. 4).

Next, singly cultured vascular endothelial cells and those cells that had been cultured with UTI were compared in terms of the change in the frequency of Bax and Bcl-2 expression in response to exposure to the stress donor. There were no prominent changes in the frequency of Bax expression in response to stress in the two groups of cells; but Bcl-2 expression was eminently reduced in the singly cultured cells. In those cells co-cultured with UTI, the reduction in Bcl-2 expression was markedly suppressed (Fig. 5). When Bax and Bcl-2 expressions were examined in relation to the concentration of the stress donor, the changes in these expressions in the singly cultured cells described above were absent in the UTI co-cultured cells (Fig. 6). Similarly, the Bax / Bcl-2 ratio did not show prominent increases, as seen in the singly cultured cells (Fig. 7).

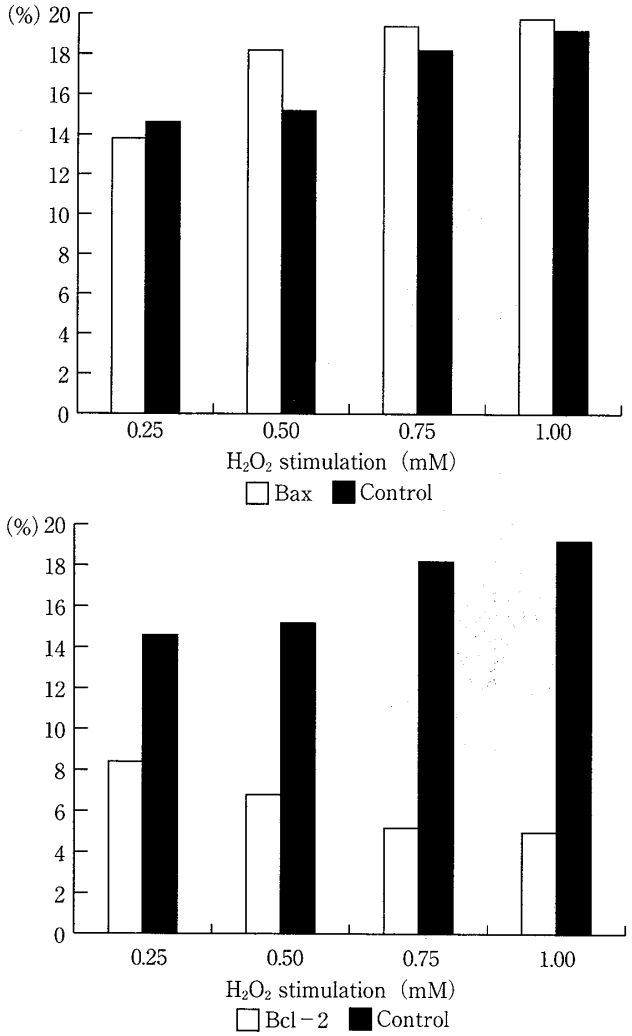

Fig. 3 Relation between Bax and Bcl-2
It is dependent on H_2O_2 concentration, when the relation between H_2O_2 concentration and Bax and Bcl-2 was measured, and Bcl-2 manifestation frequency decreased.

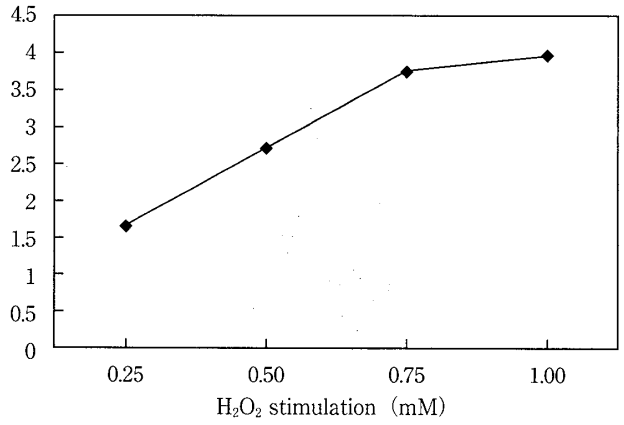

Fig. 4 Bax/Bcl-2 ratio
The Bax/Bcl-2 ratio in the cultured HUVECs and its dependancy on the H_2O_2 concentration: the ratio increases depending on the inhibitor dose.

Stress and the actual DNA fragmentation were examined by propidium iodide (PI) staining. As expected, DNA was markedly damaged by stress stimulation in the singly cultured cells, while in the UTI-co-cultured cells, DNA was relatively well-protected, with minimal damage (Fig. 8).

Discussion

The disruption of the host defense mechanism under aggressive insult still constitutes a serious problem in emergency medicine and intensive care. Vascular endothelial cells are one of the targets where stress inflicts serious damage[8)9)]. As stated in the introduction, these cells suffer from the direct or indirect effects of stress, which trigger dysfunctioning of major organs. A number of studies have recently been introduced to show the relationship between damage to the vascular endothelial cells and apoptosis[10)11)] ; but there has not been any conclusive evidence and the mechanism governing the relationship has not been elucidated.

The present study was based on the premise that apoptosis would be related to the damage that occurred in vascular endothelial cells; and Bax and Bcl-2, apoptosis-related proteins, would be related to the mechanism. When vascular endothelial cells were stimulated by hydrogen peroxide, a stress donor, the expression of Bax and Bcl-2 showed evident changes. H_2O_2 causes apoptosis in various types of cells[12)] and Bcl-2 has been known as a protein that inhibits this apoptotic process[13)]. This Bcl-2, an apoptosis-inhibiting protein, exists in intracellular nuclear membrane, mitochondrial outer membrane, and vesicular membrane. It has been understood that apoptosis was suppressed through an anti-oxidant action against active oxygen[14)], such as hydrogen peroxide

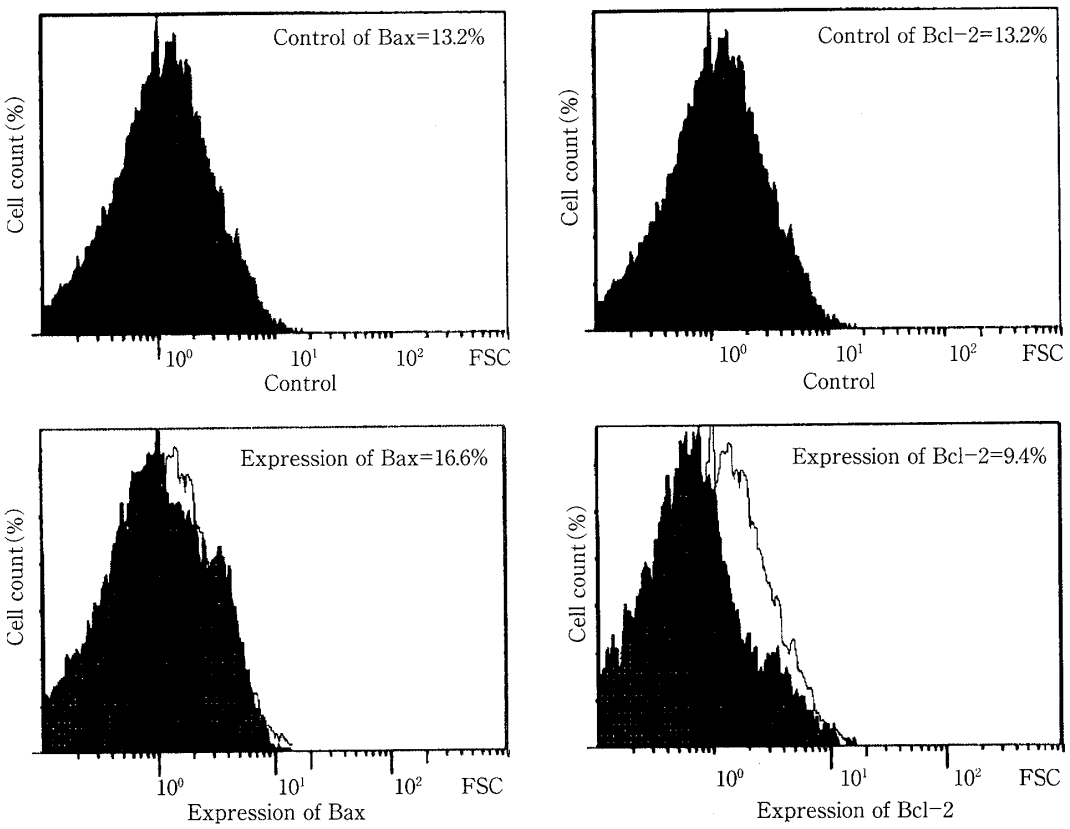

Fig. 5 Bax and Bcl-2 antigen expression
Bax and Bcl-2 expression after H_2O_2-stimulated HUVECs that were co-cultured with UTI: a prominent change in the cells without UTI is not recognized in those co-cultured with UTI.

that produced in these membranes. The results of the present study corroborated this theory by showing a marked reduction of the Bcl-2 expression of vascular endothelial cells where apoptosis had been induced by hydrogen peroxide. Bax, on the other hand, is an apoptosis-enhancing protein that co-precipitates with Bcl-2 protein during immuno-precipitation. Bax that has been expressed in excess competes against the cell death inhibiting activities of Bcl-2. It is postulated that Bax has a domain highly homologous to Bcl-2 and forms a homodimer (Bax/Bax) or heterodimer (Bax/Bcl-2) in cells; and their quantitative balance serves to make adjustments between cell death and cell survival (with Bax forming a complex with Bcl-2 to suppress the function of the latter). Thus apoptosis occurs in the cells that were forced to express Bax[15]. In the present study, the change in the Bax expression was slight in comparison with Bcl-2 expression; but prominent changes were noted in the Bax/Bcl-2 ratio in the vascular endothelial cells where apoptosis might be induced by hydrogen peroxide. These findings possibly suggest that apoptosis induction in vascular endothelial cells stimulated by hydrogen peroxide is governed by a quantitative balance between Bax and Bcl-2. It was also suggested that the reduction in the Bcl-2 content, rather than an increase in the Bax level, is more responsible for the disruption of this balance.

Protease is a general term for enzymes that hydrolyze proteins: they participate not only in digestion and absorption of proteins and peptides but are involved in almost all events that are di-

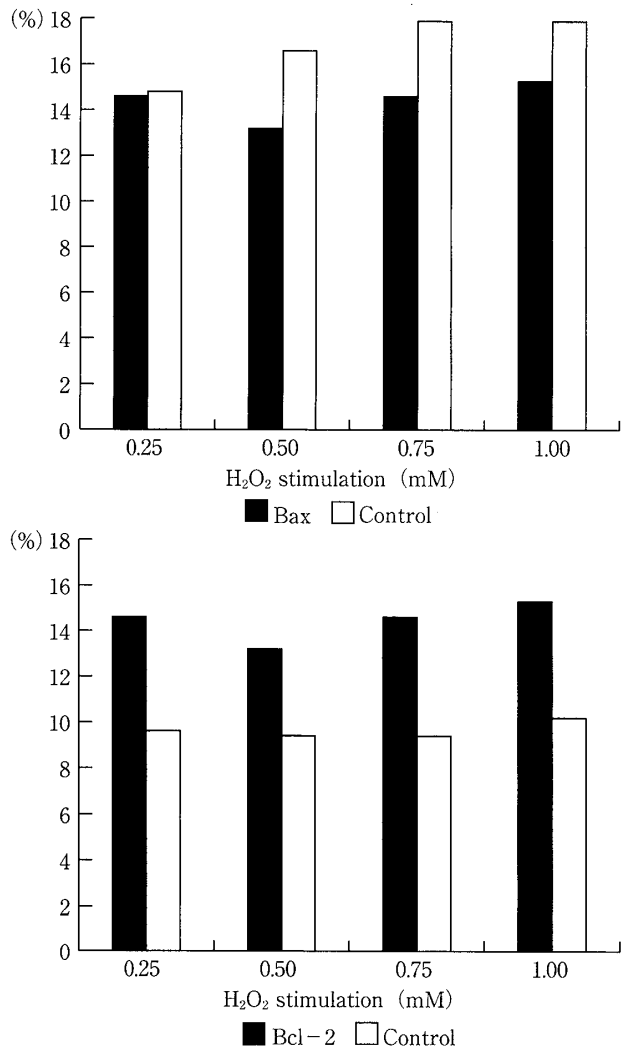

Fig. 6 Relation between Bax and Bcl-2
Comparison of the relation between H_2O_2 concentration and Bax and Bcl-2 with the independent culture group, the decrease in Bcl-2 manifestation frequency was suppressed of the UTI cocultivation group.

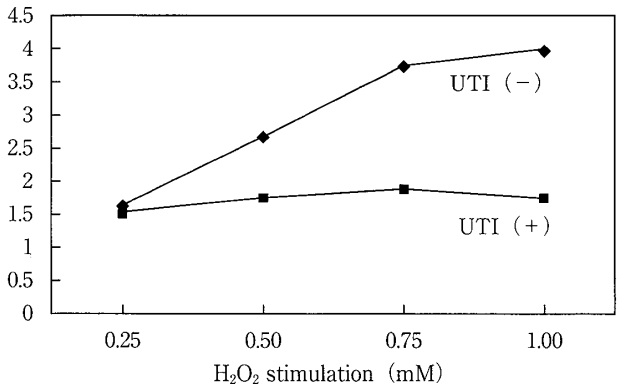

Fig. 7 Bax/Bcl-2 ratio
Comparison of the Bax/Bcl-2 ratio in HUVECs between the UTI cocultivation group and the independent culture group.

rectly related to life itself, such as the adjustment of metabolism and signal transmission[12]. The human body also possesses a potent anti-protease system, with known anti-proteases being α-1-protease inhibitor (α1-PI), inter-α-protease inhibitor (αTI), and urinary trypsin inhibitor (UTI). Among them, α1-PI performs the most functions. αTI is a protease inhibitor that ranks next to α1-PI in its serum content: it is partially decomposed by neutrophil elastase and becomes ulinastatin (UTI) with even more potent inhibitory activity.

There are many reports on the relationship between these protease inhibitors and apoptosis[16] and the importance of proteases in apoptosis is beginning to be elucidated. Based on a preliminary result that a Bax and Bcl-2 regulating action would be involved in the vascular endothelial cells in which apoptosis was induced by hydrogen peroxide, the present study was conducted on the premise that protease inhibitors would be also involved in the same regulating action. Subsequently, it was found that stimulation of the cells that had been cultured without a protease inhibitor results in a high incidence of apoptosis of vascular endothelial cells, with an associated reduction in the Bcl-2 content. When those cells co-cultured with a protease inhibitor were stimulated, the result was a reduction in the Bcl-2 content that was less prominent than that seen in cells cultured without the inhibitor. Compared with singly cultured cells, those cells co-cultured with a protease inhibitor failed to show prominent changes in the ratio of Bax/Bcl-2 expression. This experimental finding illustrated apoptosis suppression. This suggested a possibility of inhibitory effect of protease inhibitors on apoptosis regulated by Bax and Bcl-2 and that an apoptosis inhibitory action exists.

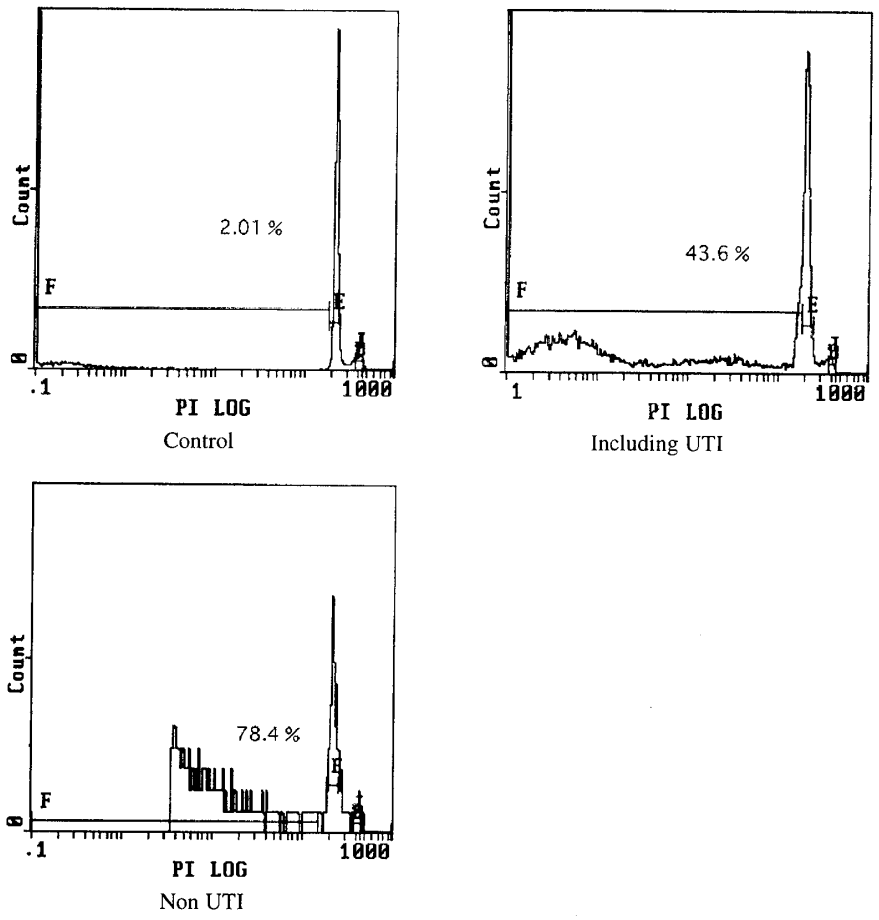

Fig. 8 Propidium iodide staining
The DNA damage shown by PI staining: oxidative stress caused DNA in the cells cultured without UTI, while in those co-cultured with UTI, DNA is relatively well protected.

The mechanism involved in the development of organ failure under stressful insult is gradually being elucidated through research. It has been said that one of the mediators directly responsible for organ dysfunction in inflammatory reactions would be proteases produced and released by neutrophils and active oxygen[17]. Protease inhibitors are known to suppress excessive host responses[18] and prevent the development of organ failure (cytoprotective action)[19]. Currently, therapy using protease inhibitors is actively practiced[20]. UTI, a protease inhibitor selected as the agents for this study, is used widely. Further investigation on host responses and the role and mechanism of apoptosis (such as in this study) will lead to the development of new therapeutic modalities.

Conclusion

1. A study was conducted on the relationship between oxidative insult and damage to the vascular endothelial cells, in connection with apoptosis. A possibility that apoptosis would be involved in the mechanism through which vascular endothelial cells were injured at the induction of oxidative stress was indicated.

2. It was suggested that Bax and Bcl-2, apoptosis-related proteins, participated in this mechanism and that the changes in this mechanism were regulated by variations in the Bax/Bcl-2 ratio through suppression of Bcl-2 expres-

sion.

3. The test results showed an interesting phenomenon: protease inhibitors suppressed apoptosis induction in vascular endothelial cells by inhibiting a reduction in Bcl-2 expression, thus acting as cytoprotective agents.

Acknowledgement

Authors are grateful gratitude to Prof. Takao NAKAGAWA for his help at the time of editing this manuscpipt.

A summary of this study was presented at the 11th Biomedical Forum (held in Tokyo in 2000).

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血管内皮細胞傷害に対するプロテアーゼインヒビターによるサイトプロテクション効果

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血管内皮細胞の機能破綻は，生体が侵襲に曝された際に引き起こされる多臓器障害等の発症の first step である．その機序には酸化ストレスによる細胞傷害が深く関与するとされている．我々は，この細胞傷害にアポトーシス関連蛋白が関与すると想定し，培養した血管内皮細胞を用いて，この内皮細胞傷害のメカニズムを解明すると共に，現在，臨床応用されているプロテアーゼインヒビター（ウリナスタチン）が細胞傷害に対してどのように作用するかを比較検討した．血管内皮細胞にストレスドナーを与え，アポトーシス関連蛋白である Bax および Bcl-2 の形質発現を，モノクロナール抗体を用いて flow cytometer で測定したところ，対照群と比較して刺激群の培養内皮細胞は，アポトーシス抑制蛋白である Bcl-2 の発現が有意に減少しており，Bax/Bcl-2 比はストレスの H_2O_2 濃度依存的に増加する傾向を示す結果を得た．この結果を踏まえ，プロテアーゼインヒビターを加えて共存培養した血管内皮細胞と単独培養した血管内皮細胞について，Bax および Bcl-2 の形質発現を比較した．単独培養群に比べて共存培養群の内皮細胞群では，Bcl-2 の発現の減少が抑制され，Bax/Bcl-2 比の増加傾向も抑制されていた．これらから血管内皮細胞の傷害にはアポトーシス関連蛋白である Bcl-2 ファミリーが関与する可能性が示唆された．また，プロテアーゼインヒビターは酸化刺激により引き起こされる Bax/Bcl-2 比の変化を正常化することにより，細胞保護的に作用する可能性が考えられた．