A20 Blocks Endothelial Cell Activation through a NF-κB-dependent Mechanism*

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The A20 gene product is a novel zinc finger protein originally described as a tumor necrosis factor α (TNF)-inducible early response gene in human umbilical vein endothelial cells (HUVEC). Its described function is to block TNF-induced apoptosis in fibroblasts and B lymphocytes, but more recently it has also been shown to play a role in lymphoid cell maturation. The mechanism of action of A20 is unknown. The aim of our study was to assess the effect of A20 upon endothelial cell activation. By transfecting bovine aortic endothelial cells (BAEC) with A20 as well as reporter constructs consisting of the promoters of genes known to be up-regulated during endothelial cell activation, i.e., E-selectin, interleukin-8 (IL-8), tissue factor (TF), and inhibitor of nuclear factor κB (IκBα), we demonstrate that A20 expression inhibits gene up-regulation associated with TNF, lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), and hydrogen peroxide (H2O2)-induced endothelial cell (EC) activation. The mechanism of action of A20 is in part, or totally, due to the blockade of nuclear factor κB (NF-κB), as shown by its ability to suppress the activity of a NF-κB reporter. This effect is specific, as A20 does not block a noninducible, constitutively expressed reporter, Rous sarcoma virus-luciferase (RSV-LUC); nor does it block the c-Tat-inducible, NF-κB-independent reporter, human immunodeficiency virus-chloramphenicol acetyltransferase (HIV-CAT). How A20 blocks NF-κB is unclear, although we demonstrate that it does not affect p65 (RelA)-mediated gene transactivation. The inhibition of endothelial cell activation by A20 is a novel function for A20.

A20, a novel zinc finger protein that was originally identified as a TNF-1-inducible gene product in HUVEC (1), is expressed in a variety of cell types (2) in response to a number of stimuli such as IL-1, CD40 cross-linking, Epstein-Barr virus latent membrane protein 1 as well as other stimuli (1, 3–5). The only described function for A20 is its ability to protect cells from TNF-induced apoptosis. The expression of A20 in fibroblasts, B lymphocytes, WEHI 164, and NIH 3T3 cells confers resistance to TNF-induced apoptosis (2, 3). Furthermore, breast carcinoma cell lines that are resistant to TNF-induced cytotoxicity express A20 constitutively (2). The mechanism by which A20 blocks apoptosis is unknown. Other suggested functions for A20 include a role in the maturation and differentiation of lymphoid cells (3).

Given that A20 blocks TNF-induced apoptosis, we hypothesized that it might also modulate other TNF responses, particularly EC activation (6). Our work demonstrates that expression of A20 dramatically inhibits TNF-induced EC activation, as shown by the inhibition of E-selectin (an endothelial cell-specific marker) (7, 8), IL-8 (9), TGF (10, 11), and pIκBα (ECI-6) (12, 13) reporter constructs. The inhibitory effect of A20 is not only associated with TNF, but also affects LPS, PMA, and H2O2-induced EC activation. In addition, we show that A20 blocks the induction of a reporter construct that is dependent solely on NF-κB for its expression, demonstrating that the effect of A20 is mediated via the blockade of this transcription factor (14, 15). We suggest that, in EC, A20 functions to protect the cells not only from apoptosis but also from the potentially untoward effects of unfettered activation (16, 17).

MATERIALS AND METHODS

Cell Culture Conditions and Treatment

Bovine aortic endothelial cells were isolated and cultured in Dulbecco’s modified Eagle’s medium, supplemented with L-glutamine (2 mM), penicillin G (100 units/ml), and fetal calf serum (10%). Cells were grown at 37 °C in a humidified incubator with a 5% CO2 atmosphere. Cells were stimulated with either 100 ng/ml LPS (Sigma Escherichia coli 055B5), 100 units/ml of recombinant human TNF (kind gift of Sandoz Pharmaceuticals, East Hanover, NJ), 5 × 10^-8 M of PMA (Sigma), or 300 μM of H2O2 (Sigma).

Metabolic Radiolabeling and Immunoprecipitation of A20

To check for A20 expression in BAEC, transfected cells were washed twice with cysteine and methionine-free medium (ICN, Lisle, IL), and then placed in the same medium supplemented with 100 μCi/ml of L-[35S]methionine and incubated at room temperature for 30 min before being added to the cells in triplicate. In all experiments (except those with HIV-wild type [wt] reporter) 0.3 μg of the β-galactosidase (β-gal) reporter was used, with 0.5 to 0.7 μg of A20 or pAC, and 0.6 to 0.7 μg of the E-selectin, IL-8, TGF, EC1-6, NF-κB, or RSV (Rous sarcoma virus)-luciferase (LUC) reporters.

In those experiments involving the induction of the ECI-6 (IκBα) reporter...
β-Galactosidase, Luciferase, and CAT Assays

Cellular extracts were assayed for β-gal activity per the Tropix, Inc. Galacto-Light protocol (Tropix, Inc, Bedford MA). Luciferase activity was assayed by adding 10 µl of cellular extract to 90 µl of a solution containing 24 µM glycglyglycine (pH 7.8), 2 mM ATP (pH 7.5), and 10 mM MgSO4. Samples were read on the Microlumat LB 96P luminometer (EG & G Berthold) using an injection mix consisting of 24 mM glycglyglycine and 0.1 mM luciferin (Boehringer Mannheim).

The CAT assay was performed for the HIV-wt reporter by means of a standard method using a Promega kit (Promega, Madison, WI). Briefly, cell extracts were incubated in a reaction mix containing [14C]chloramphenicol and 0.1 mM luciferin (Boehringer Mannheim). The reaction products were then extracted with a small volume of xylene. After two back-extractions, a portion of the xylene phase was mixed with scintillation liquid and counted in a scintillation counter (1900TR, Packard, Downes Grove, IL).

Reporter Constructs

E-selectin Reporter—The reporter construct used was made by one of the authors (C. B.) and represents bp -1286 to +484 of the porcine E-selectin promoter. This region includes the first complete intron and exon, as well as the beginning of the 2nd exon up to the ATG site. -1286 represents a NdeI site; +484 marks the position of the porcine (p) E-selectin translation start site. Just 3’ to the start ATG site a 3-bp insertion was made creating an additional NdeI site. The promoter was cloned into the pMAMneo-LUC plasmid vector by replacing the mmTV promoter (Clontech, Palo Alto, CA).

IL-8 Reporter—A gift from E. Hofer, VIRCC, Vienna, it represents the human IL-8 promoter linked to the luciferase gene (p-UBT LUC).

Tissue Factor Reporter—The construction of this reporter has been previously described (11), and represents a 4000 to +34 fragment of the porcine TF promoter cloned into a luciferase expression vector (p-UBT LUC).

ECI-6 Reporter—The construction of this reporter has been previously described (13). It represents a 600-bp fragment of the porcine ECI-6 promoter ligated into the luciferase expression vector p-UBT (p-UBT-LUC) with the creation of an additional HindIII site.

NF-κB Reporter—This reporter was constructed by one of the authors (A. P.). It consists of four copies of NF-κB elements taken from the porcine E-selectin promoter inserted upstream of a TK minimal promoter driving a luciferase gene. The backbone is a Bluescript KS+ plasmid (Strategene, La Jolla, CA).

HIV-Long Terminal Repeat (LTR)-wt Reporter—The construction of this reporter has been previously described (18). It represents -117 bp to the TATA box start of the HIV-LTR, cloned upstream of the CAT gene (CAT3N polycliniker).

Rsv-β-Gal Reporter—The full-length E. coli β-gal gene (Clontech) was inserted into the pPRUSV vector (Invitrogen, San Diego, CA) at the NotI site.

Rsv-Luciferase Reporter—This reporter was constructed by A. P. and represents the pRC/RSV vector (Invitrogen) associated with the full-length luciferase gene derived from pT3/T7-luc (Clontech).

Expression Plasmids

A20 and pAC—The A20 gene was obtained from V. Dixit and subcloned into the pAC expression vector at the XbaI restriction site. pAC is a 18-kb plasmid vector containing a CMV promoter, a pUC19 polylinker site, and a SV40 splice/poly(A) site (a kind gift of Robert Gerard, University of Texas, Southwestern).

p65—The p65 expression plasmid is a kind gift of Dr. J. Anrather and represents the human RelA (from amino acid 2 to 551) fused to a N-terminal c-myc Tag and cloned into the pcDNA 3 expression plasmid (Invitrogen, San Diego, CA) at the HindIII/XbaI polylinkering sites.

C-Tat—C-Tat is an expression vector encoding the HIV-1 Tat protein that induces in a NF-κB independent manner, HIV-1 LTR-directed transcription (18).
co-transfected with the porcine E-selectin reporter construct as well as either the A20 expression plasmid or the pAC control plasmid in conjunction with the RSV β-gal plasmid. A titration curve was performed of the A20 plasmid with amounts of A20 ranging from 0.125 μg to 0.7 μg/5 × 10^5 BAEC. A20 overexpression led to a significant decrease in the luciferase activity of the E-selectin reporter after both TNF and LPS stimulation (number of experiments, n = 9). In the pAC control, induction with either TNF or LPS led to an 8- and 14-fold increase in the activity of the E-selectin reporter, respectively. A20 expression inhibited TNF- and LPS-induced luciferase activity in a dose-dependent manner (Fig. 2). When 0.125 μg of A20 was used, the inhibition of induction reached 53% (p = 0.03) and 78% (p = 0.01) with TNF and LPS stimulation, respectively (Fig. 2, lane 5 versus 6, 9 versus 10). Inhibition was complete when the amount of A20 used was 0.5 μg and higher, if compared to the basal levels detected in the nonstimulated BAEC transfected with the empty vector (Fig. 2, lane 1 versus lanes 7, 8, 11, and 12). In addition, A20 expression decreased the basal, unstimulated luciferase activity of the E-selectin reporter by 2-fold when the amount of A20 used was 0.5 μg or higher (p = 0.02). These results show that 0.5-0.7 μg/5 × 10^5 BAEC of A20 is the optimal amount needed for maximal inhibition. The A20 dose used to analyze the other reporters was 0.5 μg/5 × 10^5 BAEC.

In the next set of experiments we chose reporters constructed with promoters of genes that, like E-selectin, are up-regulated during EC activation: IL-8, porcine IκBα (ECI-6), and TF. Results demonstrate that A20 expression inhibited the TNF and LPS induction of all three reporters. The luciferase activity of the IL-8 reporter, when co-transfected with pAC alone, increased 2.5- and 2.7-fold after stimulation with TNF and LPS, respectively (Fig. 3A, lane 1 versus 3 and 5). A20 expression inhibited the induction of IL-8 reporter activity after TNF and LPS stimulation to levels below that seen with nonstimulated, pAC-transfected cells (60% below the luciferase activity of unstimulated cells, lane 1 versus 4 and 6 (p = 0.02)) (n = 5). Furthermore, A20 overexpression decreased the basal luciferase activity of the IL-8 reporter by 3-fold (Fig. 3A, lane 1 versus 2) (p < 0.0001).

The results of the co-transfections performed using the porcine IκBα (ECI-6) reporter construct were similar to those seen with IL-8 (Fig. 3B). Induction with TNF and LPS reached 1.6- and 3.6-fold, respectively. Inhibition was complete when A20 was co-transfected. TNF- and LPS-induced luciferase activities were also lower than the basal levels noted with the empty vector (n = 6) (Fig. 3B, lane 1 versus 4 and 6) (p < 0.0001). A20 expression decreased by 5-fold the basal level of ECI-6 luciferase activity (Fig. 3B, lane 1 versus 2) (p < 0.0001). In a comparable manner, A20 expression completely inhibited the 3.5- and 4.5-fold induction of TF reporter activity reached after TNF and LPS stimulation, respectively (Fig. 3C, lanes 3-6) (n = 6). However, unlike the IL-8 and ECI-6 reporters, there was no decrease in basal TF reporter activity with A20 co-expression (Fig. 3C, lane 1 versus 2).

We further demonstrate using the E-selectin reporter construct that A20 expression inhibits both H2O2 and PMA-in-
duced EC activation (Fig. 4A). BAEC were transfected as described previously with the A20 or pAC expression plasmids together with the E-selectin and β-gal reporters. BAEC were stimulated with either PMA at a concentration of $5 \times 10^{-8}$ M for 7 h prior to cell extraction, or with H$_2$O$_2$ at a concentration of 300 μM for 4 h prior to cell extraction. A20 expression totally inhibited the 2- and 2.5-fold induction of E-selectin reporter activity reached after H$_2$O$_2$ and PMA stimulation, respectively (Fig. 4A, lane 3 versus 4 ($p = 0.001$), 5 versus 6 ($p < 0.0001$) ($n = 3$)).

A20 Inhibition of Endothelial Cell Activation via a Mechanism Involving NF-κB Does Not Affect p65 (RelA)-mediated Transactivation of an IκBα (ECI-6) Reporter—BAEC were co-transfected with a NF-κB reporter construct that is solely dependent upon NF-κB, and either A20 or the empty vector, pAC. A20 expression abrogated the 12- and 28-fold induction of reporter activity in response to TNF and LPS, respectively (Fig. 5A, lane 3 versus 4 ($p = 0.0006$), 5 versus 6 ($p = 0.011$)).

We further demonstrate that A20-related inhibition of NF-κB does not affect p65-mediated gene transactivation, since A20 expression in BAEC did not significantly modify the induction of the IκBα reporter by p65 (Fig. 5B, lane 5 versus 6) as compared to TNF-induced activation (Fig. 5B, lane 3 versus 4).

A20 Expression Does Not Affect the Activity of the NF-κB-independent Reporters RSV-LUC and HIV-CAT—RSV-LUC is a constitutive reporter which is not dependent upon NF-κB. Basal luciferase activities of the RSV-LUC reporter were comparable in the A20 and pAC transfected BAEC. No significant induction was achieved upon TNF or LPS stimulation in either the pAC or the A20-expressing cells; luciferase values remained comparable among the 2 groups (Fig. 6A) ($n = 6$). With regard to HIV-CAT, Sp1 binding sites in the HIV-LTR reporter had been previously shown to be crucially involved in Tat-
mediated gene expression (18), representing a means of gene induction independent of NF-κB. Our results show that A20 expression affected neither the basal levels nor the 10–15-fold induction of the reporter observed upon stimulation with c-Tat (Fig. 6B, lane 1 versus lanes 2–4 and lane 1 versus lanes 6–8).

**DISCUSSION**

Endothelial cell activation refers to the changes that EC undergo as a result of cytokine stimulation (TNF, IL-1), inflammatory or infectious conditions, reperfusion injury, or reversion of an allo- or xenograft (6, 16, 17, 20, 21). With activation, the cells change their phenotype, leading to fluid leakage, transmigration of leukocytes across the endothelium, as well as thrombosis. Activation involves the induction of a number of genes including adhesion molecules, cytokines, and prothrombotic molecules, many of which are dependent upon the action of NF-κB (8, 11, 13, 15, 16, 22–24). TNF induces the aforementioned genes of the activated EC, as well as other genes that confer protection against programmed cell death or apoptosis, such as A20 (3, 2, 25). We hypothesized that these genes might also affect other TNF-induced responses, in particular EC activation. To test our hypothesis, we transfected BAEC with reporter constructs consisting of promoters of genes known to be up-regulated during EC activation, i.e. E-selectin, IκBα, IL-8, and TF. Our results demonstrate that expression of A20 prevents gene induction associated with EC activation as shown by the total inhibition of all the above-mentioned reporters. Inhibition was seen when either TNF, LPS, H2O2, or PMA was used to stimulate the EC, pointing to the broad effect of A20 upon inhibiting gene induction. The comparable effect on TNF, LPS, H2O2, and PMA-induced signaling excludes any specific association of the action of A20 with the TNF response or the TNF receptor per se.

The transcriptional factor NF-κB plays a major role in the up-regulation of the above tested reporters (8, 11, 13, 15, 22–24). NF-κB is a ubiquitous transcription factor present in the cytoplasm of resting cells in association with its inhibitor, IκBα, and is involved in the acute phase response of inflammation (26). However other transcriptional factors can bind to the promoters of these genes and are involved in the regulation of their expression, e.g. activating transcription factors and cysc AMP-responsive element for E-selectin and Sp1 for TF (11, 27, 28). To evaluate the specific effect of A20 expression on NF-κB activation, we used a reporter construct dependent solely on NF-κB. Activation of this reporter by either TNF, LPS, PMA, or H2O2 was totally inhibited by expression of A20, demonstrating that the inhibitory effect of A20 on EC activation relates, at least in part and perhaps totally, to the inhibition of NF-κB.

Our results partially contradict those recently reported by Jäättelä et al. (29). In our system, A20 expression abrogates the PMA and the H2O2-induced activation of a NF-κB reporter, in contrast to her results, where no inhibition for either stimulus was seen (29). In addition, we show total inhibition of NF-κB reporter induction by A20 expression in BAEC as opposed to the only partial inhibition seen in Jäättelä’s results with the MCF-7S1 cell line. These discrepancies might relate to the type of cells used, i.e. the breast carcinoma cell line MCF-7S1 versus primary cultures of EC. A20 function may differ in different cell types. This is in keeping with reports in the literature showing that A20 protects against serum-starvation induced apoptosis in B cell lines but is not able to do so in the MCF-7S1 breast carcinoma cell line (5, 29).

The mechanism by which A20 affects NF-κB activation remains to be determined. Our data rules out an effect upon p65 (RelA)-mediated gene transactivation, since the induction of an IκBα reporter by p65 is not altered by A20 expression.

To rule out nonspecific or toxic effects of A20 upon the transcriptional machinery, we tested a constitutive, noninducible reporter, RSV-LUC, as well as an HIV-CAT reporter. The HIV-CAT reporter is induced by the viral c-Tat protein through Sp1 binding sites and does not involve NF-κB (18). Expression of A20 had no effect on either the constitutive activity of the RSV-LUC reporter or the c-Tat stimulation of the HIV-CAT reporter, which also demonstrates a lack of effect of A20 on Sp1.

We have demonstrated that, in addition to its ability to protect cells from apoptosis, expression of A20 inhibits NF-κB activation, and presumably based on this effect inhibits gene induction seen with EC activation. This new function places A20 in the same category as IκBα, i.e. genes that are dependent on NF-κB for their induction, but that subsequently inhibit NF-κB and thus EC activation (30–32). Such genes presumably function in negative-regulatory loops to regulate the extent and duration of EC activation.

The molecular basis of the inhibitory effect of A20 on EC activation is unknown. Based on the presence of zinc finger domains, it was previously hypothesized that A20 is a transcription factor that leads to gene induction (2, 33). However, neither nuclear localization nor DNA binding was demonstrated in support of this hypothesis.

One possible mechanism by which A20 could function is as an antioxidant. That A20 could be an antioxidant is consistent with its chemical structure, as the full-length human A20 cDNA encodes 7 Cys2/Cys2 repeats, which give it a high zinc binding capacity (25). Furthermore, zinc can act as an antioxidant (34, 35), and antioxidants such as pyrrolidine dithiocarbamate can prevent the gene induction associated with EC activation through the inhibition of NF-κB (14, 36, 37). However, this hypothesis remains to be tested. Alternatively, the zinc associated with A20 may serve a structural role in stabilizing its functional domain(s) and facilitate its interaction with other proteins (38), e.g. those necessary for NF-κB activation.

We propose that the antiapoptotic effect of A20 and its ability to down-regulate EC activation may both relate to a broader cell “protective” function for this gene. In addition to the rather obvious disadvantages to the cell of apoptotic death, uncontrolled and ongoing activation, involving the accumulation of damaging levels of reactive oxygen species, is clearly undesirable. If this speculation has validity, the presence of such protective genes may play a key role in the homeostatic regulation of endothelium, and perhaps other cellular systems.

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