Interferon Regulatory Factors Regulate Interleukin-1β–Converting Enzyme Expression and Apoptosis in Vascular Smooth Muscle Cells

Masatsugu Horiuchi, Hiroyuki Yamada, Masahiro Akishita, Masaaki Ito, Kouichi Tamura, Victor J. Dzau

Abstract—Apoptosis has been reported to play a pivotal role in vascular remodeling. However, cellular mechanisms of apoptosis in vascular smooth muscle cells (VSMCs) have not been well defined. In this study, we focused on interleukin-1β–converting enzyme (ICE), a key protease in the induction of apoptosis in lymphocytes and fibroblasts. We observed an increase in ICE mRNA expression in rat aortic VSMCs after serum depletion, with a peak at 12 hours and then a gradual decline. This was associated with DNA fragmentation, a hallmark of apoptosis and morphological changes of apoptosis. Treatment of these VSMCs with the ICE inhibitor N-(N-acetyl-tyrosinyl-valinyl-alaninyl)-3-amino-4-oxobutanoic acid (YVAD-CHO) attenuated DNA fragmentation. The increased ICE mRNA expression was preceded by an increase in the mRNA expression of interferon regulatory factor (IRF)-1, peaking at 6 hours after serum removal, and a rapid but transient decrease in IRF-2 mRNA expression, reaching a nadir at 3 hours after serum depletion. To demonstrate that these reciprocal changes in IRF-1 and IRF-2 regulated ICE expression and induced apoptosis, we transfected antisense oligonucleotides for IRF-1 and IRF-2 into VSMCs and examined ICE mRNA expression and apoptotic changes. IRF-1 antisense pretreatment attenuated the increase in ICE expression and reduced apoptotic changes, whereas IRF-2 antisense treatment increased ICE mRNA expression and enhanced apoptotic changes. Taken together, our results suggest that serum growth factor depletion in VSMCs upregulates IRF-1 and downregulates IRF-2, thereby increasing ICE expression and inducing apoptosis. (Hypertension. 1999;33:162-166.)

Key Words: apoptosis • growth substances • interferons • interleukin-1 • muscle, smooth, vascular

Recent evidence suggests that the cellular compositions of the heart and blood vessels are determined by the balance between apoptotic cell death, cell survival, cell proliferation, and cell migration. The processes of cell survival and cell death both involve highly regulated signaling pathways that are currently the subject of intense investigation. Studies of Caenorhabditis elegans have suggested that the product of this nematode’s cell death gene ced-3 (CED-3) is a critical factor in downstream apoptotic signal transduction and that interleukin-1β–converting enzyme (ICE) is a mammalian homologue of CED-3.1-5 The fact that human atheromatous lesions express ICE mRNA, and that immunoreactive ICE is detected specifically in apoptotic macrophages and vascular smooth muscle cells (VSMCs),6 suggests that ICE plays a role in VSMC apoptosis and consequently in the pathobiology of atherosclerosis.

Tamura et al7 showed that ICE gene expression is transcriptionally upregulated by interferon regulatory factor (IRF)-1. Tanaka et al8 reported that IRF-1 may be a critical determinant of apoptosis induction in mouse embryonic fibroblasts (EFs). They demonstrated that ras signaling induces the death of wild-type EFs and of EFs from mice with a null mutation in the IRF-2 gene (IRF-2−/− mice) but not of EFs from IRF-1−/− mice and double knockout mice, under conditions of low serum or at high density of the cells or after treatment with anticancer drugs or ionizing radiation. They suggested that the lack of IRF-1 alone is sufficient to prevent ras-induced apoptosis. These observations led us to postulate that IRF-1 is pivotal in apoptosis induction in VSMCs, possibly via the upregulation of ICE. In this study, we demonstrated that IRF-1 was upregulated after serum removal from cultured rat aortic VSMCs and that this upregulation indeed induced an increase in ICE mRNA, which mediated VSMC apoptosis. In addition, we also observed a downregulation of IRF-2 that contributed to ICE expression and apoptosis.

Methods

Cell Cultures
VSMCs were isolated from adult Sprague-Dawley rat thoracic aortas9 and cultured in Waymouth’s MB 752/1 medium (Gibco BRL) supplemented with 10% calf serum, 100 U penicillin/mL, and 100 μg

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streptomycin/mL at 37°C in a humidified atmosphere of 95% air–5% CO₂. Cells at passages 4 to 8 were used for experiments.

Oligonucleotides and Transfection to VSMCs

The phosphorothioate oligonucleotides used as antisense DNA for IRF-1 and IRF-2 in the treatment of cells were as follows: IRF-1 antisense, 5′-GAAAGATGCCCGAGATGC-3′ (214/292); and IRF-2 antisense, 5′-GTGTGAGTGTTGTTAGGG-3′ (271/254). Transfection was performed with LipofectAMINE reagent (Gibco BRL) as described previously. On the day of transfection, the original medium was replaced with fresh medium and incubated for 1 hour at 37°C. Transfection was performed with LipofectAMINE reagent used according to the manufacturer’s instructions. Cells were incubated with the oligonucleotides (0.5 μmol/L) and LipofectAMINE (1:3, wt/wt) for 3 hours, and the transfection reagent was then replaced with culture medium containing the 10% serum. Twenty-four hours after transfection, the serum-containing media were removed, and the cells were incubated with serum-free media.

Northern Blot Analysis

Total RNA was prepared from cultured VSMCs with the use of RNAzol (Tel-Test). RNA (20 μg per lane) was separated by electrophoresis and transferred onto a nylon membrane (Amersham); hybridization was carried out with 32P-labeled IRF-1, IRF-2, and ICE cDNA and with a 32P-labeled 0.78-kb PstI-XbaI fragment of a human GAPDH clone. cDNAs of IRF-1 and IRF-2 were prepared by reverse transcription–polymerase chain reaction, and polymerase chain reaction amplified DNAs were subcloned into the pCRII Vector (Invitrogen). Rat ICE cDNA probe for Northern blot analysis was kindly provided by Dr Aaron J.W. Hsueh, Department of Obstetrics and Gynecology, Stanford University. Densitometric analysis of autoradiograms was performed with a scanning densitometer (GS300, Hoeffer) and NIH image software.

Immunoblot Analysis

The cells were prepared as described previously, resolved by 12% SDS–polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membrane, and immunoblotted with IRF-1 or IRF-2 antibody (Santa Cruz Biotechnology). Antibodies were detected by horseradish peroxidase–linked secondary antibody using an enhanced chemiluminescence system (Amersham).

Internucleosomal DNA Fragmentation (DNA Laddering)

DNA extraction, subsequent 3′ end-labeling of DNA, gel electrophoresis, and quantitation of DNA fragmentation were performed as described previously. In brief, 500 ng of DNA prepared from treated VSMCs was end-labeled with [α-32P]-ddATP (Amersham) and terminal transferase (Boehringer-Mannheim) for 60 minutes at 37°C. Labeled DNA was loaded onto a 2% agarose gel and separated by electrophoresis, and autoradiography was performed. The amount of radiolabeled ddATP incorporated into low-molecular-weight (<20-kb) DNA fractions was quantified by cutting of the respective fraction of DNA from the dried gel and counting in a β-counter. The

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**Figure 1.** IRF-1, IRF-2, and ICE mRNA expression after serum starvation in cultured VSMCs. Total RNA was prepared from cultured VSMCs 0, 3, 6, 12, 24, and 48 hours after serum removal. RNA (20 μg per lane) was separated by electrophoresis and hybridized sequentially with a probe for IRF-1, IRF-2, and ICE and for GAPDH as an internal control to standardize the amount of total RNA actually blotted onto the membrane. This figure shows representative data from 4 experiments (IRF-1 and ICE, 1-day autoradiographic exposure; IRF-2, 2-day autoradiographic exposure).

**Figure 2.** Quantitative analysis of IRF-1, IRF-2, and ICE mRNA expression after serum starvation in cultured VSMCs. After RNA separation and sequential hybridization as described in Figure 1, densitometric analysis of autoradiograms was performed, and the signal density of each RNA sample hybridized to IRF-1, IRF-2, and ICE was divided by that hybridized to GAPDH. The corrected density for each time point is presented as a percentage of the value obtained before serum depletion (time 0). The values shown are mean±SD of data from 4 separate experiments. *P<0.05, **P<0.01 vs time 0.
results were expressed as a percentage of the radioactive counts in the control samples.

Chromatin Binding Dye Staining
Chromatin binding dyes Hoechst 33342 and propidium iodide (Molecular Probes) were added to samples at a concentration of $5 \times 10^{-5}$ or $10^{-7}$ mol/L to examine the morphological changes of nuclei. After incubation at 37°C for 1 hour, cells were collected. After centrifugation, the pellet was resuspended in PBS, and cells were viewed under UV microscopy.

Figure 3. Effect of antisense (AS) and sense (S) oligonucleotides for IRF-1 and IRF-2 on ICE mRNA expression 12 hours after serum removal (A, B) and IRF-1 and IRF-2 protein levels 6 hours after serum removal (C) in cultured VSMCs. Panel A shows a representative result; similar results were obtained in 4 separate experiments. The signal density of each RNA sample hybridized to ICE was divided by that of hybridized GAPDH (B). The corrected density for each time point is presented as a percentage of the value obtained with serum-fed VSMCs. The values shown are mean±SD of data from 4 experiments. *$P<0.05$, **$P<0.01$ vs serum-fed cells. Panel C shows a representative result of immunoblot; similar results were obtained in 3 separate experiments.

Figure 4. Effect of antisense (AS) and sense (S) oligonucleotides for IRF-1 and IRF-2 on DNA fragmentation (A, B) and morphological changes of apoptosis (C) after serum removal in cultured VSMCs. DNA was prepared 3 days after serum removal. A representative autoradiogram of DNA laddering is shown in panel A. Quantitative analysis was performed by calculating the radioactivity incorporated into the low-molecular-weight (<20-kb) fraction divided by the radioactivity of serum-fed untreated cells (B; n=4 for each condition, mean±SD). *$P<0.05$, **$P<0.01$ vs serum-fed untreated cells. C, The number of apoptotic nuclei that showed morphological apoptotic changes including nuclear condensation and fragmentation were counted from a total of 500 nuclei. Necrotic nuclei that were stained with propidium iodide without morphological apoptotic changes were excluded. *$P<0.05$, **$P<0.01$ vs serum-fed untreated cells (n=4 for each condition, mean±SD).
Statistical Analysis

All values were expressed as mean±SD. Statistical significance was assessed by ANOVA followed by Scheffe’s test.

Results

Roles of IRF-1 and IRF-2 in ICE Expression in VSMCs After Serum Depletion

We first examined the effect of serum depletion on IRF-1 and IRF-2 gene expression in cultured rat aortic VSMCs. As shown in Figures 1 and 2A and 2B, expression of IRF-1 mRNA increased in a time-dependent manner and was maximal at 6 hours after serum removal, whereas expression of IRF-2 mRNA decreased transiently 3 hours after serum removal and returned to basal level at 6 hours. ICE mRNA increased after serum depletion, peaking at 12 hours, and then declined gradually (Figures 1 and 2C).

These results suggest that the changes in IRFs after serum removal upregulate ICE. To prove this hypothesis, we transfected antisense oligonucleotides for IRF-1 and IRF-2 into VSMCs 24 hours before serum removal. IRF-1 antisense oligonucleotide pretreatment attenuated ICE mRNA expression in VSMCs after serum removal, whereas IRF-2 antisense pretreatment enhanced ICE mRNA expression (Figure 3A and 3B). Moreover, we confirmed 6 hours after serum depletion that IRF-1 antisense oligonucleotide pretreatment attenuated the protein level of IRF-1, whereas IRF-2 antisense pretreatment enhanced IRF-2 protein level (Figure 3C). Treatment with sense oligonucleotides for IRF-1 and IRF-2 did not influence ICE mRNA expression. These data suggest that an increase in IRF-1 and a decrease in IRF-2 after serum removal exert synergistic effects on ICE gene expression.

Induction of Apoptosis by IRF-1 and ICE

As shown in Figure 4, serum depletion induced the internucleosomal cleavage of DNA, which resulted in the generation of multiple DNA fragments of ∼180 bp in size (the size of a nucleosome), a hallmark of apoptosis. The inhibition of DNA fragmentation in serum-starved VSMCs by IRF-1 antisense oligonucleotide treatment (Figure 4A and 4B) suggested that the increase in IRF-1 plays a critical role in the induction of apoptosis. In contrast, IRF-2 antisense oligonucleotide treatment enhanced the apoptosis. Sense oligonucleotides for IRF-1 and IRF-2 transfection did not affect apoptosis after serum removal. Apoptotic changes were also examined by chromatin binding dye staining (Figure 4C). We observed that IRF-1 antisense oligonucleotide pretreatment inhibited apoptosis after serum removal, whereas the treatment with IRF-2 antisense oligonucleotide enhanced the apoptotic changes. Transfection of antisense oligonucleotides for IRF-1 and IRF-2 in serum-fed VSMCs did not alter the DNA fragmentation (data not shown).

Although ICE has been shown to mediate apoptosis in lymphocytes and fibroblasts, its role in VSMC apoptosis has not been defined. To confirm that the increase in ICE mRNA following changes in IRFs in serum-starved VSMCs contributes to apoptosis induction, we treated the VSMCs with the specific ICE inhibitor N-(N-acetyl-tyrosinyl-valinyl Alaninyl)-3-amino-4-oxobutanoic acid (YVAD-CHO) and examined DNA fragmentation after serum removal. As shown in Figure 5, treatment with YVAD-CHO (10 μmol/L) attenuated DNA fragmentation in VSMCs after serum removal.

Discussion

IRF-1 manifests antiproliferative properties, whereas overexpression of the repressor IRF-2 leads to cell transformation and increased tumorigenicity, and the latter phenotype can be reversed by the concomitant overexpression of IRF-1. Tamura et al. showed that ectopic overexpression of IRF-1 transactivates ICE in T lymphocytes and enhances the sensitivity of these cells to radiation-induced apoptosis. Cysteine protease, ICE/CED-3 family, which has recently been termed “caspases,” has been shown to be an important downstream member of the protease cascade, where various cell death pathways converge into the same effector pathway. Therefore, we examined the possibility that the increase in IRF-1 in VSMCs after serum removal transactivates ICE. We observed an increase in ICE mRNA that was inhibited by IRF-1 antisense oligonucleotide pretreatment, suggesting that enhanced expression of IRF-1 in serum-starved VSMCs transactivated ICE. Furthermore, we demonstrated that a decreased expression of IRF-2 also contributed to the increased ICE mRNA expression. Indeed, IRF-2 antisense oligonucleotide pretreatment increased ICE mRNA expression. The role of ICE expression in apoptosis was documented by our experiments; we found that ICE inhibitor prevented DNA fragmentation in VSMCs after serum starvation. Taken together, these results suggest that in serum-starved VSMCs the apoptosis mediated by increased IRF-1 and/or decreased IRF-2 is at least partially due to an increase in ICE mRNA.

IRF binding consensus element was also identified in the promoter region of inducible nitric oxide synthase (iNOS). IRF-1 is essential for iNOS activation in murine macrophages. Recently, Bachmaier et al. reported that IRF-1 upregulates iNOS and NO production in autoimmune myo-

![Figure 5. Effect of ICE inhibitor on apoptosis after serum starvation in the cultured VSMCs. The cells were incubated with the ICE inhibitor YVAD-CHO (10 μmol/L) for 3 days in the absence of serum, and DNA fragmentation was examined. Quantitative analysis was performed by calculating the radioactivity incorporated into the low-molecular-weight (<20-kb) fraction divided by the radioactivity of serum-fed untreated cells (n=4, mean±SD). *P<0.05 vs serum-starved cells not treated with the ICE inhibitor.](attachment:figure5.png)
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carditis. Moreover, we have reported previously that NO donor molecules (S-nitroso-N-acetylpenicillamine or sodium nitroprusside) induced apoptosis in cultured VSMCs.17 On the other hand, Dimmeler et al33 reported that NO abrogates the apoptotic changes in human umbilical venous endothelial cells by interfering with the activation of the caspase cascade. These results led us to examine iNOS expression in VSMCs after serum removal. We observed no apparent changes in iNOS protein level after serum starvation (data not shown). It is unclear at present why the increase in IRF-1 seen in our VSMCs after serum starvation did not result in the increase in iNOS expression. One possible explanation is that iNOS is subject to cooperative gene regulation in VSMCs by IRF-1 and other transcriptional factors.

In addition to ICE, it is possible that IRFs may transactivate other genes that exert proapoptotic effects in VSMCs. Indeed, we demonstrated previously that an increase in the ratio of IRF-1 to IRF-2 in mouse fibroblast R3T3 cells after serum starvation mediated the upregulation of angiostatin II type 2 receptor,11 resulting in the enhancement of angiotensin II–mediated apoptosis.11,13 These studies support the hypothesis that IRF-1 is a unique transcription factor that functions as an apoptotic inducer and suggest that cytokines such as interferon-γ may contribute to the pathogenesis of atherosclerosis and vascular remodeling by IRF-modulated apoptosis.

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