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

Recent studies have demonstrated that CD40/CD40 ligand (CD40L) signaling pathway is a potent activator of endothelial cells (ECs) and promoter of atherosclerosis.1,2 CD40/CD40L signaling activates the transcription factor NF-κB, an early mediator of immune and inflammatory responses.3,4 In response to CD40/CD40L-induced NF-κB activation, ECs acquire a proinflammatory, proatherogenic phenotype and express leukocyte adhesion molecules and tissue factor (TF).5,6

CD40 is a member of the TNF receptor superfamily and is constitutively expressed on the EC surface.5 CD40L is a member of the TNF superfamily and is expressed on T-lymphocytes, activated platelets, and smooth muscle cells (SMCs).7,8 CD40L can also be expressed on ECs under certain proinflammatory conditions (including the shoulder region of carotid artery plaques and rejecting heart allografts), raising the possibility of autocrine and paracrine activation.9

In animal models, CD40L blockade has been shown to prevent atherosclerotic plaque progression, promote plaque stability, and prevent transplant associated vasculopathy (TAV), an accelerated form of atherosclerosis.10,11

We have demonstrated that the cytoprotective gene A20, prevents EC activation in response to a number of stimuli (including proinflammatory cytokines and oxidative damage) by blocking NF-κB activation and shown that it protects ECs from TNF/cycloheximide-induced apoptosis through inhibition of the caspase cascade.12,13 In this study, we evaluate the effect of A20 on CD40/CD40L-mediated EC signaling and activation.

Methods

Reagents
Recombinant human TNFα and IFNγ were purchased from R&D Systems. Recombinant human, soluble, trimeric CD40 ligand (sCD40L) was obtained from Leinco technologies. Factors VIIa, X/Xa, and Spectrozyme were purchased from American Diagnostics. Saponin was purchased from Sigma. Soluble CD40L ELISA kit was purchased from Chemicon International.

Cell Culture
Bovine aortic endothelial cells (BAECs) obtained from 3 different animals were cultured as described.14 Human coronary artery endothelial cells (HCAECs) from 2 different donors (Clonetics, San...
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. 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, ICAM1, NF-κB, or TF reporters linked to the luciferase gene were transfected as described. 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. 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. 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 IκBα**

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

**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.

**Adhesion Assay**

U937 cells were labeled with the fluorescent dye BCECF-AM (Molecular Probes) as described 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 24 hours. After 1 hour incubation, HCAECs were washed and attached cells lysed in 50 mmol Tris buffer with 0.1% SDS. Fluorescence intensity (FI) was measured using the Wallac 1420 multilabel counter (Perkin Elmer) set at excitation and emission wavelengths of 485 and 530 nm.

**Analysis of Apoptosis and Cell Death**

Apoptosis was quantified by FACS analysis of DNA content as described. 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, ICAM1, NF-κB, or TF reporters linked to the luciferase gene were transfected as described. 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).

**Overexpression of A20 Inhibits CD40-Mediated NF-κB Activation at a Level Upstream of IκBα 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) by A20 overexpression (Figure 1A). Moreover, A20 completely inhibited the ~3-fold induction of the E-selectin (P<0.0001, n=5) and IκBα (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 IκBα induction. 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-

![Figure 1](http://circ.ahajournals.org/)

**Analysis of TF mRNA Expression Using Semiquantitative RT-PCR**

HCAECs were stimulated for 4 hours with 100 U/mL of TNF followed by 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.

**Statistical Analysis**

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

**Results**

- **Figure 1. A20 inhibits CD40-mediated activation of ECs.** BAECs were transfected with a control, pcDNA3 plasmid, or an A20 expression plasmid. Cotransfection with 10 ng of a CD40 expression plasmid significantly induced NF-κB (A), E-selectin (B), IκBα (C), and TF (D) reporters as compared with nontreated (NT) BAECs. A20 completely inhibited (P<0.0001) induction of the NF-κB, E-selectin, and IκBα reporters but not TF reporter. Data are mean±SEM of 5 independent experiments performed in triplicate and are expressed in relative light units of luciferase activity.

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uited the function of A20 in human ECs activated by cross-linking CD40 with its physiological ligand, sCD40L. Noninfected (NI) HCAECs and HCAECs infected with rAd.A20 or rAd.β-gal at a MOI of 100:1 were stimulated with 10 μg/mL of sCD40L and NF-κB activation was evaluated. NF-κB activation requires the phosphorylation, ubiquitination, and degradation of its inhibitor, IκBα.3 Cytoplasmic extracts were recovered before and 15 minutes and 2 hours after stimulation and Western blot analysis of IκBα was conducted. A20 expression in HCAECs inhibited IκBα degradation after sCD40L stimulation, demonstrating that A20 inhibits CD40-mediated NF-κB activation upstream of IκBα degradation (Figure 2A).

We next evaluated the effect of A20 on protein expression of the NF-κB–dependent genes E-selectin, VCAM-1, and ICAM-1 after treatment with sCD40L. FACS analysis of HCAECs stimulated with 10 μg/mL of sCD40L demonstrated a significant increase in mean fluorescence intensity (MFI) of E-selectin (1.7- and 1.6-fold increase in NI and rAd.β-gal–infected HCAECs at 6 hours, respectively), VCAM-1 (3.8- and 1.8-fold increase in NI and rAd.β-gal–infected HCAECs at 24 hours, respectively), and ICAM-1 (23- and 13-fold increase in NI and rAd.β-gal–infected HCAECs at 24 hours, respectively) (Table, Figure 2B). Overexpression of A20 significantly inhibited the upregulation of E-selectin (1.2-fold increase; \( P=0.0002, n=5 \)), VCAM-1 (1.2-fold increase; \( P=0.001, n=5 \)), and ICAM-1 (3.5-fold increase; \( P<0.0001, n=5 \)). In concordance with this result, A20 significantly inhibited the adhesion of U937 cells on sCD40L-treated HCAECs (Figure 2C). Adhesion of BCECF-labeled U937 at E/T ratio of 10:1 to NI and rAd.β-gal–infected HCAECs increased by 2- to 2.5-fold after treatment with sCD40L (FI increased from 4095±546 to 7820±348 and 2428±179 to 6064±693). Expression of A20 decreased to 1.3-fold the adhesion of U937 to HCAECs (FI minimally increased from 2944±247 to 4010±434; \( n=4; P<0.0001 \)).

**Overexpression of A20 Inhibits TF Upregulation in Response to TNF and sCD40L and Prevents Upregulation of TNF-Induced CD40 Expression**

NI and rAd.β-gal– and rAd.A20-infected HCAECs were pretreated with 100 U/mL of TNF for 4 hours followed by sCD40L for 20 hours. This treatment led to a 6.7- and

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<th>Mean Fluorescence Intensity of E-Selectin, VCAM-1, and ICAM-1 Before and After Treatment With sCD40L</th>
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<tr>
<td>E-selectin</td>
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\[ P=0.0001, n=5 \]
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; \( P \leq 0.0001 \), n = 3 experiments). Because A20 did not inhibit CD40-mediated transcription of TF without altering TFPI activity. Data shown are mean±SEM of 3 independent experiments performed in sextuplicate using HCAECs isolated obtained from 2 different donors. C, A20 inhibited upregulation of TF mRNA after treatment with TNF-α for 4 hours then sCD40L for 6 hours after sCD40L. Data are representative of 3 independent experiments.

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. Cotransfection with 10 ng of a CD40L expression plasmid significantly induced NF-κB (A), E-selectin (B), IkBα (C), and TF (D) reporters as compared with nontreated (NT) BAECs. A20 completely inhibited (P<0.0001) induction of the NF-κB, E-selectin, and IkBα reporters but not TF reporter. Data are the mean±SEM of 5 independent experiments and are expressed in relative light units of luciferase activity.

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 100U/mL of TNF-α and IFNγ in the NI and rAd,β-gal-infected cells (7.4±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 (9.2±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.12 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 sCD40L. CD40 is a NF-κB–dependent gene.18 We hypothesize that by blocking cytokine-mediated upregulation of surface CD40, A20 keeps CD40 expression beneath the critical threshold required for signaling by sCD40L. 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.11 Overexpression of A20 protected HCAECs from CD40-mediated apoptosis. This agrees with previous data by Hess et al22 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)22 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.23 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, 1xκB, 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.10,11,24

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