Abstract—In endothelial cells (ECs), the transcription factor c-Jun is induced by a variety of stimuli that perturb EC function. To extend our understanding of the role of c-Jun in EC physiology, we have directed overexpression of c-Jun in human umbilical vein ECs by using a tetracycline-regulated adenoviral expression system. In this study, we report a novel observation using this system. Specific expression of c-Jun is a sufficient trigger for ECs to undergo apoptosis, as demonstrated by a set of combined assays including an ELISA specific for histone-associated DNA fragmentation, DNA laddering, and TdT-mediated dUTP nick end labeling (TUNEL). Tetracycline can effectively shut off c-Jun overexpression and prevent EC apoptosis. Cleavage of poly(ADP-ribose) polymerase was also detected in ECs overexpressing c-Jun. Moreover, inhibitors of cysteine proteases blocked the apoptosis, suggesting a caspase-associated mechanism involved in proapoptotic effects of c-Jun. To gain further insight into the role of c-Jun as a pathophysiological regulator of EC death, TAM67, a dominant-negative mutant of c-Jun, was overexpressed in human umbilical vein ECs to abrogate endogenous c-Jun/activator protein-1 activation. H2O2-triggered apoptosis was largely attenuated in ECs overexpressing TAM67. Together, these results suggest that c-Jun, as a proapoptotic molecule, may play a role in mediating the cell death program in vascular endothelium. (Circ Res. 1999;85:387-393.)

Key Words: c-Jun ■ adenovirus ■ endothelial cell ■ apoptosis ■ hydrogen peroxide

Vascular endothelium, when unperturbed, is considered to provide a relatively nonadhesive and nonthrombotic interface. This characteristic is likely essential to physiological homeostasis. Endothelial cell (EC) apoptosis has been implicated in numerous pathophysiological processes, such as angiogenesis, thrombosis, and atherosclerosis. The molecular mechanisms controlling EC apoptosis and, particularly, its transcriptional regulation remain largely unknown. Recently, the transcription factor c-Jun has been shown to have diverse roles in the apoptotic process, depending on cell type and microenvironment. Involvement of the c-jun gene in EC apoptosis has been implied by observations showing that many EC-perturbing agents, such as inflammatory cytokines, lipopolysaccharides, reactive oxygen, and oxidized LDL, induce EC apoptosis as well as c-jun expression. Because these stimuli usually have pleiotropic effects on signal transduction pathways, it is not clear whether induction of c-jun in ECs is causally related to evoking apoptosis or, instead, is protective. Indeed, c-jun may even be an innocent bystander in EC apoptosis. To clarify this potentially important control mechanism, we have used a tetracycline-regulated adenoviral expression system for c-jun. The expression of c-jun can be exquisitely controlled using this process, and the findings of its expression are reported here. In addition, to gain further insight into the role of endogenous c-jun as a pathophysiological regulator of EC death, TAM67, a dominant-negative mutant of c-jun, was overexpressed in human umbilical vein ECs (HUVECs), and its effects on H2O2-triggered apoptosis were examined. These results indicate a key role for c-jun as a mediator of EC apoptosis.

Materials and Methods

Adenoviruses and Gene Transfer

To construct the recombinant adenovirus expressing the human c-jun gene (AdJun), a cDNA fragment containing the full-length coding regions was obtained from the parental plasmid pRSV-jun (generous gift from Dr R. Tjian, University of California, Berkeley). It was subcloned into a shuttle plasmid, pAd10x, and recombined with an E1- and E3-deleted β-gal viral DNA in CRE8 cells. Expression of the inserted gene was driven by a 7× tet/minimal cytomegalovirus promoter that was further under the control of an artificial tetracycline-responsive transactivator (tTA). Adenoviruses expressing the β-galactosidase gene (Adβ-gal) and the tTA gene (AdtTA) were constructed from pUHC-13-3 and pUhD 15-1, as described previously. Dominant-negative c-jun (TAM67) was generated from wild-type c-jun by deletion of residues 3 to 122 in the amino-terminal transcriptional activation domain. The recombinant adenovirus expressing dominant-negative c-jun mutant (AdTAM67) was generated as described.
For adenovirus-mediated gene transfer, confluent HUVECs were exposed to adenoviral vectors at a multiplicity of infection (MOI) of ~100–200 for 2 hours (AdtTA was coinfected with AdJun or Adβ-gal to induce the tetracycline-controllable expression). After the viruses were washed off, infected cells were further incubated for the indicated time courses in the presence or absence of tetracycline.

**Cell Culture and Reagents**

HUVECs were harvested by collagenase treatment of umbilical cord veins and cultured on plates coated with collagen. Cells were maintained in M199 supplemented with 20% FBS, 20 mmol/L HEPES (pH 7.4), 1 ng/mL recombinant human fibroblast growth factor, 90 μg/mL heparin, and antibiotics. In all of the experiments, cells within 3 passages were used. Hydrogen peroxide (H2O2), DAPI, and MTT were purchased from Sigma. The peptide inhibitors zDEVD.fmk and zVAD.fmk were from Calbiochem.

**Assessment of Apoptosis**

**Detection of DNA Fragmentation by ELISA**

Quantitative analysis of DNA fragmentation was carried out using a histone-based ELISA system (Boehringer Mannheim). HUVECs in 6-well plates were incubated for 36 hours after infection with Adβ-gal in the presence or absence of tetracycline (0.1 μg/mL) and then lysed. The histone-associated DNA fragments were linked to a mouse anti-histone antibody, and the DNA part of the nucleosome was linked to the anti–DNA-peroxidase. The amount of peroxidase retained in the immunocomplex was photometrically determined.

**DNA Laddering**

After treatment, attached cells and floating cells were combined and lysed in 0.2 mL of lysis buffer (4 mol/L urea, 100 mmol/L Tris, 20 mmol/L NaCl, and 200 mmol/L EDTA [pH 7.4]) and 40 μL of protease K solution (20 mg/mL in 50 mmol/L Tris-HCl [pH 8.0] and 1 mmol/L CaCl2) for 1 hour at 55°C. After elution and isopropanol precipitation, DNA was resuspended in Tris-EDTA buffer, fractionated on 1.5% agarose gel in 1× Tris-boric acid-EDTA buffer, and stained with ethidium bromide.

**DAPI Staining**

For morphological evaluation of nuclei, cells were stained with DAPI solution (0.2% μg/mL) for 20 minutes and then visualized by fluorescence microscopy.

**TdT-Mediated dUTP Nick End-Labeling (TUNEL) Assay**

A TUNEL kit was used according to the manufacturer’s instruction (Oncor). Confluent HUVECs were infected with Adβ-gal at the same MOI and seeded in chamber slides (Lab-Tek) in the presence or absence of tetracycline (0.1 μg/mL) for 36 hours. Cells were fixed with 1% paraformaldehyde. The 3′-nick ends were labeled with digoxigenin-dUTP and a fluorescein-conjugated antidigoxigenin antibody. Propidium iodide was used to counterstain the nuclei.

**Determination of Cell Viability**

Cell viability was measured by means of the MTT assay. HUVECs were grown to confluence in 96-well plates and were exposed to apoptotic stimuli or control medium for 18 hours. Cells were incubated with MTT solution (0.5 mg/mL) for 4 hours at 37°C. medium was removed, and cells were lysed with 2-isopropanol containing 0.04 mol/L HCl. The metabolized MTT was determined photometrically at 570 nm, with 690 nm as reference.

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated using Trizol reagent (Life Technologies), fractionated on a formaldehyde/agarose gel, transferred to a nylon membrane, and hybridized to random-primed cDNA probes for human c-jun and Von Willebrand factor (VWF) genes.

**Protein Isolation and Western Blot Analysis**

Nuclear proteins were extracted from HUVECs and cultured on plates coated with collagen. Cells were maintained in M199 supplemented with 20% FBS, 20 mmol/L HEPES (pH 7.4), 1 ng/mL recombinant human fibroblast growth factor, 90 μg/mL heparin, and antibiotics. In all of the experiments, cells within 3 passages were used. Hydrogen peroxide (H2O2), DAPI, and MTT were purchased from Sigma. The peptide inhibitors zDEVD.fmk and zVAD.fmk were from Calbiochem.

**Statistical Analysis**

Quantitative data were expressed as mean±SEM. Statistical analysis was performed with the Student t test. Differences were considered significant when probability values were <0.05.

**Results**

**Conditional Induction of c-Jun in HUVECs**

To study the functional role of c-Jun in EC apoptosis, tetracycline-regulated adenoviral expression vectors were generated, in which the full-length coding region of human c-jun cDNA was cloned into the E1- and E3-deleted adenovirus 5. Expression of the c-jun gene, driven by a 7× tet/minimal cytomegalovirus promoter, was under the control of an artificial rtTA, which was produced by a helper adenovirus, AdtTA. The principle of this regulatable system is that an appropriate level of tetracycline can disrupt interaction between the transactivator and the rtTA-responsive element and, therefore, shut off the induced gene expression. As shown in Figure 1, c-jun expression was induced at both mRNA and protein levels in HUVECs coinfected with AdJun and AdtTA. Expression of c-jun transcripts can be detected as early as 12 hours after the transfection and remains overexpressed for the remainder of the experiments. Addition of tetracycline into the culture medium tightly controlled the induced c-jun expression at both mRNA and protein levels in a dose-dependent manner. In contrast, the abundance of VWF mRNA and JNK-1 protein was not affected by tetracycline-regulated c-Jun expression. Tetracycline, at tested concentrations (~0.1 μg/mL), did not show either cytotoxicity (eg, cell morphology, viability, and growth) or interference with expression of endogenous genes thus far tested in HUVECs.
c-Jun Triggers Apoptosis in HUVECs

Using conditional c-jun expression in HUVECs, we examined the role of c-Jun in EC apoptosis. Confluent cells were infected with AdJun and AdtTA and incubated in medium containing 20% FBS in the presence or absence of tetracycline (0.1 μg/mL). Alternatively, cells were coinfected with Adβ-gal and AdtTA as a control for adenovirus-mediated gene overexpression. Starting at 24 hours after the infection, the first morphological changes, including membrane blebbing, nuclear condensation, and cell detachment, were observed in HUVECs overexpressing c-Jun (Figure 2A). The number of apoptotic cells increased with time and accounted for ~50% of the total cell population by 48 hours after infection. In contrast, such apoptotic morphological changes did not occur in ECs overexpressing the β-galactosidase gene or those identically infected with Ad-Jun but maintained in tetracycline-supplemented medium.

A series of assays was performed to document this c-Jun–triggered EC death as an apoptotic process. These include the TUNEL assay (Figure 2B), DNA laddering (Figure 2C), and an ELISA specific for histone-associated DNA fragments (Figure 2D). These typical apoptosis determinations occurred exclusively in ECs overexpressing c-Jun. Collectively, our results point directly to a causative role for c-jun in EC apoptosis.

Caspase Activity Is Involved in c-Jun–Triggered EC Apoptosis

The caspase family of cysteine proteases is increasingly implicated in the apoptotic process for numerous cell types, including ECs. To examine whether c-Jun–triggered apoptosis involves activation of caspase, protein isolated from ECs undergoing c-Jun–triggered apoptosis was subjected to Western blot analysis using an antibody raised against PARP. As...
shown in Figure 3A, marked PARP cleavage was identified in cells overexpressing c-Jun but not in controls. This finding suggests the involvement of caspase activation. Furthermore, we tested whether c-Jun–induced EC apoptosis could be inhibited by specific peptide inhibitors of caspases. As shown in Figure 3B, c-Jun–induced EC apoptosis was largely attenuated by zDEVD.fmk, an irreversible cell-permeable inhibitor of caspase-3. Under the same conditions, the addition of zVAD.fmk, a relatively specific inhibitor of caspase-1, showed less inhibitory effect. Thus, it is highly suggested that activation of caspses, especially caspase-3, may mediate c-Jun–induced EC apoptosis.

The N-Terminal Transactivation Domain Is Required for the Proapoptotic Effect of c-Jun

As an important member of the transcription factor activator protein-1 (AP-1), c-Jun, in the form of heterodimers or homodimers, can transcriptionally regulate gene expression via its amino-terminal transactivation domain.13 To test whether the proapoptotic effect of c-Jun was associated with its transactivation ability, an adenovirus expressing TAM67, a mutant form of c-Jun lacking the transactivation domain, was used to infect ECs. The truncated c-jun gene was overexpressed in HUVECs. In contrast to wild-type c-Jun, TAM67 did not cause apoptosis in HUVECs (Figure 4). Thus, c-Jun may function as a proapoptotic molecule, likely via its transcriptional control of target genes essential to cell death.

c-Jun Is Involved in H<sub>2</sub>O<sub>2</sub>-Induced EC Apoptosis

Clinical studies as well as experimental evidence suggest a causal pathophysiological role of increased oxidative stress in endothelial dysfunction and injury. H<sub>2</sub>O<sub>2</sub> can be produced extracellularly via the respiratory burst of neutrophils or macrophages or intracellularly by the activation of the xanthine oxidase system and has been demonstrated to be a proapoptotic stimulus for ECs.14 In this study, we explored the involvement of c-Jun/AP-1 activation in H<sub>2</sub>O<sub>2</sub>-induced EC apoptosis. Incubation with H<sub>2</sub>O<sub>2</sub> (200 μmol/L) for 18 hours induced apoptosis of ECs, as previously reported. DAPI staining revealed nuclear condensation and fragmentation typical of apoptosis (Figure 5B). Western blot analysis demonstrated that upregulation of c-Jun protein was induced in response to H<sub>2</sub>O<sub>2</sub> treatment (Figure 5A). To examine whether activation of c-jun is required for H<sub>2</sub>O<sub>2</sub>-induced EC apoptosis, HUVECs were infected with AdTAM67, which selectively inactivated c-Jun/AP-1 via a trans-dominant-negative mechanism, and exposed to H<sub>2</sub>O<sub>2</sub> for 18 hours. The susceptibility of TAM67-expressing cells to H<sub>2</sub>O<sub>2</sub>-induced apoptosis was assessed and compared with ECs infected with the adenovirus expressing a wild-type c-jun or that infected with Ad-gal. As shown in Figure 5C, TAM67-expressing ECs exhibited increased resistance to H<sub>2</sub>O<sub>2</sub>-induced apoptosis, whereas ECs overexpressing wild-type c-Jun showed increased susceptibility to H<sub>2</sub>O<sub>2</sub>-induced apoptosis. Therefore, it is suggested that the proapoptotic signaling of H<sub>2</sub>O<sub>2</sub> may be mediated through an endogenous c-jun/AP-1 pathway.

Discussion

In this study, we have used a regulated adenoviral expression system to evaluate the functional role of c-Jun in HUVECs. Using this approach, we provide the first direct evidence that c-jun can be a potent apoptosis inducer in primary cultured human endothelial cells.

Because ECs, especially human primary ECs, are refractory to many transfection techniques,15,16 adenoviral vectors have been used to confer high levels of transgene expression.17,18 Nevertheless, caution must be used when interpreting the results obtained with such an approach, especially those regarding cell death. For example, in addition to the adenoviral early protein E1,19 some late proteins have recently been shown to induce apoptosis.20,21 Thus, it is critical to determine the possibility that apoptosis may be caused by the adenoviral vector itself rather than by the effect of transgene expression. In this study, we have examined this possibility in 2 ways. First, AdJun–mediated EC apoptosis could be completely abolished in identically infected cells by stopping the c-jun expression. Second, high levels of β-galactosidase expression did not induce apoptosis. Thus, EC apoptosis has been demonstrated to be a consequence of specific c-Jun induction.
c-Jun is a major component of the AP-1 transcriptional complex. It can form either homodimers or heterodimers with other AP-1 components from the Jun family (Jun B and Jun D) or the Fos family (c-Fos, Fra-1, Fra-2, and Fos B), and it binds to a palindromic sequence known as the tissue-type plasminogen activator–responsive element or AP-1 consensus site. In addition, c-Jun can also dimerize with members of the activating transcription factor family and can bind to a sequence recognized by the cAMP-responsive element binding protein family. Thus, the different c-Jun dimers can exhibit distinct transcriptional properties with diverse biological consequences. Evidence has accumulated indicating a role for c-Jun/AP-1 in apoptosis, functioning as both a positive and a negative modulator of apoptotic pathways in different cell types. For example, when rat sympathetic neurons undergo apoptosis during nerve growth factor withdrawal, the levels of c-jun mRNA and protein increase. The microinjection of neutralizing antibodies specific for c-Jun, or overexpression of a dominant-negative c-jun mutant, was able to protect nerve growth factor–deprived sympathetic neurons from apoptosis. However, somewhat contradictory evidence has been published. c-Jun has been suggested not to be essential for apoptosis in vivo during normal development, as c–jun−/− mouse embryos exhibited increased rather than reduced apoptosis in their livers. These authors conclude that AP-1 may have a protective role against apoptosis. Several studies in other cell types also showed that c-Jun induction either has no direct relationship to apoptosis or has an inhibitory role. Recently, Bossy-Wetzel et al. provided more direct observations. Conditional induction of c-Jun activity appears sufficient to trigger apoptotic cell death in the NIH 3T3 murine fibroblast cell line. However, similarly generated BALB/c 3T3 cell lines expressed high levels of c-Jun but did not undergo apoptosis, which underscores the necessity of considering the genetic background and cellular context when evaluating the role of c-Jun in apoptosis. Therefore, we present not only the first direct evidence implicating c-Jun as a proapoptotic molecule in vascular endothelial cells, but also the first report extending the finding from a rodent cell line to human primary cells. Hence, this observation may have unique and important implications for human vascular biology.

Increasing evidence has emerged linking EC apoptosis to various vascular pathologic conditions, including atherogenic and thrombotic processes. Apoptotic ECs have been detected at the luminal surface of atherosclerotic coronary vessels. Circulating ECs, suggesting EC death, were detected in hypertension and homocysteinemia. Additionally, the molecular markers of apoptosis, such as Fas, were found on ECs in transplant atherosclerosis. In vitro studies show cultured ECs undergoing apoptosis on exposure to various stimuli, which are shown to promote atherogenesis or its complications. EC proapoptotic factors include proinflammatory cytokines, lipopolysaccharides, LDL oxidative products, cholesterol metabolites, homocysteine, high levels of proinsulin and glucose, angiotensin II, activated leukocytes, and oxidative stress. Furthermore, it has been demonstrated that apoptotic vascular ECs become procoagulant and hyperadhesive for mononuclear cells. It is indicated that EC apoptosis, as a pathological mechanism, may convert ECs to a prothrombotic and proinflammatory state. This in turn may contribute to the pathogeneses of important vascular processes, including angiogenesis, inflammation, thrombosis, and atherosclerosis.

Recent studies have suggested that H2O2, a reactive compound formed endogenously in the breakdown of superoxide or generated by inflammatory cells, may mediate the induction of apoptosis in various cell types, including ECs. Despite its importance in the physiopathology of the vascular endothelium, the molecular mechanisms regulating the H2O2 injury of ECs are currently not well understood. The results obtained from other types of cells indirectly lead to speculation that c-Jun activation may be involved in H2O2-initiated EC apoptosis. First, c-Jun/AP-1 has been known as a major transcription factor responsive to the cellular redox state. Second, both c-Jun expression and AP-1 binding activity can be induced by H2O2. In addition, in glomerular mesangial cells, disruption of c-Jun/AP-1 inhibited H2O2-initiated apo-
In this study, immunoblot analysis revealed an up-regulation of c-Jun in HUVECs after H$_2$O$_2$ treatment. This is consistent with a previous observation in human microvascular ECs that H$_2$O$_2$ induced an AP-1 binding complex containing c-Jun. A functional role of c-Jun/AP-1 activation in H$_2$O$_2$-induced apoptosis has been further established by using adenovirus-mediated expression of c-jun or its dominant-negative mutant, TAM67. When c-Jun is prevented, ECs appear to be predisposed to H$_2$O$_2$ damage, whereas disrupting c-Jun activity by TAM67 renders ECs resistant to the H$_2$O$_2$ proapoptotic effect. Together, these data strongly indicate a pathophysiological role for c-Jun in EC apoptosis triggered by oxidative stress. On the other hand, as shown in Figure 5C, the H$_2$O$_2$ proapoptotic effect was largely but not completely abolished in TAM67-overexpressing cells. It is likely that an alternative pathway, other than c-Jun/AP-1, may also mediate the H$_2$O$_2$ damage to ECs.

How might c-Jun trigger EC apoptosis? First, overexpression of c-Jun leads to specific cleavage of PARP (Figure 3), which is a nuclear protein and a major substrate for caspase-3. Its characteristic cleavage is an indicator of caspase-3 activation. Moreover, the proapoptotic effect of c-Jun can be effectively attenuated by peptide inhibitors of caspases, particularly zDEVD.fmk, which is a selective inhibitor of caspase-3. Thus, a caspase cascade may be responsible for the c-Jun–triggered apoptotic program. However, the downstream mechanisms by which c-Jun initiates the caspase cascade remain to be identified. c-Jun is a transcriptional regulator of gene expression. Hypothetically, c-Jun may trigger EC apoptosis by activating a variety of “death genes,” including members of the caspase family. Alternatively, c-Jun may repress some “protective genes.” Such regulatory mechanisms may be achieved either directly, via the AP-1 cis elements in target genes, or indirectly, via the interaction with other transcription factors. The fact that deletion of the transactivation domain of c-Jun abolished this proapoptotic effect (Figure 4) supports such a hypothesis. Interestingly, a recent report described overexpression in rat arterial endothelial cells by common and unique mechanisms.

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