Communication

Endothelial Cell Death Induced by Tumor Necrosis Factor-α Is Inhibited by the Bcl-2 Family Member, A1

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Endothelial cells play a central role in the inflammatory process. Tumor necrosis factor-α (TNF) is a multifunctional cytokine which elicits many of the inflammatory responses of endothelial cells. While TNF directly causes apoptosis of tumor cells and virally infected cells, normal cells are generally resistant. However, most resistant cells, including human endothelial cells, can be rendered susceptible to TNF by inhibiting RNA or protein synthesis. This finding suggests that TNF provides a cell survival signal in addition to a death signal. We have previously cloned a human Bcl-2 homologue, A1, and shown that it is specifically induced by proinflammatory cytokines but not by endothelial growth factors.

In this study, we show that retroviral-mediated transfer of the A1 cDNA to a human microvascular endothelial cell line provides protection against cell death initiated by TNF in the presence of actinomycin D. The induction of A1 by TNF in this system is mediated via a protein kinase C pathway. Since TNF signaling has also been shown to proceed via ceramides, we tested whether exogenous ceramides could induce A1. Our findings indicate that ceramides do not induce A1 but do up-regulate c-jun and induce endothelial death. Ceramide-activated endothelial death is also inhibited by A1, suggesting that TNF may initiate divergent survival and death pathways via separate lipid second messengers.

TNF is an inflammatory cytokine originally defined by its tumoricidal activity (1, 2). Subsequently, it has been shown to evoke multiple biological responses affecting virtually every cell type (3). Considerable attention has recently been paid to the apoptotic pathway elicited by TNF. A series of elegant experiments have defined a death pathway emanating from the TNF receptor 1. Engagement of the TNF receptor results in cell death by recruitment of a complex of proteins to the cell mem-
supplemented with insulin, transferrin, sodium selenite (Sigma), and 0.1% bovine serum albumin. The PA317 and PE501 packaging lines (27, 29) (provided by A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. All cells were maintained at 37 °C in 5% CO2.

**Gene Transfer**—The coding region of Bcl-XL cDNA (25) (provided by L. Boise and C. B. Thompson, University of Chicago) was ligated into the HindIII/HpaI sites of the replication-deficient retroviral vector pLNCX (27) (provided by A. D. Miller). A FLAG octapeptide N-terminal-tagged human A1 was generated by PCR using the following primers: sense, 5'-CCAGCTAAGCTTCCACCATGGACTACAAGGACGACGATGACAGACGACTGTAATTTGAT-3', antisense, 5'-GGTAAAGAATTCCTCCTGTTCAACGATGTGCT-3', and ligated into the HindIII/HpaI site of pLNCX. The PCR product was sequenced on both strands to confirm the authenticity of FLAG-A1. The viral long terminal repeat drives expression of NeoR while the cytomegalovirus promoter drives transgene expression in pLNCX. Generation of packaging cell lines was performed as described (29). The pLNCBcl-XL, pLNCFLAG-A1 constructs, or pLNCX were transiently transfected into the ecotropic packaging line, PES501, by calcium-phosphate precipitation. Viral supernatants were harvested and used to transduce the amphotropic line PA317 in the presence of 4 μg/ml Polybrene. Polyclonal retrovirus-producing cell lines were obtained by selection in 1 mg/ml G418 (Life Technologies, Inc.). Retroviral supernatants from the PA317 cell lines were used to transduce HMEC-1 cells. Following selection in 200 μg/ml G418 and expansion, HMEC-1 cells were used in survival studies. Polyclonal HMEC-1 lines were used in order to avoid artifacts due to retroviral integration and also because this cell line does not grow in colonies.

**Western Blotting**—Total cellular extracts from the transduced cells were prepared by lysing cells in 1 M Tris, 1 M NaCl, 1% Triton X-100, 1 mM EDTA, pH 7.4, at 4 °C. Samples were solubilized with dimethyl sulfoxide. Absorbance at 630 nM was subtracted with medium containing 1 mg/ml MTT (Sigma) and incubated (30). Briefly, medium was removed and replaced with medium containing 1 mg/ml MTT (Sigma) and incubated for 5 h. The medium was then aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbance at 570 nm was subtracted (to reduce background absorbance) from absorbance at 570 nm for each well.

**Northern Analysis**—Endothelial cells were stimulated for 3 h with the various factors as indicated. Total cellular RNA (15 μg) was separated on agarose-formaldehyde gels, blotted onto nylon filters, and hybridized overnight with random-primed 32P-labeled probes as indicated. The A1 probe was generated by reverse transcription-PCR as described previously (24). The c-jun probe was an approximately 700-bp HindIII/SacII fragment of the murine cDNA (gift of D. Morris, University of Washington, Seattle, WA). The final washing conditions were 0.1 × SSC, 0.1% SDS for 15 min at room temperature. Blots were stripped in boiling water prior to reprobing. A β-actin probe (Clontech) was used to confirm equivalent loading of RNA samples.

**RESULTS AND DISCUSSION**

By virtue of their location between blood and tissue, endothelial cells play a central role in the inflammatory process (31, 32). TNF has multiple effects on endothelial cells ranging from structural reorganization to the up-regulation of adhesion molecules and the elaboration of chemotactic factors (31, 32). Thus, the integrity of the endothelium during inflammation is of great importance. It has previously been shown that human endothelial cells are not susceptible to TNF toxicity unless cocultivated with either cycloheximide or actinomycin D (14). To determine whether human A1, which is inducible by TNF, can abrogate TNF-mediated death, we generated polyclonal FLAG-A1 overexpressing HMEC-1 cell lines. We chose retroviral transduction to achieve overexpression because human endothelial cells are extremely difficult to transfect using standard methods. As a control, we chose to overexpress Bel-2 (25), another member of the Bcl-2 family, since it is constitutively expressed in cultured endothelial cells (Fig. 1B) and may be one of the constitutive TNF cytoprotectants. Also, Bel-2 has been shown to protect the MCF7 breast carcinoma cell line from TNF-induced apoptosis (33). Attempts were made to generate clonal cell lines as well, but HMEC-1 cells do not proliferate in colonies, so all experiments on transduced cells were done with polyclonal cell populations.

When a FLAG-A1 construct was overexpressed in HMEC-1 cells, these cells were protected from TNF cytotoxicity. As shown in Fig. 1A, 18-h incubation of HMEC-1 cells in the presence of TNF at various concentrations and actinomycin D resulted in dose-dependent cell death. The fact that about half of the vector-only transduced cells (HMEC-Neo) were still viable in the presence of TNF at various concentrations and actinomycin D only. Results are the mean ± S.E. of an experiment done in triplicate which is representative of four separate experiments. B, Western blots of the cell lines shown above were probed with the FLAG M2 monoclonal antibody or a Bel-2 monoclonal antibody. C, HMEC-FLAG-A1 or HMEC-Bcl-2L cells were exposed to 50 μg/ml cycloheximide, and levels of the overexpressed protein were monitored at various times by Western blot.

![Figure 1](image-url)
overexpression of Bcl-XL resulted in virtually complete protection against TNF (Fig. 1A). The partial protection conferred by A1 may, in part, be due to the shorter half-life of A1 protein. When HMEC-FLAG-A1 or HMEC-Bcl-XL cells were exposed to cycloheximide (50 μg/ml) to inhibit new protein synthesis, for up to 12 h, FLAG-A1 protein was not detectable after 3 to 6 h whereas Bcl-XL levels remained virtually unchanged (Fig. 1C). Thus, after the first few hours of treatment with TNF and actinomycin D, following degradation of existing RNA and protein, there are probably insufficient levels of A1 to provide protection. Although others have shown that murine A1 inhibits apoptosis following IL-3 withdrawal in an IL-3-dependent myeloid cell line (94), human A1 is not induced by endothelial growth factors but rather by proinflammatory cytokines (24).

Because PMA also induces A1 (24), we wondered whether PKC was involved in transducing the TNF signal to up-regulate A1 expression. Several TNF responses have previously been shown to be mediated via PKC (3). Fig. 2 shows that PMA and TNF induced A1 independently. The highly specific PKC inhibitor, calphostin C (0.1 μM), completely blocked TNF-mediated A1 induction. Additionally, H7, another PKC inhibitor, also partially blocked A1 induction, but HA1004, which at the concentrations used (30 μM) blocks PKA and PKG but not PKC (35), had no effect. These findings suggest that PKC is involved in the signal to up-regulate A1 expression.

TNF can also signal through another lipid second messenger, ceramide, generated by the hydrolysis of sphingolipids (3). Ceramides also have pleiotropic effects on cells, dependent on cell type and the context in which ceramide is generated. In fact, ceramides have been reported to induce both apoptosis and mitogenesis (11, 36, 37). Besides TNF, IL-1, ionizing radiation, and chemotherapeutic agents have also been reported to generate ceramides (3, 38–41). Multiple potential targets of ceramide stimulation have been identified including activation of the atypical PKCζ (36, 42). Hence, we investigated whether exogenous membrane-permeable ceramides could result in A1 mRNA accumulation. Fig. 3 shows that PMA and the non-phorbol PKC activator, mezerein, both induced A1, but the inactive phorbol analogue, 4-b-phorbol, did not. Again this suggests that PKC is involved in A1 induction. The addition of either C2-ceramide or the inactive analogue, C2-dihydroceramide, did not result in A1 accumulation when tested over a wide range of concentrations. Recent studies have shown that ceramide-induced apoptosis is associated with induction of c-jun and activation of c-jun N-terminal kinase in human myeloid cell lines and bovine endothelial cells (41, 43, 44). To determine if c-jun is also up-regulated by ceramides in human endothelial
Haimovitz-Friedman et al. (38) have shown that ionizing radiation acts on bovine aortic endothelial cell membranes to generate ceramides and initiate apoptosis. Others have shown that TNF and IL-1 can generate ceramides in human endothelial cells. Wild-type HMEC-1 cells were exposed to C2- and C2-dihydroceramide for 24 h, and viability was assessed by MTT assays. As described for bovine endothelial cells (39, 40), the accumulation of C2-ceramide or C2-dihydroceramide for 24 h, and viability was assessed by MTT assays. As described for bovine endothelial cells, ceramide induced human microvascular endothelial cell death in this system, the Northern blots were probed with a Jun cDNA, a gift from Dr. H. M. Shepard, H. M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3893–3897.

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