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*J Immunol* 1998; 161:4572-4582; ;
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Adenovirus-Mediated Expression of a Dominant Negative Mutant of p65/RelA Inhibits Proinflammatory Gene Expression in Endothelial Cells Without Sensitizing to Apoptosis

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We hypothesized that blocking the induction of proinflammatory genes associated with endothelial cell (EC) activation, by inhibiting the transcription factor nuclear factor κB (NF-κB), would prolong survival of vascularized xenografts. Our previous studies have shown that inhibition of NF-κB by adenovirus-mediated overexpression of IκBa suppresses the induction of proinflammatory genes in EC. However, IκBa sensitizes EC to TNF-α-mediated apoptosis, presumably by suppressing the induction of the NF-κB-dependent anti-apoptotic genes A20, A1, manganese superoxide dismutase (MnSOD), and cellular inhibitor of apoptosis 2. We report here that adenovirus mediated expression of a dominant negative C-terminal truncation mutant of p65/RelA (p65RHD) inhibits the induction of proinflammatory genes, such as E-selectin, ICAM-1, VCAM-1, IL-8, and inducible nitric oxide synthase, in EC as efficiently as does IκBa. However, contrary to IκBa, p65RHD does not sensitize EC to TNF-α-mediated apoptosis although both inhibitors suppressed the induction of the anti-apoptotic genes A20, A1, and MnSOD equally well. We present evidence that this difference in sensitization of EC to apoptosis is due to the ability of p65RHD, but not IκBa, to inhibit the constitutive expression of c-myc, a gene involved in the regulation of TNF-α-mediated apoptosis. These data demonstrate that it is possible to block the expression of proinflammatory genes during EC activation by targeting NF-κB, without sensitizing EC to apoptosis and establishes the role of c-myc in controlling induction of apoptosis during EC activation. Finally, these data provide the basis for a potential approach to suppress EC activation in vivo in transgenic pigs to be used as donors for xenotransplantation. The Journal of Immunology, 1998, 161: 4572–4582.

Quiescent endothelial cells (EC) maintain a selective barrier between blood and tissue while preventing coagulation. Inflammatory stimuli, such as TNF-α, bacterial LPS, IL-1-α, IL-1-β, or IFN-γ, activate EC. EC activation is characterized by induction of proinflammatory genes, including those encoding adhesion molecules (e.g., E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1)), cytokines/chemokines (e.g., monocyte chemotactic protein-1, RANTES, IL-1, IL-6, and IL-8), and procoagulant factors (e.g., tissue factor, plasminogen activator inhibitor-1) (1, 2). Under physiologic conditions the expression of these proinflammatory genes plays a central role in triggering immune responses by selectively recruiting circulating leukocytes to sites of inflammation. However, uncontrolled expression of proinflammatory genes by EC can result in vascular thrombosis and tissue necrosis such as observed during chronic inflammation, ischemia-reperfusion injury, septic shock, and allograft or xenograft rejection (2–4).

Induction of proinflammatory genes by EC is often triggered by proinflammatory cytokines such as TNF-α and is largely dependent on the activation of members of the Rel nuclear factor κB (NF-κB) family of transcription factors (5). In addition to the signaling cascade leading to NF-κB activation, TNF-α activates members of the Caspase family of proteases inducing apoptosis (6). Most cells, however, do not undergo apoptosis when stimulated by TNF-α, likely based on the induction by TNF-α of anti-apoptotic genes such as A20 (7), the Bcl-2 family member A1 (8), manganese superoxide dismutase (MnSOD) (9) and cellular inhibitor of apoptosis-2 (c-IAP-2), a member of the family of inhibitors of apoptosis (10). Most of these anti-apoptotic genes, i.e., A20, MnSOD, and A1 also act as inhibitors of NF-κB and thus may control the expression of proinflammatory genes during EC activation (3, 11, 12). We refer to these anti-apoptotic genes as “protective genes” given their dual role in protecting EC from apoptosis and blocking NF-κB. We have proposed a model for EC...
activation in which the expression of protective genes down-modulates the proinflammatory response while preventing EC apoptosis (3). We have shown that long term survival of vascularized xenografts is associated with the expression of protective genes by xenograft EC, whereas xenografts undergoing rejection do not express these genes (3,11).

Our hypothesis is that EC activation with expression of NF-κB-dependent proinflammatory genes underlies delayed xenograft rejection by providing the proinflammatory environment necessary for the activation and infiltration of recipient NK cells and monocytes, and the development of thrombosis: hallmarks of this type of rejection (4). Given the above, we have conceived strategies to prevent xenograft rejection based on genetic engineering of EC and aimed to inhibit EC activation through suppression of the transcription factor NF-κB (13). One such approach was to overexpress IκBα, the natural inhibitor of NF-κB (14). While highly effective at blocking NF-κB and suppressing the induction of the proinflammatory genes, IκBα overexpression sensitized EC to TNF-α-mediated apoptosis (14,15), presumably by inhibiting up-regulation of NF-κB-dependent anti-apoptotic genes, a finding that has been described in other cell types as well (16–18). These findings, if also relevant in vivo, would render this approach useless for xenotransplantation. An alternative approach to block NF-κB is based on the overexpression of a dominant negative C-terminal truncation mutant of p65RelA in EC (13). This mutant, p65RHD, lacks the transactivation domain of p65RelA but retains the N-terminal Rel homology domain (RHD), which is necessary and sufficient for DNA binding as well for dimerization to other members of the Rel family (p50/NF-κB1, c-Rel/Rel) (13). We report here that recombinant adenovirus-mediated overexpression of p65RHD in EC blocks NF-κB activity and inhibits proinflammatory gene induction equally as well as does IκBα. However, contrary to IκBα, p65RHD does not sensitize EC to TNF-α-mediated apoptosis, despite the fact that it blocks the induction of the anti-apoptotic genes A20, A1, and MnSOD. We explain this difference by the ability of p65RHD, but not IκBα, to repress the expression of c-myc, a gene previously reported to be involved in TNF-α-mediated apoptosis (19–21).

Materials and Methods

Cell culture

Porcine aortic EC (PAEC) were isolated and grown on 0.2% bovine gelatin (Sigma, Saint Louis, MO)-coated cell culture flasks in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 100 U/ml penicillin G (50 μg/ml) (Life Technologies) and streptomycin (50 μg/ml) (Life Technologies) as described before (22). Human umbilical vein EC (HUVEC) were isolated and grown in M199 medium (Life Technologies) supplemented with 15% FBS, NaHCO3 (20 mM), HEPE (25 mM), glutamine (5 mM, Life Technologies), heparin (100 μg/ml), gentamicin (50 μg/ml), and endothelial growth factor (50 μg/ml) (Sigma) as described before (23). The murine 2F–2B cell line and the human 293 embryonic kidney cell line (American Type Culture Collection (ATCC), Rockville, MD) were grown in DMEM, 10% FBS, penicillin G (50 μg/ml), and streptomycin (50 μg/ml).

Recombinant adenoviruses

The p65RHD-dominant-negative mutant has been described before (13). Briefly, this construct encodes for amino acids (aa) 2–320 of the human p65RelA preceded by a 13-amino acid sequence containing 10 aa from the human c-myc gene used as a recognition sequence by anti-C-terminal human c-myc mAb 9E10 (hybridoma CRL-1729) (24). The p65RHD recombinant adenovirus was constructed as described before for the IκBα-recombinant adenovirus (14). Briefly, cDNA coding for p65RHD was cloned in to the pAC.CMV-PlpLAsR+ and the resulting p65RHD/pAC.CMV-PlpLAsR+ vector was cotransfected into 293 cells with the pM17 recombination plasmid containing the full length adenoviral genome with a deletion in the E1 region. Adenovirus clones obtained by limiting dilution in 293 cells were tested for p65RHD expression by Western blotting as described below. The recombinant β-galactosidase adenovirus was a kind gift of Dr. Robert Gerard (University of Texas Southwest Medical Center, Dallas, TX). The recombinant IκBα adenovirus expressing the porcine IκBα gene (ECI-6) was generated in our laboratory by C. J. Wrighton and has been described elsewhere (14). All recombinant adenoviruses were produced in 293 cells, extracted, purified through two cesium chloride gradient ultracentrifugations, and their titers were determined by limiting dilution in 293 cells as described before (14).

Adenovirus-mediated gene transfer to EC

Pre-confluent PAEC were infected with a multiplicity of infection (MOI) of 500 plaque-forming units per cell (pfu/cell) and HUVEC were infected at 100 pfu/cell, respectively. Adenoviral infection was carried out in 1% FBS DMEM for 1.5 h at 37°C, 5% CO2, and 95% humidity under agitation. The FBS concentration was then adjusted to 10%, transduced EC were kept in presence of the recombinant adenovirus for 24 h, and the medium was replaced by fresh supplemented DMEM for an additional 24 h before being assessed for the expression and the function of the transferred gene. Unless otherwise indicated, all experiments were carried out with cells infected at the MOI giving maximal expression 2 days after infection.

Crystal violet vital staining

Cell viability was assessed by a colorimetric assay based on the uptake of the vital dye, Crystal violet, as described elsewhere (25). Briefly, cells were stained (2 min, room temperature) with Crystal violet solution, washed under tap water, and blue crystals were dissolved in 10% acetic acid (5 min, room temperature). OD was measured at λ = 405 nm. For each recombinant adenovirus infection, cells in culture medium were considered to reflect 100% cell viability.

Transient transfection and plasmid constructs

The cDNAs encoding for human β-galactosidase, p65, p65RHD and a degradation-resistant mutant (26) of porcine IκBα (ECI-6, a kind gift from Dr. R. de Martin, University of Vienna, Vienna International Research Cooperation Center, Vienna, Austria) were cloned into pcDNA3 vector (Invitrogen, Carlsbad, CA). The DNA-binding-deficient mutants of p65 and p65RHD were derived through PCR-based approach from p65 and p65RHD, respectively, as described elsewhere (13,27) and were expressed from NC4 (nDNA3) vector. The full length human IκBα was expressed by PCR from HeLa cell cDNA and expressed from the pcDNA3 vector. All constructs were verified by dsDNA sequencing. All transient transfection experiments were carried out using the murine EC line 2F–2B. All constructs were co-transfected with β-galactosidase and the amount of DNA was maintained constant using the pcDNA3 vector. Cells were seeded at 3 × 105 cells in 35-mm wells and transfected 20 to 24 h later using Lipofectamine (Life Technologies) according to the manufacturer’s suggestions (2–3 μg DNA/6–9 μl Lipofectamine, 5–6 h). Twenty-four hours after transfection, cells were stimulated with human rTNF-α (100 U/ml, 16 h) (R&D Systems, Minneapolis, MN) and cell viability was evaluated through detection of β-galactosidase activity using 0.1% X-Gal (Life Technologies) in 0.1 NaP (pH 7.2), 1.3 mM MgCl2, 3 mM potassium ferrocyanide, and 3 mM potassium ferricyanide. Cell viability was assessed by counting the number of “blue-stained” transfected cells that retained normal EC morphology. The percentage of viable cells was normalized for each DNA preparation to the number of transfected cells counted in the absence of TNF-α treatment. All experiments were performed three times in duplicate.

Cell extracts and Western blot analysis

Cells were washed in PBS (pH 7.2), harvested by scraping, centrifuged (300 g, 5 min, 4°C) and total protein was extracted (30 min, 4°C) in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Nonidet P-40 (Sigma), 0.5% deoxycholate (Sigma), 0.1% SDS supplemented with 0.1 mM TPCK (Sigma), 0.1 mM TLCK (Sigma), 0.5 mM PMSF (Sigma), 1 μg/ml aprotinin (Boehringer Mannheim, Indianapolis, IN), and 1 μg/ml Leupeptin (Boehringer Mannheim)). Protein extracts were centrifuged (12,000 g, 30 min, 4°C) and protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein were boiled in Laemmli buffer, and electrophoresis was carried out under denaturing conditions using 10% polyacrylamide gels. Proteins were transferred onto a polyvinylidifluoride membrane (Immobilon P, Millipore, Bedford, MA) by electroblotting and detected using rabbit polyclonal Abs directed against the N-terminal region of the human p65RelA (Santa Cruz Biotechnology, Santa Cruz, CA), the C-terminal region of human c-myc (Upstate Biochemicals, Lake Placid, NY) or IκBα (Santa Cruz Biotechnology). Proteins were visualized using horseradish peroxidase-conjugated...
donkey anti-rabbit IgG (Pierce, Rockford, IL) and the enhanced chemiluminescence assay (Amersham Life Science, Arlington Heights, IL) according to the manufacturer’s instructions.

**Flow cytometry**

EC were treated with human rTNF-α (100 U/ml, 6 h) (R&D Systems) and harvested by trypsin digestion (0.05% in PBS). Cells were washed in PBS (pH 7.2), 5% FBS, 0.1% NaN₃, and incubated (1 × 10⁶ cells in 100 μl, 30 min, 4°C) with a mouse anti-human VCAM-1 mAb (Genzyme, Cambridge, MA), 1 μg/ml in PBS, 5% FBS, 0.1% NaN₃. After washing in PBS, 5% FBS, 0.1% NaN₃ (300 × g, 5 min) cells were stained (30 min, 4°C) with a FITC-labeled polyclonal goat anti-mouse IgG Ab (Sigma). Fluorescent labeling was evaluated using a FACSort equipped with Cell Quest Software (Becton Dickinson, Palo Alto, CA). Specific labeling was compared with nonspecific staining using a FITC-labeled isotype-matched control mAb. Modulation of VCAM-1 expression was assessed by comparing the expression on treated vs untreated cells. Detection of phosphatidylserine in the outer leaflet of the plasma membrane of apoptotic EC was carried out using FITC-labeled annexin V according to the manufacturer’s suggestions (R&D Systems).

**Cell cycle**

PAEC were left untreated or infected with β-galactosidase, IxBα, or p65RHD recombinant adenoviruses as described above and analyzed 48 h after infection. Cell cycle progression was assayed by propidium iodide DNA staining as described elsewhere (28). Fluorescent labeling was evaluated using a FACSort equipped with Cell Quest Software (Becton Dickinson). Experiments were carried out in triplicate.

**Immunocytochemistry**

Confluent HUVEC or PAEC were trypsinized, centrifuged onto glass coverslips, fixed with 0.05% glutaraldehyde (15 min, room temperature), and permeabilized with Triton X-100 (Sigma). The presence of p65RHD was revealed by the anti-c-myc-specific mAb 9E10 using biotinylated goat anti-mouse IgG and horseradish peroxidase-coupled streptavidin (Pierce) as detection system for which AEC (3-amino-9-ethylcarbazole, Sigma) was used as substrate.

**Northern blot analysis**

PAEC and HUVEC were treated with TNF-α (2 h, 500 U/ml) and RNA was extracted using TRIzol, according to the manufacturer’s suggestions (Life Technologies). Total RNA was separated on a 1.3% agarose formaldehyde gel, transferred overnight to Hybond-N nylon membranes (Amersham Life Science), and analyzed by specific hybridization to radioabeled cDNA probes for human A20 (kind gift from Dr. V. Dixit, Genentech, South San Francisco, CA), bcl-xL cloned in our laboratory by A. Badrichani, human c-myc (as described above), MnSOD (ATCC), VCAM-1 (a kind gift from T. Collins Brigham and Women’s Hospital, Boston, MA), p65/RelA (a kind gift from W. C. Greene, University of California, San Francisco, CA), rat inducible nitric oxide synthase (iNOS).

**Electrophoretic mobility shift assay (EMSA)**

The p65RHD recombinant adenovirus was grown as described in Materials and Methods. The p65RHD dominant negative mutant encodes for aa 2–320 of the human p65/RelA preceded by a 13-aa sequence containing 10 aa from the human c-myc gene used as a recognition sequence by anti-C-terminal human c-myc mAb 9E10. This mutant lacks the transactivation domain of p65/RelA but retains the N-terminal RHD, which is necessary and sufficient for DNA binding as well for dimerization to other members of the Rel family. Expression of p65RHD is driven by a minimal CMV promoter (CMV).

**FIGURE 2.** Recombinant adenovirus-mediated expression of p65RHD in EC. A. Dose response: PAEC were transduced with p65RHD recombinant adenovirus at a multiplicity of infection (MOI) ranging from 0.05 pfu/cell to 500 pfu/cell, and expression of p65RHD was analyzed 48 h after infection by Western blot using a polyclonal Ab recognizing the N-terminal region of human p65/RelA as described in Materials and Methods. At its maximal level of expression, there was a 50 to 100-fold excess of p65RHD over endogenous RelA as estimated by Western blot (data not shown). B. Time course: PAEC were transduced with p65RHD recombinant adenovirus at a MOI of 500 pfu/cell and analyzed by Western blot for expression of p65RHD 1, 2, 3, 4, and 5 days after infection as described in Materials and Methods. C. Immunocytochemistry: HUVEC were transduced with p65RHD recombinant adenovirus at a MOI of 100 pfu/cell and p65RHD expression was analyzed 48 h after infection by immunocytochemistry as described in Materials and Methods. Shown in the figure are: 1) negative control for detection of p65RHD in β-galactosidase-transduced HUVEC (similar staining was obtained in noninfected cells); 2) negative control using an isotype-matched mAb in p65RHD-transduced HUVEC; and 3) detection of p65RHD protein expression using an anti-c-myc C-terminal mAb (9E10) in p65RHD-transduced HUVEC. Notice that 100% of HUVEC express p65RHD, which localizes mainly to the nucleus.

**FIGURE 1.** Schematic representation of the p65RHD recombinant adenovirus. Recombinant adenovirus was generated as described in Materials and Methods. The p65RHD dominant negative mutant encodes for aa 2–320 of the human p65/RelA preceded by a 13 aa sequence containing 10 aa from the human c-myc gene used as a recognition sequence by anti-C-terminal human c-myc mAb 9E10. This mutant lacks the transactivation domain of p65/RelA but retains the N-terminal RHD, which is necessary and sufficient for DNA binding as well as dimerization to other members of the Rel family. Expression of p65RHD is driven by a minimal CMV promoter (CMV).
temperature) with 100,000 cpm of double-stranded, [α-32P]dATP radiolabeled NF-κB-specific oligonucleotide (5′-AAATTCAGAGGGGATT TCCCAAGG-3′) derived from the human κ light chain promoter or with [γ-32P]dATP radiolabeled NF-κB oligonucleotide (5′-AGTTGA GAATTTCCAGGG-3′) and the resulting DNA/protein complexes were separated on a 5% polyacrylamide gel in Tris/glycine/EDTA buffer at pH 8.5. Binding of AP-1 to the AP-1 DNA-binding consensus was carried out using 100,000 cpm of double-stranded [γ-32P]dATP radiolabeled AP-1 oligonucleotide (5′-CTGACTCATCTCGA-3′). The mass of the probes was approximately 20 fmol.

FIGURE 3. Inhibition of NF-κB binding to NF-κB oligonucleotides in nuclear extracts from recombinant adenovirus-transduced HUVEC. A, Expression of IκBα in recombinant adenovirus-transduced HUVEC. IκBα was detected by Western blot in whole cell extracts of noninfected (NI), β-galactosidase (β-gal), IκBα (IκBα), and p65RHD (p65RHD)-recombinant adenovirus-transduced HUVEC, unstimulated (−) or 30 min after TNF-α stimulation (+), as described in Materials and Methods. Notice the overexpression of IκBα protein in IκBα-transduced HUVEC. Notice as well that overexpressed IκBα protein was not degraded upon TNF-α stimulation. B, Detection of NF-κB and AP-1 consensus-specific DNA binding in adenovirus-transduced HUVEC. Specific NF-κB and AP-1 consensus DNA binding was detected by EMSA in noninfected (NI), β-galactosidase (β-gal), IκBα (IκBα), and p65RHD (p65RHD)-recombinant adenovirus-transduced HUVEC, unstimulated (−) or 30 min after TNF-α stimulation (+), as described in Materials and Methods. Specificity of the assay for the consensus analyzed was tested by incubation of free radio labeled consensus oligonucleotide in the absence of nuclear extracts (lane I), by DNA-binding competition with 50 ng of nonradiolabeled oligonucleotide (lane II), and by competition with 50 ng of nonradiolabeled irrelevant oligonucleotide (lane III). Competition controls were carried out using nuclear extracts from TNF-α-stimulated (50 U/ml, 30 min) HUVEC. Notice specific DNA binding of endogenous NF-κB in noninfected or β-galactosidase-transduced HUVEC after TNF-α stimulation. Identity of the different NF-κB heterodimeric proteins was carried out by incubation of the protein extracts with Abs specific for p65, p50, or c-Rel as previously described (13) (data not shown). The higher m.w. protein/DNA complex was composed mainly by p65/p50 heterodimers and the lower m.w. complex by p50 homodimers. Notice the suppression of endogenous NF-κB translocation in HUVEC transduced with recombinant IκBα adenovirus and the constitutive binding of p65RHD to NF-κB but not AP-1 DNA-binding consensus in HUVEC transduced with recombinant p65RHD adenovirus. Notice, as well, that DNA binding of endogenous NF-κB was not observed under overexpression of p65RHD. As previously described, p65RHD binds to DNA mainly in the form of the p65RHD homodimers (13).

Results

Evaluation of effects of IκBα and p65RHD-recombinant adenovirus over expression

In order to achieve expression of p65RHD in vitro in essentially all EC, we constructed a recombinant adenovirus for p65RHD, as previously described for IκBα (Fig. 1) (14). Transduction of PAEC with increasing numbers of plaque-forming units per cell (pfu/cell) of the p65RHD-recombinant adenovirus led to a dose-dependent expression of p65RHD protein (Fig. 2A). Maximal expression was obtained at a MOI of 500 pfu/cell (Fig. 2A) and of 100 pfu/cell in HUVEC (data not shown). p65RHD protein was detected 1 day after infection and reached maximal levels at 2 days, declining thereafter but persisting at significant levels at least until day 5, the last time point analyzed (Fig. 2B). Adenovirus-mediated transduction of p65RHD resulted in protein expression in the cytoplasm and in the nucleus of virtually 100% of HUVEC, as analyzed by immunocytochemistry (Fig. 2C). Similar results were obtained in PAEC (data not shown).

We compared the effects of adenovirus-mediated p65RHD or IκBα overexpression on IκBα degradation and endogenous NF-κB DNA binding following stimulation of HUVEC with TNF-α. We have previously suggested that p65RHD interferes with NF-κB function by competitive inhibition of DNA binding and have shown that expression of p65RHD does not interfere with the degradation of IκBα (13). As such, stimulation with TNF-α resulted in rapid proteolysis of IκBα (Fig. 3A) in noninfected β-galactosidase or p65RHD-transduced HUVEC. This was correlated with rapid nuclear translocation and DNA binding of endogenous NF-κB as tested by EMSA (Fig. 3B) and by Western blot (data not shown). In HUVEC overexpressing IκBα, there was no...
detectable IκB degradation upon TNF-α stimulation and, consequently, no nuclear translocation of endogenous NF-κB (Fig. 3A). Overexpression of p65RHD in HUVEC resulted in specific DNA binding of p65RHD, which was not significantly altered following TNF-α stimulation (Fig. 3B). Presence of high levels of p65RHD in these cells presumably competes with endogenous NF-κB for DNA binding since there was no detectable DNA binding of endogenous nuclear NF-κB in HUVEC transfected with p65RHD, while endogenous NF-κB was detected by Western blot (data not shown). Overexpression of IκBα or p65RHD did not alter DNA binding of AP-1, another transcription factor present in HUVEC (Fig. 3B).

Adenovirus-mediated overexpression of p65RHD was as effective as IκBα at inhibiting the up-regulation of several NF-κB-dependent proinflammatory genes induced by TNF-α in PAEC (Fig. 4). Induction of E-selectin, ICAM-1, IL-8, and iNOS was inhibited by more than 90 to 95% upon overexpression of either p65RHD or IκBα, as compared with nontransduced or β-galactosidase adenovirus-transduced PAEC. Inhibition of endogenous IκBα induction, which is also NF-κB dependent, by p65RHD was

**FIGURE 5.** Inhibition of NF-κB-dependent gene expression by p65RHD does not sensitize EC to undergo TNF-α-mediated apoptosis. A. Inhibition of NF-κB by p65RHD adenovirus-mediated transduction does not sensitize to TNF-α-mediated apoptosis. Expression of VCAM-1 was used as a marker of NF-κB-dependent gene expression to assess the level of NF-κB-mediated gene transcription upon adenovirus-mediated overexpression of p65RHD or IκBα in HUVEC. Detection of VCAM-1 was carried out by flow cytometry as described in Materials and Methods. Open histograms represent nonstimulated HUVEC and hatched histograms represent HUVEC stimulated with 50 U/ml of TNF-α for 6 h. Notice the complete inhibition of VCAM-1 expression in TNF-α-stimulated p65RHD and IκBα-transduced HUVEC as compared with TNF-α-stimulated β-galactosidase or noninfected cells. Expression of phosphatidyl-serine on the surface of apoptotic PAEC was used as an early marker of apoptosis. Detection of phosphatidyl-serine was carried out using FITC-labeled annexin V by flow cytometry as described in Materials and Methods. Open histograms represent nonstimulated PAEC and hatched histograms represent PAEC stimulated with 50 U/ml of TNF-α for 6 h. Notice induction of phosphatidyl-serine expression in TNF-α-stimulated IκBα adenovirus-transduced PAEC as compared with p65RHD, β-galactosidase, or noninfected PAEC. B. Comparison of p65RHD vs IκBα overexpression in terms of sensitization of EC to TNF-α-mediated apoptosis. Viability of recombinant adenovirus-transduced PAEC or HUVEC was measured by a colorimetric assay based on the uptake of the Crystal Violet dye as described in Materials and Methods. Gray histograms represent untreated cells and black histograms represent cells treated with TNF-α (50 U/ml, 6 h). Results shown are mean ± SD. C. Transient overexpression of IκBα, but not p65RHD, sensitizes 2F-2B EC to TNF-α-mediated apoptosis. Transient transfections were carried out as described in Materials and Methods. All cells were transfected with β-galactosidase (300 ng). The p65RHD and IκBα vectors were used at 1000 ng and total DNA was adjusted to 2 μg using the pCDNA3 vector. Transfected cells expressing the β-galactosidase gene were stained with X-Gal and the number of “blue cells” retaining a normal morphology was counted as described in Materials and Methods. Gray histograms represent untreated cells and black histograms represent cells treated with TNF-α (50 U/ml, 16 h). Results shown are mean ± SD.
also greater than 95% as compared with nontransduced or β-galactosidase adenovirus-transduced PAEC (Fig. 4).

IkBa but not p65RHD sensitizes to TNF-α-induced apoptosis

Overexpression of either p65RHD or IkBa in HUVEC resulted in total repression of proinflammatory gene induction by TNF-α at both the mRNA level (Fig. 4) and at the protein level, as demonstrated for VCAM-1 (Fig. 5A). Similar results were obtained at the protein level for other proinflammatory genes such as E-selectin (data not shown). Given the apparent equality of p65RHD and IkBa in terms of inhibiting NF-κB-dependent gene expression, we tested the ability of these inhibitors to sensitize PAEC to TNF-α-mediated apoptosis by analyzing translocation of phosphatidylserine into the outer leaflet of the plasma membrane of apoptotic EC, an early marker of EC apoptosis (32). Transduction of PAEC with IkBa-recombinant adenovirus-sensitized cells to TNF-α-mediated apoptosis, while nontransduced β-galactosidase or p65RHD-transduced cells did not undergo apoptosis when stimulated with TNF-α (Fig. 5A). Similar results were obtained using a viability assay based on the staining of living EC with Crystal Violet (Fig. 5B). PAEC transduced with p65RHD did not undergo apoptosis when stimulated with TNF-α, while PAEC transduced with the IkBa-recombinant adenovirus did undergo apoptosis (Fig. 5B). Similar results were obtained using a transient transfection assay in 2F-2B EC line. Only about 30% of 2F-2B EC transfected with IkBa survived TNF-α challenge, whereas essentially all cells transfected with the p65RHD or an empty control plasmid survived the same treatment (Fig. 5C). Similar results were obtained using HUVEC (Fig. 5B). However, contrary to PAEC and 2F2B, HUVEC transduced with p65RHD showed some level of cell death upon TNF-α stimulation, which was, nevertheless, threefold lower as compared with HUVEC transduced with the IkBa adenovirus (Fig. 5B).

Both IkBa and p65RHD inhibit the induction of anti-apoptotic genes

The presumed reason why expression of IkBa sensitizes EC to TNF-α-mediated apoptosis is that NF-κB-dependent anti-apoptotic genes, such as A20, A1, MnSOD, and c-IAP-2, are repressed through blockade of NF-κB-mediated gene transcription. A differential effect of IkBa and p65RHD on blocking the up-regulation of these anti-apoptotic genes would explain the differential effects of these two inhibitors on sensitizing EC to apoptosis. However, overexpression of p65RHD was equally effective as compared with IkBa at inhibiting the up-regulation of the NF-κB-dependent anti-apoptotic genes A20, A1, and MnSOD in HUVEC (Fig. 6). In addition, Stroka et al. in our laboratories have recently shown that the inducible anti-apoptotic gene, A1, is NF-κB-dependent and we have found that the induction of A1 is equally well suppressed by p65RHD and IkBa (Fig. 6). The expression of other anti-apoptotic genes such as bcl-xL, which is constitutively expressed in HUVEC, was not significantly affected by either p65RHD or IkBa overexpression (Fig. 6). We have also tested the effect of p65RHD and IkBa overexpression on the expression of the proapoptotic genes bax and bak, which are constitutively expressed in HUVEC as tested by Western blot: the expression of these genes was unaffected by either p65RHD or IkBa overexpression (data not shown).

p65RHD inhibits sensitization to TNF-α-mediated apoptosis by IkBa

To probe whether the lack of sensitization to TNF-α-mediated apoptosis involved an active “protective mechanism” induced by overexpression of p65RHD, we tested whether expression of p65RHD would protect EC from sensitization to apoptosis by overexpressing IkBa. Our hypothesis was that if overexpression of p65RHD was regulating an anti-apoptotic mechanism (such as by altering the expression of a given anti- or pro-apoptotic gene) then overexpression of p65RHD should protect EC transfectected with IkBa from undergoing apoptosis when stimulated by TNF-α. We first tested if cotransfection of full length p65 with IkBa, at a ratio allowing nuclear translocation, DNA binding, and transcriptional activity of p65, rescued EC from TNF-α-mediated apoptosis. As illustrated in Figure 7A, p65 rescued EC-overexpressing IkBa from TNF-α-mediated apoptosis. This result was consistent with both nuclear localization (Fig. 7B) and DNA binding (Fig. 7C) of p65, despite the presence of high levels of IkBa in the cytoplasm (Fig. 7B). These data show that 2F-2B EC behave in a manner similar to other cell types with respect to induction of apoptosis by TNF-α in the absence of NF-κB (16–18).

As suggested above, the likely interpretation of this result is that p65 restores the expression of NF-κB-dependent anti-apoptotic genes and thus protects EC from TNF-α-mediated apoptosis. Transient cotransfection of a DNA-binding-deficient mutant of p65 with IkBa failed to protect EC from TNF-α-induced apoptosis (Fig. 7A). These data suggest that p65 DNA binding and/or transcriptional activity are necessary to rescue EC from TNF-α-induced apoptosis. We then tested the ability of p65RHD to rescue 2F2B cells overexpressing IkBa from undergoing TNF-α-induced apoptosis. When overexpressed with IkBa, at a ratio allowing nuclear localization and DNA binding, p65RHD rescued EC overexpressing IkBa from TNF-α-induced apoptosis (Fig. 7). These data show that the anti-apoptotic effect (protective activity to apoptosis of p65RHD) is dominant over the IkBa-mediated sensitization. The findings also suggest that the reason p65RHD does not sensitize EC to apoptosis is not because it fails to block an anti-apoptotic gene that is inhibited by IkBa. Rather, they suggest...
that p65RHD has an additional active function as compared with IκBα, and that it is this additional function that is responsible for p65RHD-mediated protection from apoptosis. Transient cotransfection of a DNA-binding-deficient derivative of p65RHD with IκBα failed to protect EC from TNF-α-induced apoptosis (Fig. 7A). These data suggest that the ability of p65RHD to protect EC from apoptosis involves DNA binding but not transcriptional activation of anti-apoptotic genes.

**p65RHD, but not IκBα, represses basal c-myc expression**

In an effort to explain these findings, we studied the effects of p65RHD and IκBα on expression of c-myc, which has been implicated in the regulation of apoptosis in various cell types (19–21). In our studies, overexpression of p65RHD inhibited c-myc as studied at the mRNA level, whereas IκBα did not (Fig. 8A). This suppressive effect of p65RHD on c-myc expression was dose dependent (Fig. 8B). It is possible that p65RHD suppresses the expression of c-myc at the transcriptional level through direct binding of p65RHD to NF-κB consensus sites in the c-myc promoter (33). Binding of p65RHD may compete with other transcription factors involved in the basal expression of this gene and thus would result in down-regulation of c-myc mRNA expression. This hypothesis is currently being tested.

**Ectopic expression of c-myc in EC in which NF-κB is inhibited sensitizes these EC to TNF-α-mediated apoptosis**

Given that expression of c-myc in some cell types correlates with the induction of apoptosis (19–21), we questioned if the ability of p65RHD to suppress c-myc might be responsible for protection from TNF-α-mediated apoptosis. The data shown in Figure 9A support this notion. Murine 2F-2B EC are resistant to TNF-α-induced apoptosis when transiently transfected with empty plasmid (pCDNA3) or with p65RHD. Expression of c-myc alone in 2F-2B cells failed to sensitize to TNF-α-mediated apoptosis (Fig. 9A). However, when both p65RHD, which inhibits up-regulation of the anti-apoptotic genes (Fig. 6), and c-myc were transiently overexpressed, 2F-2B EC were sensitized to TNF-α-mediated apoptosis (Fig. 9A). This effect was dose dependent, i.e., higher amounts of c-myc induced higher levels of sensitization to TNF-α-mediated apoptosis (Fig. 9B). We conclude that c-myc expression may be necessary for the induction of apoptosis by TNF-α in EC under conditions in which NF-κB activity is repressed and anti-apoptotic genes are not induced.

We also tested whether the mechanism by which p65RHD protected EC from sensitization to apoptosis by IκBα involved down-regulation of c-myc. The data presented in Figure 9C demonstrate...
that this is the case. Murine 2F-2B EC were transiently transfected with IxBo and p65RHD, with or without ectopic expression of c-myc. In the absence of c-myc overexpression, p65RHD protected EC from sensitization to apoptosis by IxBo (Fig. 9C). However, when c-myc was overexpressed, EC became sensitive to apoptosis when challenged with TNF-α (Fig. 9C). This suggests that under conditions that suppress NF-κB, such as by overexpressing IxBo plus p65RHD, expression of c-myc is necessary for the induction of apoptosis by TNF-α through a mechanism that we still do not understand. This finding has potential general relevance to the use of inhibitors of NF-κB in situations in which apoptosis is undesirable.

Overexpression of p65RHD does not alter cell cycle progression in confluent EC

We tested whether suppression of expression of c-myc protected EC from TNF-α-mediated apoptosis by influencing a gene involved in cell-cycle regulation, which has been demonstrated to be critical for induction of apoptosis in EC (34); if p65RHD blocked progression through the cell cycle, this would presumably protect EC from apoptosis. Given the above we tested the effect of p65RHD overexpression on cell-cycle progression. As expected, the majority of nontransduced confluent PAEC was found to be in the G0/G1 phase of the cell cycle (>90%), as tested by nuclear propidium iodide staining (Fig. 10). Transduction of PAEC with β-galactosidase, IxBo, or p65RHD-recombinant adenoviruses did not alter significantly the percentage of cells in each phase of the cell cycle, i.e., G0/G1, S, G2/mitosis (Fig. 10). These data indicate that differential sensitization of EC transduced with IxBo or p65RHD to undergo apoptosis likely does not involve differential cell-cycle progression.

Discussion

The interrelationship between the proinflammatory aspects of EC activation, involving the action of NF-κB, and apoptosis has to
incubation, one would wish to inhibit NF-κB, which we and others have previously shown is key to the induction of the proinflammatory genes in EC (14, 38). However, when IκBα is used to suppress this aspect of EC activation, the EC are sensitized to TNF-α-mediated apoptosis (Figs. 5 and 7) (15). Recent experiments involving inhibition of NF-κB by IκBα in other cell systems have yielded similar results (17). Apoptosis of EC, which is involved in the pathogenesis of septic shock (39) and is a common feature of the pathology of xenograft rejection as well as xenograft arteriosclerosis (15), is highly undesirable in a clinical setting. Induction of EC apoptosis leads to cell retraction and exposure of the subendothelium, which promotes platelet aggregation and thrombosis. Furthermore, apoptotic EC have been shown to become prothrombotic (32) and may activate complement through C1q, further enhancing a prothrombotic environment (40).

As an alternative to IκBα, we have developed a dominant negative mutant of p65/RelA, one member of the Rel/NF-κB family that is intimately involved in proinflammatory gene induction during EC activation. We refer to the mutant as p65RHD, since the C-terminal (amino acid aa) 321–551 transactivation domain was deleted, but the RHD (aa 2–320) has been retained. The p65RHD mutant contains the region from the wild-type p65/RelA that is responsible for binding to IκBα. p65RHD retains the ability to bind to IκBα (data not shown). Detection of high levels of nuclear p65RHD in nonstimulated endothelial cells (Fig. 2) probably results from “saturation” of the “available pool” of endogenous IκBα molecules present in the cytoplasm of these cells (Fig. 2). Under these conditions, the “excess” of p65RHD; which is not retained in the cytoplasm, translocates to the nucleus (Fig. 2). In the presence of nuclear p65RHD, DNA binding of endogenous NF-κB is inhibited (Fig. 3). Given that overexpression of p65RHD does not inhibit IκBα degradation (Fig. 3) or nuclear NF-κB translocation (data not shown), we conclude that the presence of high levels of nuclear p65RHD competes with endogenous NF-κB for DNA binding, resulting in suppression of DNA binding of endogenous NF-κB.

Overexpression of p65RHD suppresses the transcriptional activity of NF-κB and inhibits the induction of proinflammatory genes equally as well as does IκBα (13) (Figs. 4 and 5). However, overexpression of p65RHD does not sensitize EC to TNF-α-mediated apoptosis (Fig. 5) despite the finding that p65RHD, like IκBα, suppresses the induction of the anti-apoptotic genes, A20, A1, and MnSOD (Fig. 6) (Stroka et al., unpublished data). There are at least two possible interpretations for the difference between IκBα and p65RHD in terms of sensitizing EC to TNF-α-mediated apoptosis. Expression of p65RHD may fail to suppress one or more anti-apoptotic genes, known or unknown, which are suppressed by IκBα, and thus EC would still remain resistant to TNF-α-mediated apoptosis. Alternatively, it may be that p65RHD itself exerts an additional function, as compared with IκBα, which results in protection against apoptosis. To test these possibilities, we coexpressed p65RHD and IκBα in EC and analyzed the ability of TNF-α to induce EC apoptosis. The fact that in this situation the cells remained resistant to apoptosis (Figs. 5, 7, and 9) suggests that the difference is not related to the failure by p65RHD to suppress anti-apoptotic genes and supports the concept that p65RHD actively provides protection. We have previously shown that overexpression of p65RHD does not bind all of the available IκBα that is in the cytoplasm, and allows IκBα to still prevent nuclear translocation of the NF-κB heterodimer, p50/p65 (13).
observation that lymphocytes lacking the TNF receptor-associated molecule TRAF-2 retain the ability to activate NF-κB, and presumably to induce the expression of NF-κB-dependent anti-apoptotic genes, but undergo apoptosis in the presence of TNF-α (41, 42). These authors thus suggest that NF-κB-dependent anti-apoptotic genes may regulate induction of apoptosis by TNF-α. Our data indicate that, at least in EC, expression of c-myc regulates the induction of apoptosis by TNF-α, providing a “go-signal” that allows the induction of apoptosis in the absence of NF-κB-dependent anti-apoptotic genes. The exact mechanism by which c-myc promotes TNF-α-mediated apoptosis remains to be established. While several reports have suggested that this mechanism may involve the up-regulation of the proto-oncogene p53 (21, 43), it has also been suggested that c-myc may be directly or indirectly involved in the up-regulation and surface expression of the TNF receptor family member, Fas ligand (FasL/CD95L) (44, 45). In cells in which Fas (CD95) is constitutively expressed, up-regulation of FasL would lead to apoptosis through Fas ligation (44, 45). However, ligation of Fas has been suggested to be insufficient to induce apoptosis in EC (46) and thus sensitization of EC to TNF-α-mediated apoptosis by c-myc may not involve the Fas pathway. It has also been hypothesized that the expression of c-myc in certain cell types may constitutively down-regulate the expression of one or several anti-apoptotic genes regulating the induction of apoptosis by members of the TNF receptor family (44). Taking this into consideration, down-regulation of c-myc would allow these anti-apoptotic genes to be expressed and thus would be associated with a protective phenotype. We favor this hypothesis as the mechanism involved in protection of EC from TNF-α-mediated apoptosis by c-myc down-regulation. Our preliminary data indicate that, contrary to other cell types, c-myc expression in EC may not be regulated by NF-κB. This is supported by the observation that c-myc is constitutively expressed in quiescent EC in the absence of nuclear NF-κB. Furthermore, TNF-α, which mediates nuclear NF-κB translocation, does not up-regulate c-myc expression in EC (data not shown). However, the c-myc promoter contains two κB-binding sites to which p65RHD may potentially bind. It is therefore possible that binding of p65RHD to these κB sites may compete with other transcription factors involved in the constitutive expression of c-myc in EC and thus would result in c-myc down-regulation.

In conclusion, our data demonstrate that p65RHD is a dominant-negative mutant that can be used to suppress NF-κB specifically without the undesirable effect of sensitizing EC to TNF-α-mediated apoptosis. This makes p65RHD a candidate transgene that could be expressed in EC of a porcine organ that is to be transplanted to a human. Expression of p65RHD would prevent the response caused by induction of the proinflammatory genes involved in the pathogenesis of delayed xenograft rejection without the complications of rendering the EC susceptible to apoptosis induced by TNF-α, a cytokine present at essentially all sites of inflammation.

Acknowledgments

We thank D. Rainer de Martin, D. stroka, and A. Badrichami for supplying reagents used in this study; Vilmos Csizmadia and members of our laboratory for helpful discussion; Eva Csizmadia for assistance in cell culture; and Dr. Gail Sonenschein for advice and helpful discussion.

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