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# Type 1 IFN Inhibits the Growth Factor Deprived Apoptosis of Cultured Human Aortic Endothelial Cells and Protects the Cells From Chemically Induced Oxidative Cytotoxicity

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## ABSTRACT

It has been shown that the genesis of atherosclerotic lesions is resulted from the injury of vascular endothelial cells and the cell damage is triggered by oxygen radicals generated from various tissues. Human vascular endothelial cells can survive and proliferate depending on growth factors such as VEGF or basic FGF and are induced apoptosis by the deprivation of growth factor or serum. It was found that type 1 IFN inhibits the growth factor deprived cell death of human aortic endothelial cells (HAEC) and protects the cells from chemically induced oxidative cytotoxicity. The anti-apoptotic effects of type 1 IFN were certified by flow cytometry using annexin-V-FITC/PI double staining and cell cycle analysis, fluorescence microscopy using Hoechst33342 and PI, colorimetric assay for caspase-3 activity, p53 and bax mRNA expressions, and cell counts. It was considered that IFN-β inhibits the executive late stage apoptosis from the results of annexin-V-FITC/PI double staining and the inhibition of caspase-3 activity, and that the anti-apoptotic effect might be owing to the direct inhibition of the apoptotic pathway mediated by p53 from the transient down-regulation of bax mRNA expression. Whereas, type 1 IFN protected the cells from the oxidative cytotoxicity induced by tertiary butylhydroperoxide (TBH) under the presence of $Ca^{2+}$. The effects of IFN-β is more potent inhibitor of cell death than IFN-α. These results indicate that type 1 IFN, especially IFN-β may be useful for the diseases with vascular endothelium damage such as atherosclerosis or restenosis after angioplasty as a medical treatment or a prophylactic. J. Cell. Biochem. 113: 3823–3834, 2012. 

**KEY WORDS:** HUMAN ENDOTHELIAL CELLS; TYPE 1 IFN; ANTI-APOPTOTIC EFFECT; GROWTH FACTOR DEPRIVATION; TERTIARY BUTYLHYDROPEROXIDE

Vascular endothelial cells have a significant role to keep the homeostasis of blood vessels. It has been known that vascular endothelial cell injury occurs in atherosclerotic lesions or in restenosis of coronary arteries after angioplasty [Ross and Glomset, 1973] and that the occurrence of cell damage is triggered by abruptly generation of oxygen radicals during the artery occlusion and recirculation [Forman et al., 1989]. Therefore, the development of a useful drug which can increase the survival of endothelial cells in vascular diseases has been expected. It has been demonstrated that human vascular endothelial cells can proliferate and survive only in the presence of specific growth factor such as VEGF or basic FGF (bFGF) [Gospodarowicz et al., 1986; Leung et al., 1989] and that the cells are induced apoptosis by the deprivation of growth factors [Araki et al., 1990] or serum [Chen et al., 2010]. Therefore, the growth factor or serum deprivation is considered as a useful model for evaluating the effects of various substances on the endothelial cell survival.

Apoptosis plays a fundamental role in many normal biological processes as well as several diseases states [Ellis et al., 1991]. Apoptosis can be induced by various stimuli and all produce the same end result. For the detection of early stage of apoptosis, the changes in the position of phosphatidylserine (PS) in the cell membrane have been used. In non-apoptotic cells, most PS molecules are localized at the inner layer of the plasma membrane, but soon after inducing apoptosis, PS redistributes to the outer layer of the membrane, and becomes exposed to the extracellular

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environment [Fadok et al., 1992; Martin et al., 1995]. The PS externalization occurs irrespective of the kinds of stimuli and the exposed PS can be easily detected with annexin V [Martin et al., 1995]. While, propidium iodide (PI) is used as a DNA stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis and as a counterstain in multicolor fluorescent techniques [Moore et al., 1998; Lecoeur, 2002]. PI is membrane impermeable and generally excluded from viable cells. Therefore, it is commonly used for identifying dead cells in a population. Hoechst 33342 has been also used for specifically staining the nuclei of living or fixed cells and tissues [Parrilla et al., 2004]. A combination of Hoechst 33342 and PI have been extensively used for simultaneous flow cytometric and fluorescence imaging analysis of the stages of apoptosis and cell-cycle distribution. Whereas, caspase-3 activities have been examined for the decisive confirmation of apoptosis. Caspase-3 is an active cell death protease involved in the execution phase of apoptosis [Green and Reed, 1998].

Interferons (IFNs) are a family of related cytokines that mediate a wide range of diverse functions including antiviral, anti-proliferative, antitumor, and immuno-modulatory activities. IFNs are currently classified into two major groups of type I and type II [Gresser, 1990]. IFN-α (leukocyte), -β (fibroblast), and -ω (trophoblast) are included in type I IFN. The type II IFN is designated IFN-γ, also known as immune IFN. These IFN types bind distinct cellular receptors and activate both individual and overlapping pathways. There are a lot of reports relative to anti-virus and anti-tumor effects of IFN, however, very little is known about the cyto-protective effects although the protection on T-cell apoptosis [Zipp et al., 2000; Chang et al., 2001] or cyto-protective effect on the hepatocyte apoptosis with virus infection by IFN-α [Saile et al., 2003; Kocic et al., 2007] has been reported.

IFN has been known to induce cell death by direct cytotoxic effect [Grander et al., 1993] or by apoptosis [De Marco et al., 1999; Panaretakis et al., 2008] in many cell types. Some molecular mechanisms relating IFN inducing apoptosis have been clarified [Thyrell et al., 2004; Dedoni et al., 2010]. It was shown that type 1 IFN can induce apoptosis by the up-regulation of bax gene expression [Wittnebel et al., 2005] which is directly activated by p53 [Miyashita and Reed, 1995] in various types of cancer cells. Dedoni et al. [2010] reported apoptotic effect of IFN-β through activation of JAK-STAT signaling and down-regulation of PI3K/Akt pathway in human neuroblastoma. Whereas, there are some reports relating anti-apoptotic effects of type 1 IFN. Saile et al. [2003] showed the cyto-protective effect of IFN-α on the hepatocyte apoptosis with virus infection. The effect was considered to be due to anti-virus effect of IFN. Further, the inhibition of human t-cell apoptosis by IFN-β was demonstrated by Chang et al. [2001]. It was considered that the main mechanism of apoptosis inhibition is immunomodulation mediated by IFN-β rather than the direct inhibition of the apoptotic pathway in human T cells. Recently, it was shown that IFN-α can protect the anti-Fas-antibody induced hepatocyte apoptosis using animal models and in vitro cultures with acute viral hepatitis [Kocic et al., 2007]. The report demonstrated that IFN-α increased the number of viable cells by the increase of bcl-2 positive cells and the decrease of bax positive cells. It was also made clear that IFN-α contributes to late phase apoptosis. Recently, it was demonstrated that IFN-β modulates endothelial damage in patient with cardiac persistence of human parvovirus B19 infection [Schmidt-Lucke et al., 2010]. However, the effects of IFN on the endothelial cell functions or survival have not been examined sufficiently until now although the anti-proliferative effect [Mintzer et al., 1998] or the enhancement of tube formation [Hammar et al., 1992] has been investigated.

Tertiary butylhydroperoxide (TBH) has been used in the various in vitro experiments for the induction of oxidative cell damage [Cheng et al., 2007]. The oxidative injury of endothelial cells has been known to bring the loss of various endothelium functions [Ham et al., 1982; Shatos et al., 1990] and TBH has been also demonstrated to inhibit the endothelial cell function [Whorton et al., 1985]. It has been reported that the induction of oxidative cell damage is concerned with cellular $Ca^{2+}$ homeostasis [Lounsbury et al., 2000] and that the increase of intra-cellular free $Ca^{2+}$ concentration resulted from the overload of extra-cellular $Ca^{2+}$ [Ashraf, 1987]. Hirosumi et al. [1988] examined the effect of superoxide and lipid peroxide on cytosolic free calcium concentration and demonstrated calcium ion antagonists can suppress the endothelium damage. It was also shown that the induction of endothelial cell damage by TBH is concerned with the inhibition of calcium signaling [Elliott et al., 1993]. Therefore, TBH is available for evaluating the effects of substances against oxidative cytotoxic damage.

We were interested in investigating the effects of various IFNs on the endothelial cell death induced by oxidative stress or growth factor deprivation using human aortic endothelial cells (HAEC). In this report, we could reveal for the first time that type 1 IFN can inhibit the growth factor deprived apoptosis of human aortic endothelial cells and can prevent the cell death from the chemically induced oxidative cytotoxicity.

## MATERIALS AND METHODS

### MATERIALS

Natural type of human IFN-α (Sumitomo Pharmaseuticals, Osaka, Japan) and IFN-β (Toray Industries, Tokyo, Japan) and recombinant IFN-γ (Genentech, South San Francisco, CA) derived from *E. coli* were used for experiments. Bovine serum albumin (BSA) was purchased from Sigma Chemical (St Louis, MO). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical. Tertiary butylhydroperoxide (TBH) was purchased from Sigma–Aldrich (St. Louis, MO).

### CELL AND CELL CULTURE

Human aortic endothelial cells (HAEC) were purchased from Kurabo (Osaka, Japan) and the cells of 8–15 PDL were used for the experiments. HAEC were cultured routinely in M199 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% of fetal calf serum (FCS, Life Technologies, Grand Island, NY) and 10 ng/ml of bFGF (Pepro Tech, Inc., Rocky Hill, NJ) or 20 ng/ml of $VEGF_{165}$ (Immuno-Biological Laboratories, Gunma, Japan) using 25 $cm^2$ collagen coated flasks (Iwaki, Chiba, Japan). The confluent culture was passaged using 0.25% trypsin-1 mM EDTA-4Na solution (Invitrogen, San Diego, CA).

### DETERMINATION OF CELL NUMBER

The determination of living or dead cell number was carried out using 24 well collagen coated plate (Iwaki). The number of the cells was counted using coulter counter (Coulter counter Z1, Beckman Coulter, Fullerton, CA). The viable cells were distinguished from dead cells stained with 0.45% trypan blue (Sigma–Aldrich). Dead cell number was determined by counting floating cells because all the detached cells were stained by trypan blue solution.

### CELL CYCLE DISTRIBUTION ANALYSIS

The apoptotic cell population with degraded DNA was determined by the cell cycle distribution analysis by FACS. Propidium iodide (PI, Molecular Probes, Eugene, OR) was used as a DNA stain. After the treatment with IFN in the medium supplemented with 2% FCS without growth factor, the cells were harvested by trypsinization and fixed in ice-cold 70% ethanol. The fixed cells were treated with staining solution containing 0.2 mg/ml DNase free RNase A (Takara-Bio, Mountain View, CA) and 50 μg/ml PI for 30 min at 4°C. Then, their fluorescence were measured using FACS-Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The DNA histograms were generated using CellQuest software (Becton Dickinson).

### FLUORESCENCE MICROSCOPIC ANALYSIS

The cells were cultured in 25 $cm^2$ collagen coated flasks (Iwaki) until confluent. The cells were treated with IFN-β in the growth factor deprived medium supplemented with 2% FCS. After rinsing the cells with PBS (+) supplemented with 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$, the cells were double-stained with Hoechst-33342 (Thermo Scientific, Rockfold, IL) and PI (Molecular Probes) for 15 min at room temperature according to the manufacture's instruction and observed under a fluorescence microscope (Olympus IX70, Olympus, Shinjyuku, Japan). Hoechst 33342 was exited at 352 nm by UV light and emitted blue fluorescence at 461 nm. It is used for specifically staining the nuclei of living cells. PI is also a fluorescent molecule. When excited at 488 nm, it fluoresced red and could be detected at 588 nm. PI is commonly used for identifying dead cells in a population.

### FLOW CYTOMETRIC ANALYSIS BY ANNEXIN-V-FITC/ PI DUAL STAINING

The cells were cultured in 25 $cm^2$ collagen coated flasks (Iwaki) until confluent. After treating the cells with IFN-β, the cells were assessed for apoptosis using annexin-V-FITC and PI double staining kit (Clontech, Mountain View, CA) according to the manufacturer's instruction. The cells were analyzed by FACS-Calibur and the histogram was analyzed by Flowjo software (BioLegend, San Diego, CA). The cells were excited with a 488 nm argon laser line and the fluorescence of annexin-V-FITC and PI was analyzes on FL1 (525 nm) and FL3 (610 nm), respectively.

### DETERMINATION OF CASPASE-3 ACTIVITY

The caspase-3 activity was determined using the ApoAlert caspase colorimetric assay kit (Clontech) according to the manufacturer's instruction. Briefly, the cells were collected and lysed with lysis buffer for 10 min on ice. The cell debris was centrifuged at 12,000 rpm for 15 min at 4°C. The colorimetric assay was performed through the spectrophotometric detection of the chromophore p-nitroaniline (pNA) generated from the cleavage of the labeled caspase-specific substrates (DEVD–pNA) by caspase-3. After addition of reaction buffer, caspase-3 substrate (DEVD–pNA) was added and incubated at 37°C for 1.5 h. The absorbance at 380 nm was measured by NanoDrop-2000C (Thermo Scientific, Wilmington, DE). The activities were determined from the pNA calibration curve prepared according to the manufacture's instruction. All the activities were subtracted the value in the IFN-β un-treated cells without substrate, and the relative activities were shown as fold-increase by comparing each value with those in the positive control cells in bFGF added culture.

### QUANTITATIVE RT-PCR

HAEC were cultured in 25 $cm^2$ collagen coated flasaks. After treatment of IFN-β, total RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacture's instruction. RNA was reverse-transcribed to cDNA with SuperScript Reverse Transcriptase II (Invitrogen, Carlsbad, CA). Real time PCR was performed on the ABI 7900HT (Applied Biosystems, Tokyo, Japan) with SYBR green PCR mix (Takara-Bio, Mountain View, CA). The sequences introduced by Ismail et al. [2007] were referred for making the following primer pairs: p53 forward; 5′-TCTCCCCAGCCAAAGAAGAAA-3′, reverse; 5′-TTCCAAGGCCTCATTCAGCTC-3′, bax forward; 5′-TGGAGCTGCA-GAGGATGATTG-3′, reverse; 5′-CCAGTTGAAGTTGccGTCAGA-3′. Fold changes of relative mRNA expression of p53 and bax were calculated using the comparative $2^{-\Delta\Delta Ct}$ method using β-actin (Takara-Bio, Tokyo, Japan) to normalize mRNA level as previously described [Livak and Schmittgen, 2001] and were expressed as the means ± SE. Experiments were performed three times independently.

### EVALUATION OF OXIDATIVE CELL DAMAGE

A system to evaluate the oxidative cytotoxicity was devised using TBH. The cells were seeded in collagen coated 96 well plate (Iwaki) and cultured until sufficiently confluent. The confluent cells were treated with 10 mM TBH prepared using PBS(−) supplemented with 0.1% BSA and 1 mM $CaCl_2$. The cells were incubated at 37°C for 40–50 min for causing oxidative cell damage. The adherent living cells are changed to floating dead cells with time elapse. After the proportion of viable adherent cells was reduced to about 50% by the observation under a microscope (Olympus CK-40, Tokyo, Japan), the culture medium was changed to 10% FCS containing medium. Immediately after the medium change, the viability was analyzed by the MTT assay reflected the dehydrogenase activity of mitochondria according to the slightly modified method described by Mitic-Culafic et al. [2009]. Briefly, MTT solution was added to each well for another 4 h. Then, the supernatant was gently aspirated and DMSO was added to each well. After incubation with shaking for 10 min, the OD value was measured at 490 nm.

### STATISTICAL ANALYSIS

All of the experiments related to the cell viability were replicated over three times. Average numbers of living or dead cells and

standard errors of the mean were calculated using Excel software. Appropriate comparisons were made by the Tukey–Kramer method for multiple comparisons using JMP software (Ver. 3, SAS Institute, Cary, NC). All the differences were considered significant with $P < 0.05$.

## RESULTS

### EFFECTS OF VARIOUS IFNs ON THE SURVIVAL OF THE CELLS

The effect of IFN-α, -β, and -γ on HAEC survival was examined under growth factor deprivation. The cells of $1 \times 10^5$ were seeded in each well of collagen coated 24 well plate and cultured in the medium supplemented with 10% FCS and 10 ng/ml bFGF until sufficiently confluent. The cells were treated with IFNs of 0.1–1,000 IU/ml in the medium supplemented with 2% FCS without growth factor. The adherent living cells were tripsinized and counted after 48 h of IFN treatment. As shown in Figure 1A, the number of living cells was increased by the treatment of IFN-α or IFN-β in a dose dependent manner. IFN-β was more effective than IFN-α. On the contrary, IFN-γ decreased the living cells indicating that only type 1 IFN can prevent the cell death. Then, the effects of various IFNs on the cell death were examined in the presence or absence of bFGF. The cells were cultured in 25 $cm^2$ collagen coated flasks until confluent in the presence of 10 ng/ml bFGF and 10% FCS before IFN treatment. After confluent, the cells were treated with 100 IU/ml of IFNs in the presence or absence of 10 ng/ml bFGF for 48 h. The dead cells were confirmed using 0.4% trypan blue. As shown in Figure 1B, IFN-α and -β suppressed the formation of dead cells dyed with trypan blue in the absence of bFGF. The obvious inhibition of cell death was not found in the groups supplemented with bFGF suggesting that growth factor contributes to the survival of the cells. IFN-γ increased death cells in the growth factor deprived medium. These results indicate that type 1 IFN may suppress the programmed death of HAEC under growth factor deprivation.

### CELL CYCLE DISTRIBUTION ANALYSIS FOR THE DETERMINATION OF APOPTOSIS IN VARIOUS IFN TREATED CELLS

The effects of IFNs on the apoptosis were examined by cell cycle distribution analysis using flow cytometry. PI was used as a DNA stain. HAEC were grown in 25 $cm^2$ collagen coated flasks until confluent. The cells were treated with 1,000 IU/ml of IFN-α, -β or -γ for 24–48 h in the growth factor deprived medium. The cells were harvested by trypsinization and rinsed with PBS (−) without $Ca^{2+}$ and $Mg^{2+}$ before fixation by 70% ethanol. The fixed cells were treated with PI staining solution for 30 min at 4°C. Then, the fluorescence was measured by FACS. Based on the DNA histograms obtained from CellQuest software, the ratio of apoptotic cell population was determined. The histograms were shown in Figure 2. The apoptotic cells with degraded DNA were increased with time elapse in the culture without IFN. However, the proportions of apoptotic cells were decreased in the cultures treated with IFN-α or -β. However, the apoptotic cells in the IFN-γ treated culture were increased. These results indicate that type 1 IFN causes an anti-apoptotic effect on HAEC.

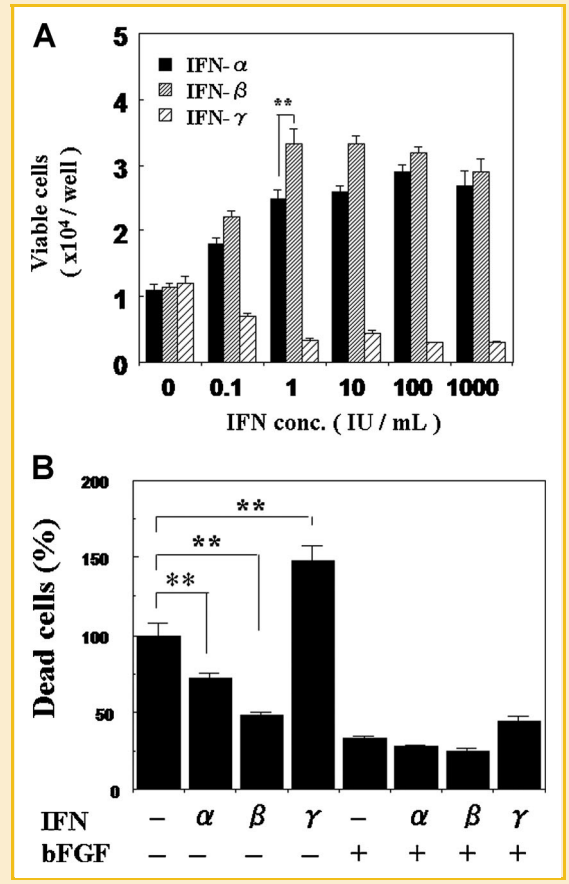


Fig. 1. Effects of IFNs on the survival of HAEC. A: Effects of various IFNs on cell viability. HAEC of $1 \times 10^5$ were seeded in each well of collagen coated 24 well plates and cultured until confluent using the medium supplemented with 10% FCS and 10 ng/ml of bFGF. The cells were rinsed once with serum and growth factor deprived medium and treated with 0.1–1,000 IU/ml of IFN-α, -β, and -γ for 48 h in the medium supplemented with 2% FCS. The adherent living cells were counted by coulter counter. Average cell number ± SE in four wells was shown in the figure. $^{**}P < 0.01$, compared with IFN- α and -β. B: Effects of IFNs and bFGF on the cell death. The cells were cultured in 25 $cm^2$ collagen coated flasks until confluent and were treated with 100 IU/ml of various types of IFNs for 48 h in the presence or absence of 10 ng/ml of bFGF. The dead cells dyed with 0.4% trypan blue were counted by coulter counter. The average cell numbers ± SE in three flasks were calculated and the relative values against the number of the cells in the culture without bFGF and IFN (100%) were illustrated in the figure. $^{**}P < 0.01$, compared with IFN minus and various IFNs in the absence of bFGF.

### FLUORESCENCE MICROSCOPIC ANALYSIS FOR THE DETECTION OF APOPTOSIS IN IFN-β TREATED CELLS

HAEC were cultured in 25 $cm^2$ collagen coated flasks until confluent. The cells were treated with 1,000 IU/ml of IFN-β for 48 h in the medium without growth factor. After rinsing the adherent cells with PBS (+) supplemented with 0.9 mM $Ca^{2+}$ and 0.33 mM $Mg^{2+}$, the cells were double-stained with Hoechst 33342 and PI for 15 min at room temperature. The cells were inspected by fluorescence microscopy under the following conditions. Hoechst 33342 was used for specifically staining the nuclei of living cells and was excited at 352 nm and emitted at 461 nm. PI was used for identifying dead cells in the cultures and was excited at 488 nm and

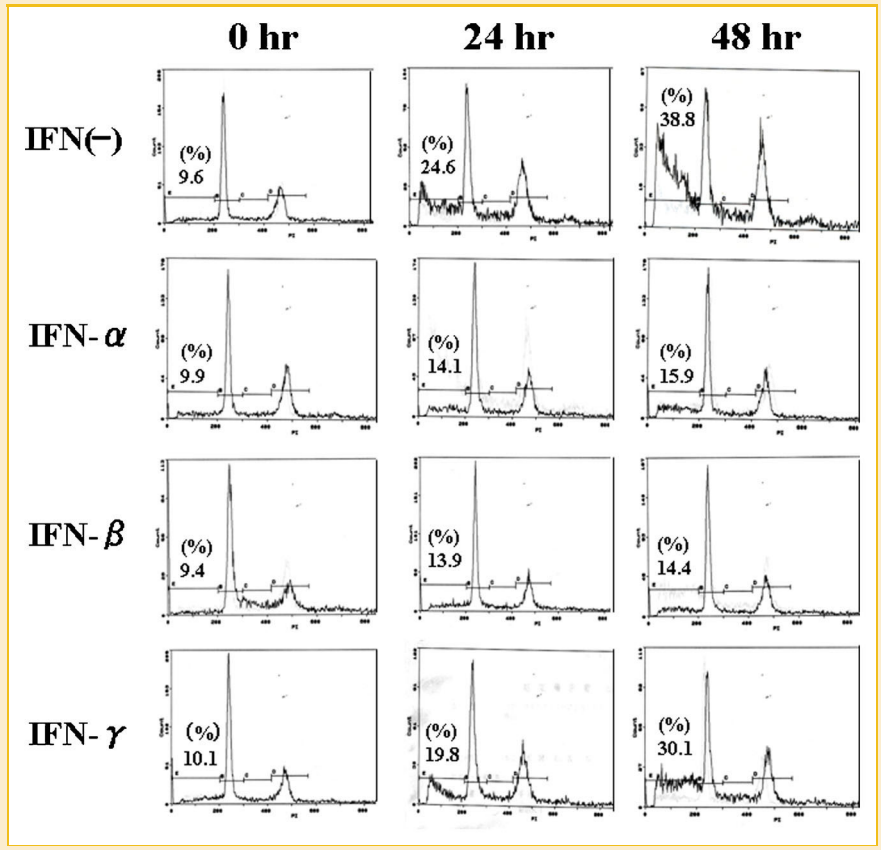


Fig. 2. Cell cycle distribution analysis. HAEC were cultured in collagen coated 25 $cm^2$ flasks until confluent in the medium supplemented with 10% FCS and 10 ng/ml of bFGF. After rinsing the cells with serum and growth factor deprived medium, the cells were treated with IFN-α, -β, and -γ of 1,000 IU/ml for 24–48 h in 2% FCS containing medium without growth factor. The cells were fixed by ethanol and analyzed according to the method described in Materials and Methods using PI. The proportion (%) of the cells with apoptotic DNA was shown in the figure.

emitted at 588 nm. As shown in Figure 3, most of the cell nuclei were stained with Hoechst33342 in both the control and IFN-β treated cultures, however, the nuclei stained with PI were decreased by the treatment of IFN-β. This indicates that IFN-β can prevent the death of HAEC induced by growth factor deprivation.

### FLOW CYTOMETRIC ANALYSIS FOR ANTI-APOPTOTIC EFFECT OF IFN-β USING ANNEXIN-V-FITC/PI DOUBLE STAINING

The effect of IFN-β on the HAEC apoptosis was analyzed by FACS using annexin-V-FITC/PI dual staining. The cells cultured in 25 $cm^2$ collagen coated flasks were treated with 1,000 IU/ml of IFN-β for 24–48 h in the growth factor deprived medium supplemented with 2% FCS. The cells were harvested by trypsinization and rinsed with binding buffer. The cell pellet was re-suspended with the binding buffer and treated with annexin-V-FITC and PI reagents for 15 min at room temperature according to the manufacturer's protocol. The stained cells were analyzed by FACS-Calibur flow cytometer. From the result of PI stain in Figure 4A, the proportion of executed apoptotic cells was decreased by the treatment of IFN-β. However, the early stage apoptosis detected by annexin-V-FITC staining was not affected by the IFN. To confirm the effect of IFN-β on the early stage apoptosis, various concentrations of IFN-β were treated to the confluent cells. The cells were treated with 10–1,000 IU/ml of IFN-β and cultured for 72 h in the absence of growth factor. The cells were double-stained with annexin-V-FITC/PI. As shown in Figure 4B, the executed late stage apoptosis demonstrated by PI staining was inhibited by IFN-β in a dose dependent manner, however, the proportion of the early stage apoptotic cells dyed with annexin-V-FITC was not altered. The relative proportion of early and late stage apoptotic cells obtained from three time experiments under the same conditions as Figure 4B was demonstrated in Figure 4C. The apoptotic cells detected by PI were significantly decreased by IFN-β in a dose dependent manner although those by annexin-V-FITC were not changed. These results indicate that IFN-β does not affect on early stage apoptosis, but affect on the executed late stage apoptosis.

### EFFECT OF IFN-β ON CASPASE-3 ACTIVITY

The effect of IFN-β on caspase-3 activity was examined using a colorimetric assay. HAEC were cultured in 25 $cm^2$ collagen coated flasks. After confluent ($1.0–1.3 \times 10^6$ cells/flask), the cells were cultured in the absence or presence of 1,000 IU/ml of IFN-β for 48 h in the medium supplemented with 2% FCS without growth factor. The cells were collected and lysed for the colorimetric assay. The caspase-3 activities were determined by the spectrophotometric detection of the chromophore p-nitroaniline (pNA) cleaved by

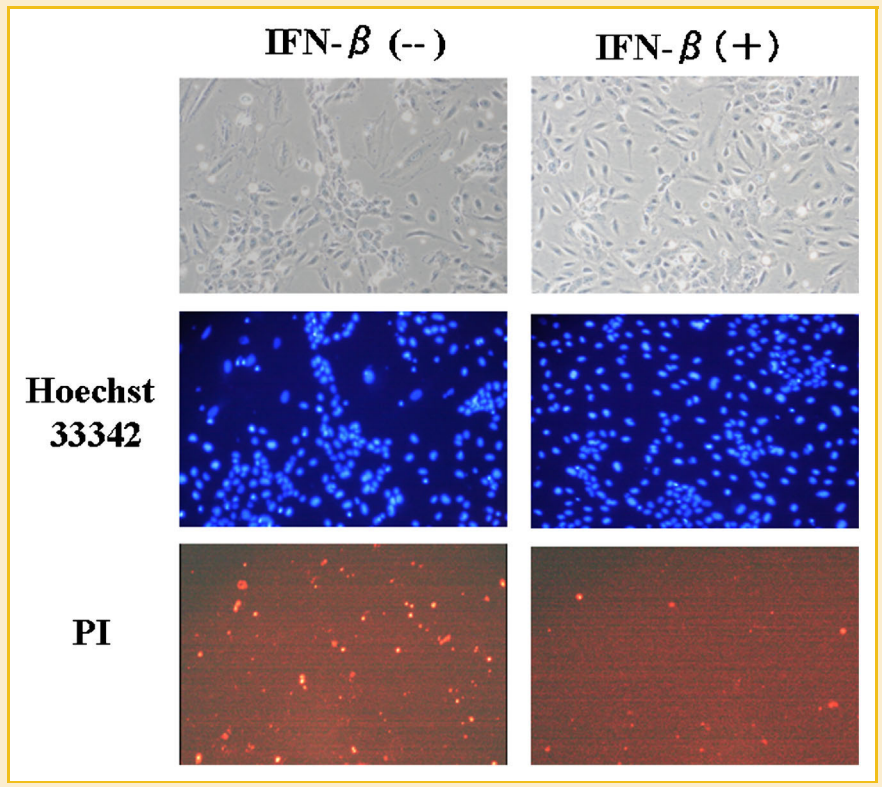


Fig. 3. Fluorescence microscopic analysis. The confluent cells grown in 25 $cm^2$ collagen coated flasks were treated with 1,000 IU/ml of IFN-β for 48 h. After rinsing the cells with PBS(+) supplemented with $Ca^{++}$ and $Mg^{++}$, the cells were directly stained with Hoechst 33342 and PI for 15 min in the flasks. The cells were observed under a fluorescent microscope. Hoechst 33342 for living cell nuclei was exited at 352 nm by UV light and emitted blue fluorescence at 461 nm. PI for dead cell nuclei is excited at 488 nm and detected at 588 nm.

caspase from the labeled caspase-specific substrates (DEVD-pNA). As shown in Figure 5, IFN-β inhibited the caspase-3 activity enhanced by the deprivation of growth factor. The activity was confirmed to be inhibited by DEVD-fmk of caspase-3 inhibitor. The protease activities in the cells were demonstrated as the fold-increase comparing with the values of positive control cells cultured in bFGF (10 ng/ml) supplemented medium. This result proves that IFN-β can inhibit the HAEC apoptosis caused by growth factor deprivation.

### EFFECT OF IFN-β ON THE EXPRESSION OF p53 AND BAX mRNA

The molecular mechanisms of anti-apoptotic effect of IFN-β were examined using a quantitative RT-PCR. The effect of IFN-β on the apoptotic pathway mediated by p53 was investigated. HAEC were cultured in collagen coated 25 $cm^2$ flasks until confluent and treated with 1,000 IU/ml of IFN-β for 4–24 h. The cells were harvested by trypsinization for the extraction of total RNA. The extracted total RNA was reverse transcribed to cDNA. Relative mRNA transcription levels of p53 and bax gene were determined with quantitative RT-PCR according to the method described in Materials and Methods Section. The fold change calculated using the $2^{-\Delta\Delta Ct}$ method was demonstrated in Figure 6A,B. The relative transcription level of bax gene was transiently down-regulated at 4 h after IFN-β treatment. Thereafter, the expression level was restored. Whereas, p53 mRNA level was up-regulated after 8 h of IFN-β treatment suggesting that the p53 mRNA synthesis might be stimulated after the activation by IFN-β. These results indicate that the anti-apoptotic effect of type1 IFN on human vascular endothelial cells may be resulted from the direct inhibition of apoptotic path-way of p53.

### CONSTRUCTION OF AN ASSAY SYSTEM FOR EVALUATING THE EFFECT OF IFNs ON TBH INDUCED CYTOTOXICITY

TBH was used for the induction of oxidative cell damage. At first, the effect of $Ca^{2+}$ concentration on the TBH induced cytotoxicity was examined for evaluating cell damage. The cells were cultured in collagen coated 96 well plates using the medium supplemented with 10% FCS and 10 ng/ml of bFGF. The culture medium was changed to the growth factor deprived medium supplemented with 2% FCS at 4 days and the cells were cultivated until confluent. The cells were rinsed with fresh M199 medium without serum and growth factor, and were treated with TBH of 0.4–50 mM in PBS(−) supplemented with 0.1% BSA and various concentrations of $CaCl_2$ for 40 min at 37°C. After confirming optimum cytotoxic damage which is about 50% adherent cells under a microscope, the viability was assayed by MTT method. As shown in Figure 7A, the concentration dependent cytotoxic damage was observed in the presence of 1–2 mM $CaCl_2$. Then, the optimum treating time was examined. The confluent cells were treated with 10 mM TBH solution with 1 mM $CaCl_2$ for 10–60 min at 37°C in $CO_2$ incubator. The proportion of viable adherent cells was decreased with time lapse as shown in Figure 7B. The

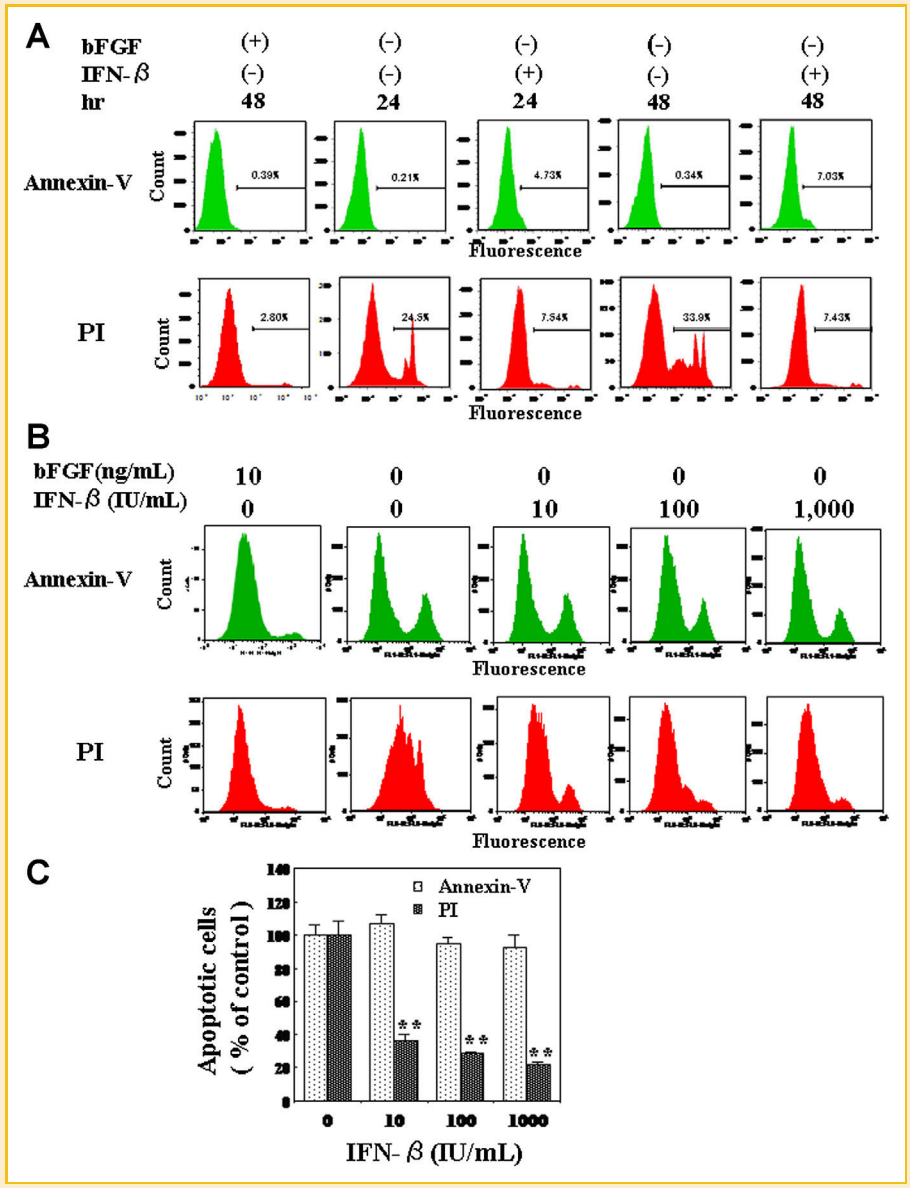


Fig. 4. Flow cytometric analysis by annexin-V-FITC/PI dual staining. A: Effect of IFN-β on apoptosis. The confluent cells grown in 25 $cm^2$ collagen coated flasks were treated with 1,000 IU/ml of IFN-β for 24–48 h. The cell pellets harvested by trypsinization were re-suspended in the binding buffer and treated with annexin-V-FITC and PI reagents for 15 min at room temperature according to the manufacturer's instruction. The stained cells were dual-analyzed by FACS using FL-1 and FL-3. B: Dose dependent effect of IFN-β on apoptosis. The cells were cultured in 25 $cm^2$ collagen coated flasks until confluent. The cells were treated with IFN-β of 10–1,000 IU/ml for 72 h and dual-stained with annexin-V-FITC and PI reagents in binding buffer. The fluorescence of dual-stained cells was analyzed by FACS using FL-1 and FL-3. C: The relative proportion of early and late stage apoptotic cells. The proportion of early stage apoptotic cells stained with annexin-V-FITC was compared with those in the executed late stage apoptosis dyed with PI. The figure was plotted based on the results in three time experiments carried out under the same conditions as (B). $^{**}P < 0.01$, compared with IFN-β minus and IFN-β plus in PI stained cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

optimum cytotoxicity was appeared after 30–40 min of TBH treatment. As the result, 10 mM TBH was treated for 30–40 min in the presence of 1 mM $CaCl_2$ for evaluating cytotoxicity.

Then, the effect of growth factors on the cytotoxic damage by TBH was examined. The cells which were cultured in the medium supplemented with 10 ng/ml of bFGF or 20 ng/ml of $VEGF_{165}$ until confluent were compared with those replaced with growth factor deprived medium at 4 days for the induction of cell damage by TBH. The concentration dependent viabilities in both the cultures were shown in Figure 7C,D. The presence of growth factors attenuated the cytotoxic damage by TBH. Therefore, for the evaluation of cell damage, the growth factors were eliminated from the culture medium used in the last medium change of the cultures.

### EFFECT OF IFNs ON TBH INDUCED CYTOTOXICITY

Using the optimized evaluation system, the effect of IFNs on the TBH induced cytotoxicity was examined. Confluent HAEC were treated with 1,000 IU/ml of IFN-α, -β, or -γ for 24 h in the medium

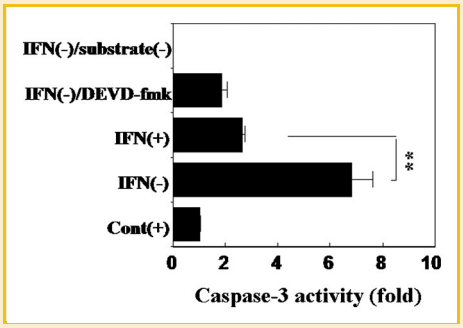


Fig. 5. Effect of IFN-β on caspase-3 activity. The cells were cultured in 25 $cm^2$ collagen coated flasks until confluent (1.0–1.3 × $10^6$ cells/flask). The cells were treated with 1,000 IU/ml of IFN-β for 48 h in the medium supplemented with 2% FCS without growth factor. The cells were also cultured in the medium supplemented with 10 ng/ml bFGF as positive control (Control (+)). The activities were determined by subtracting the value obtained from substrate free samples in the IFN-β un-treated cells (IFN (−)/substrate (−)) from each sample value and were shown as fold- increase by comparing each value with those in positive control cells. IFN (−)/DEVD-fmk means the activity in the IFN-β un-treated cells in the presence of caspase-3 inhibitor, DEVD-fmk. The relative activities in the figure show the average value ± SE in three independent experiments. $^{**}P < 0.01$, compared with IFN-β plus and IFN-β minus.

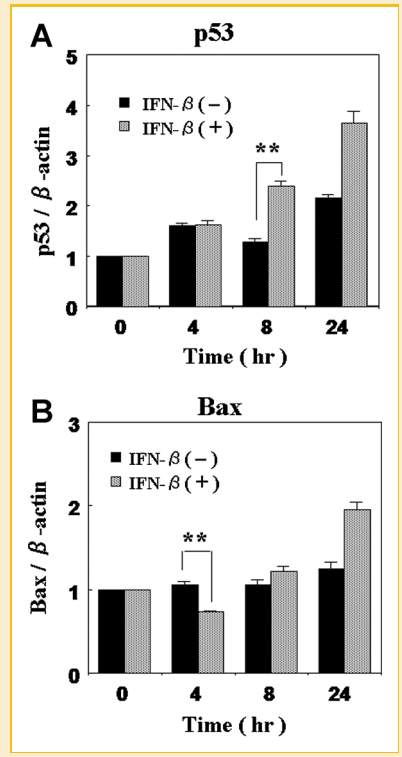


Fig. 6. Effect of IFN-β on the expression of p53 and bax mRNA. A,B: The mRNA expression levels were determined by quantitative RT-PCR. HAEC were cultured in collagen coated 25 $cm^2$ flasks and treated with 1,000 IU/ml of IFN-β in the growth factor deprived medium. The cells were harvested at 4–24 h after IFN-β treatment for the real time RT-PCR analysis. Total RNA from 1 × $10^6$ cells were extracted and reverse transcribed to cDNA. RT-PCR reaction was carried out according to the method described in Materials and Methods. The average fold changes in three time experiments were calculated using the $2^{-\Delta\Delta Ct}$ method and were plotted in the figures. $^{**}P < 0.01$, compared with IFN-β plus and IFN-β minus.

supplemented with 2% FCS without growth factor. The cells were treated with various concentrations of TBH for 40 min. The cytotoxic damage by TBH was occurred in a dose-dependent manner in all cultures as shown in Figure 8A. The cell damage was inhibited by the treatment of IFN-α and -β. Whereas, the viability was decreased by the treatment of IFN-γ. The damage was observed markedly in over 0.8 mM of TBH. Subsequently, the dose dependency of each IFN effect on the TBH induced cytotoxicity was examined. The cells were treated with IFNs of 1–10,000 IU/ml for 24 h and were treated with 10 mM TBH for 40 min at 37°C. Thereafter, the viability was examined by MTT method. As shown in Figure 8B,C, IFN-α and -β increased the proportion of viable cells by the treatment of over 1 IU/ml. However, IFN-γ did not support the cell survival obviously although the cell damage was slightly increased by the treatment of 10,000 IU/ml. These results indicate that type 1 IFN might be able to inhibit the oxidative endothelium damage in vascular vessel.

## DISCUSSION

In this report, we have shown for the first time that type 1 IFN can inhibit the growth factor deprived apoptosis of human vascular endothelial cells and protects the cells from TBH induced oxidative damage.

Up to now, IFN has been documented to induce apoptosis in many cell types. Yano et al. [2005] demonstrated IFN-α induced apoptosis in human liver cancer cells and De Marco et al. [1999] reported IFN-β specific apoptosis induction using human keratinocytes transformed with HPV-16 DNA. While, Tagawa et al. [1997] demonstrated apoptosis activation by IFN-γ in mouse Con A-induced hepatitis. Some molecular mechanisms relating apoptosis induction or activation by IFNs have been also investigated. Thyrell et al. [2004] showed IFN-α induced apoptosis is mediated through PI3K in rapamycin signaling pathway and Dedoni et al. [2010] demonstrated the apoptosis of human neuroblastoma cells by IFN-β is induced through the activation of JAK-STAT signaling and down-regulation of PI3K/Akt pathway. Castelli et al. [1997] reported the apoptosis by IFNs is activated by 2–5 A system. Whereas, there are some reports as to anti-apoptotic effect of IFN. Saile et al. [2003] demonstrated that IFN-α can suppress the hepatocyte apoptosis with virus infection. It was considered that the effect is mainly resulted from the anti-virus effect of IFN-α. Kocic et al. [2007] has shown that the protective effect of IFN-α on anti-Fas-antibody induced apoptosis in the hepatocyte with acute viral hepatitis was resulted from the decrease of the bax positive cells. Further, the inhibition of human t-cell apoptosis by IFN-β was reported by Chang et al. [2001]. In this case, the main mechanism of apoptosis inhibition is considered to be immunomodulation mediated by IFN-β rather than the direct inhibition of the apoptotic pathway in human T cells.

We have shown the anti-apoptotic effects of type 1 IFN on HAEC using flow cytometry, fluorescence microscopy, caspase-3 colorimetric assay and cell count. IFN-β was most effective to the apoptosis inhibition. For the reliable experiments, we used natural

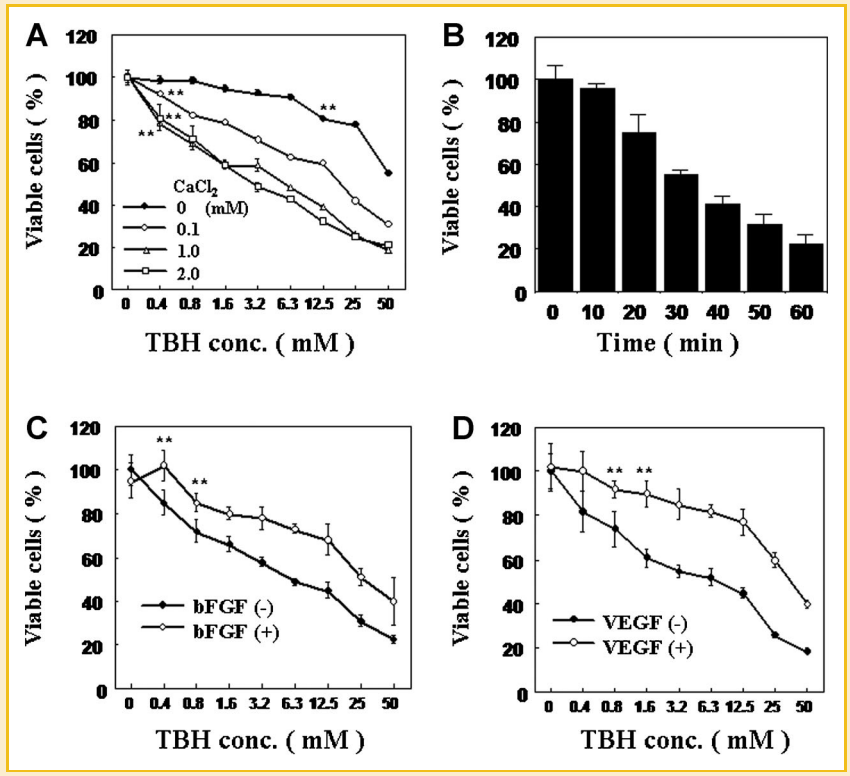


Fig. 7. Construction of evaluating system for oxidative cell damage using TBH. A: Effect of $Ca^{++}$ on oxidative cytotoxicity by TBH. The confluent cultures in 96 well collagen coated flasks were treated with various concentrations of TBH in PBS (−) supplemented with 0.1% BSA and 0.1–2.0 mM of $CaCl_2$ for 40 min at 37°C. The cell viability was determined by MTT assay. The viable cell proportions were calculated from the relative absorbance and compared with those in the absence of TBH (100%). The plots in the figure represent the average ± SE in three plates. $^{**}P < 0.01$, compared with $Ca^{++}$ minus and 0.1–2 mM of $CaCl_2$. B: Time dependent cytotoxicity of TBH. The confluent cells were treated with 10 mM TBH for 10–60 min at 37°C in a $CO_2$ incubator. The proportion of viable cells was determined by MTT assay. The viabilities in each treating time were calculated from absorbance and compared with those in TBH un-treated starting cultures. The result shows the average ± SE obtained from three plates. C and D: Effects of growth factors on TBH induced cytotoxicity. The viabilities of the cells grown in the medium supplemented with bFGF (10 ng/ml) or $VEGF_{165}$ (20 ng/ml) were compared with those in the cultures changed to growth factor deprived medium at just before confluent. The plots in the figures represent the average ± SE in three plates. $P < 0.01$, compared with growth factor minus and plus.

type IFN-α (Sumitomo Pharmaseuticals) and IFN-β (Toray Industries) with high specific activities of 0.8 to $2.0 \times 10^8$ IU/mg, and recombinant IFN-γ manufactured by Genentech. The anti-apoptotic effect by IFN-β was considered to be resulted from the direct inhibition of p53 mediated apoptotic pathway (Fig. 6). The expression of bax mRNA was transiently down-regulated at 4 h by IFN-β and the synthesis of p53 mRNA was enhanced after 8 h of IFN-β treatment suggesting that the synthesis of p53 mRNA might be promoted after the phosphorylation for the activation of the p53-related genes. To clarify the mechanisms of this anti-apoptotic effect more precisely, the additional experiments are in progress. It is very interested in the different regulation of p53 mediated apoptotic pathway by type 1 IFN depending on the cell types.

It was found the different patterns in the histograms between annexin-V-FITC and PI (Fig. 4). PI is commonly used for identifying dead cell DNA and annexin-V binds with the PS distributed to the outer layer of membrane in early stage of apoptosis. IFN-β suppressed executive late stage apoptosis expressed by PI, however, did not inhibit the early stage apoptosis demonstrated by annexin-V. The decrease of apoptotic DNA was also shown by a fluorescence microscopy using Hoechst33342 and PI double staining (Fig. 3). The effects of IFN-β on the execution phase of apoptosis were also confirmed by the suppression of caspase-3 activities as shown in Figure 5. These results indicate that the anti-apoptotic effects of type 1 IFN may be resulted from the inhibition of late stage apoptosis similar to the result of Kocic et al. [2007].

Extensive evidence suggests that reactive oxgen species are critically involved in the pathogenesis of cardiovascular diseases, such as atherosclerosis and myocardial ischemia-reperfusion injury [Olafsson et al., 1987]. There are several physiological and pathophysiological sources of tissue reactive oxygen formation [Dhalla et al., 2000]. Reactive oxygen species are known to induce the endothelial cell disfunction [Whorton et al., 1985; Brutsaert, 2003] and the cell damage [Forman et al., 1989]. Different reactive oxygen species are derived from reduction of molecular oxygen resulting in formation of superoxide, hydrogen peroxide, and hydroxyl radical operating in multiple pathways [Fernandes et al., 2010]. Although there were many reports relating with the signaling pathways and molecular actions of reactive oxygen radicals, they often involved the change in cellular $Ca^{2+}$ homeostasis [Kourie, 1998; Lounsbury et al., 2000]. TBH has been known to inhibit the endothelial cell function [Whorton et al., 1985] and calcium

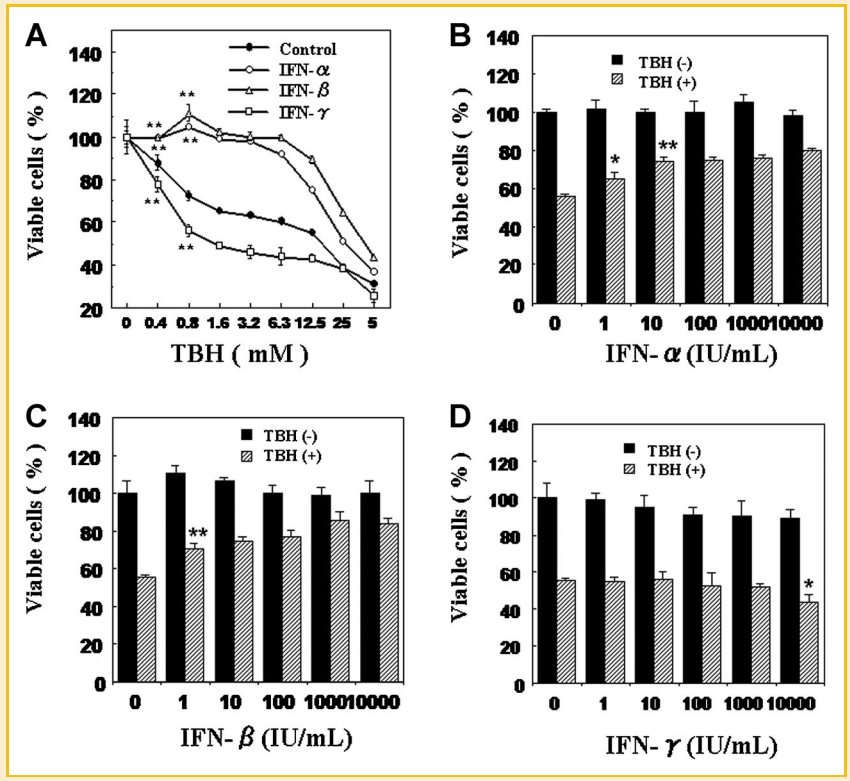


Fig. 8. Effect of IFNs on TBH induced cytotoxicity. A: Effect of IFNs on the concentration dependent TBH cytotoxicity. The confluent cells in 96 well collagen coated plates were treated with 1,000 IU/ml of IFNs for 24 h in the 2% FCS supplemented medium without growth factor. Thereafter, the cells were treated with various concentrations of TBH for 40 min. The viability of the cells was determined by MTT assay. The viable cell proportion in the presence of various concentrations of TBH was compared with those of viable cells in the absence of TBH (100%). The values expressed in the figure show the average ± SE obtained from three plates. $^{**}P < 0.01$, compared with IFN minus and each IFN plus. B–D: Effects of IFN concentrations on TBH induced cytotoxicity. The confluent cells were treated with various concentrations of IFNs in growth factor deprived medium for 24 h before TBH treatment. The cells were treated with 10 mM TBH for 40 min at 37°C. The control cultures without TBH were also incubated at 37°C. The viabilities were determined by MTT assay. The proportion of viable cells was determined by comparing the values in IFN treated cells with the values in the cultures without TBH and IFN (100%). The proportion of viable cells in each culture condition was shown as the average ± SE obtained from three plates. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with IFN minus and each IFN plus under the presence of TBH.

signaling [Elliott and Doan, 1993; Florea and Blatter, 2008]. We could also confirm that the cytotoxic damage by TBH is induced depending on extra-cellular $Ca^{++}$ concentration (Fig. 7). An evaluating system for oxidative injury by TBH was constructed by optimizing $Ca^{2+}$ concentration and by analyzing by MTT assay. Type 1 IFN could prevent the cell damage by TBH (Fig. 8). TBH has been also shown to give a genotoxicity to various cells [Mitic-Culafic et al., 2009] and to activate apoptotic bax gene [Haidara et al., 2002]. This suggests that the cyto-protective effect of type 1 IFN on TBH induced cell damage might be also resulted from the inhibition of apoptotic pathway mediated by p53 since IFN-β down-regulated the expression of bax gene under growth factor deprivation (Fig. 6).

We have demonstrated the different effects between type 1 IFN and IFN-γ on the cell survival. Further, we could also find that IFN-β was more potent inhibitor of cell death than IFN-α (Fig. 1A) although the receptor of these IFNs is common. It has been known that the homology of amino acid sequence between IFN-α and -β is about 29% and natural type IFN-β is glycosylated different from IFN-α. The primary structure and receptor of IFN-γ are quite different from those of type 1 IFN although the same intra-cellular signaling pathway as type 1 IFN is used for anti-virus effect. Since there are some reports demonstrating different effects between IFN-α and -β [De Marco et al., 1999], the experiments to examine the different potency of these IFNs are under consideration.

In this study, we could demonstrate that type 1 IFN, especially IFN-β might be useful for the diseases with vascular endothelium damage such as atherosclerosis or restenosis after angioplasty as a medical treatment or a prophylactic.

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