A20 Protects From CD40-CD40 Ligand-Mediated Endothelial Cell Activation and Apoptosis

Christopher R. Longo, MD; Maria B. Arvelo, MD; Virendra I. Patel, MD; Soizic Daniel, PhD; Jerome Mahiou, PhD; Shane T. Grey, PhD; Christiane Ferran, MD, PhD

Background—CD40/CD40 ligand (CD40L) signaling is a potent activator of endothelial cells (ECs) and promoter of atherosclerosis. In this study, we investigate whether A20 (a gene we have shown to be antiinflammatory and antiapoptotic in ECs) can protect from CD40/CD40L-mediated EC activation.

Methods and Results—Overexpression of CD40, in a transient transfection system, activates the transcription factor NF-κB and upregulates IκBα, E-selectin, and tissue factor (TF) reporter activity. Coexpression of A20 inhibits NF-κB and upregulation of IκBα and E-Selectin but not TF, suggesting that CD40 induces TF in a non–NF-κB–dependent manner. In human coronary artery ECs (HCAECs), adenovirus-mediated overexpression of A20 blocks physiological, CD40-induced activation of NF-κB, upstream of IκBα degradation (Western blot) and subsequently upregulation of ICAM-1, VCAM-1, and E-selectin (flow cytometry). Although A20 does not block TF transcription its expression in HCAECs inhibits TF induction (colorimetric assay and RT-PCR) by blunting CD40 upregulation. We demonstrate that CD40 signaling induces apoptosis in a proinflammatory microenvironment. A20 overexpression protects from CD40-mediated EC apoptosis (DNA content analysis and trypan blue exclusion). We also demonstrate that signaling through CD40L activates NF-κB and induces apoptosis in ECs, both of which are inhibited by A20 overexpression.

Conclusion—A20 works at multiple levels to protect ECs from CD40/CD40L mediated activation and apoptosis. A20-based therapy could be beneficial for the treatment of vascular diseases such as atherosclerosis and transplant-associated vasculopathy. (Circulation. 2003;108:1113-1118.)

Key Words: endothelium ■ atherosclerosis ■ gene therapy ■ thrombosis ■ inflammation
Diego, Calif) were grown in EGM-2 MV media at 37°C with a 5% CO2 atmosphere and used between passage 4 and 6. The U937 monocytic cell line was cultured as recommended (American Tissue Culture Collection, Manassas, Va).

Lipofection Protocol

BAECs were grown in 6-well plates and transfected as described.13 In all experiments, 0.25 μg of the β-galactosidase (β-gal) reporter, 0.7 μg of the expression plasmid for human A20 (kind gift of Dr Vishva Dixit, Genentech), or the control, empty plasmid (pAC), and 0.65 μg of the E-selectin, IkBα, NF-κB, or TF reporters linked to the luciferase gene were transfected as described.13 In some experiments, human (hu) CD40 or CD40L expression plasmids were cotransfected at different concentrations. The huCD40, huCD40L, and control pcMV β vectors were kindly given by Ellen Garber (Biogen, Inc, Cambridge, Mass).

β-Galactosidase and Luciferase Assays

Cellular extracts were assayed for β-galactosidase (β-gal) and luciferase activity as described.13 Luciferase activity was normalized for β-gal using the formula (luciferase activity/β-gal activity×1000) and reported as relative light units (RLU).

Recombinant Adenoviruses

The rAd.A20 was produced in our laboratory as described.12 The control rAd.β-gal was a kind gift of Dr R. Gerard (University of Texas Southwestern). In HCAECs, a multiplicity of infection (MOI) of 100:1 provided strong expression in more than 98% of infected cells, 48 hours after infection with negligible toxicity.

Western Blot Analysis of IkBα

Expression of cytoplasmic IkBα was detected by Western blot analysis using the anti-IkBα rabbit IgG C-21 (Santa Cruz Biotechnology, Santa Cruz, Calif).12

Flow Cytometry

HCAECs were analyzed for surface expression of CD40, CD40L (Pharmingen), E-selectin, ICAM-1, and VCAM-1 (R&D Systems) using a FACScan (Becton Dickinson) as described.12

Adhesion Assay

U937 cells were labeled with the fluorescent dye BCECF-AM (Molecular Probes) as described14 and added at an effector to target (E/T) ratio of 10:1 to HCAECs treated or not with 10 μg/mL of sCD40L for 6 hours. Total mRNA was isolated (RNeasy Mini Protocol, Qiagen) and cdNA synthesized (Superscript Pre-amplification System for First Strand cdNA Synthesis, Life Technologies, Gibco BRL) according to the manufacturer instructions. PCR analysis of TF and β-actin mRNA expression was conducted as described.6

Statistical Analysis

Statistical analysis was performed using the two-tailed unpaired t test and the INSTAT software.

Results

Overexpression of A20 Inhibits CD40-Mediated NF-κB Activation at a Level Upstream of IkBα Degradation and Prevents the Acquisition of a Proinflammatory EC Phenotype

Transfection of BAECs with human CD40 led to an 11-fold induction of NF-κB reporter activity that was completely inhibited (P<0.0001, n=5) by A20 overexpression (Figure 1A). Moreover, A20 completely inhibited the ~3-fold induction of the E-selectin (P<0.0001, n=5) and IkBα (P<0.0001, n=4) reporters by CD40 (Figures 1B and 1C). This is concordant with the strict NF-κB dependency of E-selectin and IkBα induction.12 A20 did not abrogate a 2.5-fold, CD40-mediated induction of the TF reporter (Figure 1D). These data indicate that, in ECs, NF-κB activation is not required for CD40-mediated TF induction. Given limitations of transient transfection systems, we eval-
Table 2. A20 blocks CD40-mediated activation of NF-κB at a level upstream of IκBα degradation and inhibits acquisition of a proinflammatory EC phenotype.

A. NI HCAECs and HCAECs infected with rAd.A20 or rAd.β-gal were stimulated with 10 μg/mL of sCD40L to induce NF-κB activation, evidenced by IκBα degradation 15 minutes after stimulation. A20 completely inhibited IκBα degradation. Data are representative of 3 independent experiments. B. NI HCAECs or HCAECs infected with rAd.A20 or rAd.β-gal were stimulated with 10 μg/mL of sCD40L. Expression of E-selectin, VCAM-1, and ICAM-1 was assessed by flow cytometry. Solid histograms represent untreated cells and empty histograms represent cells stimulated with sCD40L. A20 significantly (P<0.001) inhibited sCD40L-mediated induction of E-selectin, ICAM-1, and VCAM-1. Data are representative of 5 independent experiments. C. Expression of A20 inhibited the adhesion of BCECF-labeled U937 cells on HCAECs infected with rAd.A20 for 24 hours with sCD40L. Data are mean±SEM of sextuplicates and are representative of 4 independent experiments.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>sCD40L−</th>
<th>sCD40L+</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-selectin</td>
<td>7±0.4</td>
<td>12±0.2</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>53±3.3</td>
<td>20±0.5</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>31±3</td>
<td>862±29</td>
</tr>
</tbody>
</table>
6-fold increase in TF activity in NI and rAd.β-gal–infected HCAECs, respectively (Figure 3A). A20 overexpression blocked the induction of TF activity (1.3-fold induction; \( P < 0.0001, n = 3 \) experiments). Because A20 did not inhibit CD40-mediated transcription of TF in transient transfection assays, we investigated whether A20 affected other parameters such as the TFPI. Our results demonstrate that A20 overexpression did not modify TFPI levels (Figure 3B). Overexpression of A20 did, however, inhibit the upregulation of CD40 surface expression. CD40 expression was upregulated in NI and rAd.β-gal–infected HCAECs 24 hours after TNF treatment (MFI increased from 9±0.7 to 48±2 and from 26±1.4 to 71±1.7, respectively). A20 overexpression significantly (\( P < 0.0001, n = 5 \)) inhibited TNF-mediated CD40 upregulation (15±0.7 to 26±0.8 MFI) (Figure 4). Upregulation of CD40 is prerequisite for induction of TF by sCD40L in HCAECs. Failure to upregulate CD40 surface expression in A20-expressing HCAECs prevented TF mRNA induction after treatment with TNF and sCD40L. In contrast, TF mRNA was readily increased in NI and rAd.β-gal–infected HCAECs \( (n = 3, \) Figure 3C). No evidence of cell death was detected in TNF/sCD40L-treated HCAECs up to 24 hours (data not shown).

HCAECs expressing high levels of CD40L was observed in both control groups after stimulation. Data are representative of 5 independent experiments.
A20 Protects EC from Cytokines/CD40-Mediated Apoptosis

NI and rAd,β-gal–infected HCAECs treated with TNF-α, IFNγ, and sCD40L for 32 hours underwent apoptotic cell death. The percentage of apoptotic HCAECs rose from 2.3±0.3% to 16.3±0.9% in NI HCAECs and from 4.6±0.3% to 21±1.5% in rAd,β-gal–infected HCAECs. Overexpression of A20 completely (P<0.0001, n=3) protected HCAECs from apoptosis (Figure 5A). Similar data were obtained when analyzing the percentage of dead cells by trypan blue exclusion. The percentage of dead blue cells rose from 3.2±0.3% to 16.5±0.6% and from 5±0.2% to 20.3±1.1% in NI and rAd,β-gal–infected HCAECs, respectively, whereas no increase in dead blue cells was recorded in A20 expressing HCAECs (Figure 5B). This result could relate to decreased CD40 expression in A20-expressing HCAECs. To determine whether A20 also blocks intracellular apoptotic signaling by CD40, BAECs were cotransfected with increasing amounts of CD40 expression plasmid and the RSV-β-gal plasmid. The RSV-β-gal plasmid and cell death quantified by the number of live blue cells/well. We observed a dose-dependent relationship between overexpression of CD40 and reduced blue cell numbers (Figure 6A). Coexpression of A20 totally abrogated cell death when HCAECs were transfected with 50 ng of CD40 plasmid while reducing cell death when the higher 250 ng concentration of CD40 plasmid was used (P<0.0001; n=4). The baseline number of blue cells in A20 expressing HCAECs was significantly higher than that of control, probably relating to protection by A20 from transfection toxicity. These data demonstrate that expression of A20 protects ECs from CD40-mediated apoptosis by blocking intracellular apoptotic pathways and surface expression of CD40.

A20 Inhibits CD40L Expression and Signaling

We chose to work with HCAECs for their ability to express CD40L in vivo. HCAECs expressed low levels of constitutive CD40L that were significantly upregulated 24 hours after addition of 100 U/mL of TNF-α and IFNγ in the NI and rAd,β-gal–infected cells (7±0.5 to 49.4±1.6 and 20.6±6.7 to 42.2±8.0 MFI, respectively) (Figure 4). Overexpression of A20 significantly (P<0.0001, n=5) blocked CD40L upregulation (9±0.7 to 17±1.3 MFI). Cross-linking of CD40L in HCAECs proved difficult due to lack of effective reagents. We investigated CD40L signaling in EC by overexpressing human CD40L on BAECs in a transient transfection system. Overexpression of CD40L led to cell death in a dose-dependent manner. BAECs cotransfected with increasing amounts of CD40L expression plasmid and the RSV-β-gal plasmid demonstrated a dose-dependent relationship between CD40L concentrations and reduced living transfected blue cell number/well (Figure 6B). Coexpression of A20 significantly decreased cell death for each dose of CD40L (P<0.0001, n=4), although this effect was more evident at the lowest concentration of CD40L (50 ng of plasmid).

Transfection with 10 ng of huCD40L plasmid (that does not lead to cell death) led to a 26-fold induction of NF-κB reporter activity that was completely inhibited (P<0.0001, n=6) by A20 overexpression (Figure 7A). Soluble CD40L was not detected (ELISA) in the supernatant, eliminating shed CD40L as the possible signal source (data not shown). A20 overexpression also completely inhibited CD40L-mediated 3.6-fold (P<0.0001, n=5) induction of the E-selectin reporter and 7.7-fold (P<0.0001, n=4) induction of the IkBα reporter (Figures 7B and 7C). Overexpression of A20 did not abrogate a 4-fold, CD40L-mediated, induction of the TF reporter (Figure 7D). These data indicate that, in ECs, NF-κB is not required for CD40L-mediated induction of TF.

Discussion

Signaling through CD40 is proinflammatory in ECs via activation of NF-κB and other transcription factors including AP-1, STAT-1, and Egr-1.2 CD40-activated ECs acquire a proinflammatory and prothrombotic phenotype, expressing leukocyte...
adhesion molecules and TF. We demonstrate that overexpression of A20 blocks CD40-mediated NF-κB activation and subsequent upregulation of adhesion molecules both at the protein and transcription levels.

Expression of A20 did not affect CD40-mediated activation of a TF reporter, suggesting that NF-κB activation is not necessary for TF induction by CD40. Analysis of the TF promoter identified response elements for several transcription factors including NF-κB, AP-1, Sp1, and Egr-1. In saphenous ECs, CD40-mediated transactivation of TF requires AP-1 and Egr-1 as well as NF-κB. We have shown that A20 does not affect AP-1-mediated transactivation. Our data in HCAECs demonstrate that A20 completely blocks the induction of TF mRNA and activity by physiological CD40L signaling after treatment with TNF-α. Pretreatment with TNF-α increases CD40 surface expression in HCAECs, a prerequisite for TF upregulation on addition of CD40L. CD40 is a NF-κB-dependent gene. We hypothesize that by blocking cytokine-mediated upregulation of surface CD40, A20 keeps CD40 expression beneath the critical threshold required for signaling by CD40L. These data demonstrate that overexpression of A20 in ECs blocks CD40-mediated inflammation and thrombosis at multiple levels including surface expression and signaling pathways. To the extent that CD40 cross-linking upregulates A20 in a NF-κB-dependent manner, one could easily speculate that A20 is part of a regulatory feedback loop modulating signaling through CD40.19,20 We demonstrate that CD40-mediated signaling of cytokine-activated HCAECs leads to apoptosis contrasting with data in HUVECs. Overexpression of A20 protected HCAECs from CD40-mediated apoptosis. This agrees with previous data by Hess et al demonstrating that A20 protects the fibroblast SV80 cell line against apoptotic cell death induced by TNF. Protection from CD40-mediated apoptosis by A20 could relate to blockade of apoptotic pathways and caspase activation (as shown for TNF and Fas) and/or a decrease in cytokine-induced surface expression of CD40. Having demonstrated the latter, we overexpressed CD40 in BAECs and evaluated the effect of A20 on CD40-mediated death pathways. Our data demonstrate that overexpression of CD40 led to a dose-dependent increase in cell death, significantly, although not completely inhibited by overexpression of A20, demonstrating that A20 also blocks cell death pathways triggered by CD40 signaling. Under inflammatory conditions, some EC subtypes, such as HCAECs, express CD40L as well as CD40 on their surface. CD40L expression is NF-κB dependent. We demonstrate that HCAECs upregulate CD40L after treatment with inflammatory cytokines and that A20 blocks this upregulation. Overexpression of A20 in BAECs inhibited CD40L-mediated activation of the NF-κB, IκBα, and E-selectin reporters and protected from a CD40L dose-dependent increase in EC death. A20 expression did not block CD40L-mediated upregulation of a TF reporter, but we anticipate that it may inhibit TF activity in a physiological setting by inhibiting cytokine-mediated upregulation of CD40L to the critical threshold required for signaling. This is the first demonstration that A20 blocks CD40/CD40L-mediated inflammatory, procoagulant, and apoptotic responses in ECs. By specifically interrupting this dyad, A20-based gene therapy in EC could, as demonstrated using anti-CD40L Ab therapy, significantly impact on the initiation and progression of atherosclerosis and TAV.
A20 Protects From CD40-CD40 Ligand-Mediated Endothelial Cell Activation and Apoptosis
Christopher R. Longo, Maria B. Arvelo, Virendra I. Patel, Soizic Daniel, Jerome Mahiou, Shane T. Grey and Christiane Ferran

Circulation. 2003;108:1113-1118; originally published online July 28, 2003;
doi: 10.1161/01.CIR.000083718.76889.D0
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/9/1113

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/