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The Cell Death Regulatory Protein Bak Is Expressed in Endothelial Cells in Inflamed Tissues and Is Induced by IFN-γ *in Vitro*

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In the present report, we examined the endothelial expression of the anti- and pro-apoptotic proteins Bcl-2 and Bak *in situ* and *in vitro.* Endothelial cells (EC) in regular tissue of the bowel and the skin were essentially negative for both Bcl-2 and Bak. In contrast, EC within the walls of fistulas and abscesses in these organs stained distinctly for Bak, but remained Bcl-2-negative. In tissue culture both unstimulated human dermal microvascular endothelial cells (HDMEC) and human umbilical vein endothelial cells (HUVEC) expressed Bcl-2 and Bak constitutively. Exposure of EC to 200–1000 IU IFN-γ downregulated Bcl-2 but upregulated Bak. This opposing regulation of Bcl-2 and Bak *in vitro* and the expression of Bak in EC adjacent to necrotic tissue areas suggests that this pro-apoptotic protein may play a decisive role in regulation of EC survival *in vivo.*

Apoptosis, a morphologically distinct form of programmed cell death, is critical during embryonal development as well as in tissue renewal and homeostasis [1]. In the induction of apoptosis a chain of tightly regulated events which results in the activation of cellular proteases and endonucleases is triggered by external or internal stimuli [2]. Early steps in this process are regulated by members of the bcl-2 family of genes, which may determine the fate of cells by forming homo- and heterodimers at different relative concentrations causing either pro- or anti-apoptotic effects [3, 4].

Apoptosis of EC occurs in blood vessel regression during scar formation [5], in inflammatory processes such as atherosclerosis [6] and progressive glomerulonephritis [7] as well as during transplant rejection [8] and in luteolysis of the ovary [9]. Induction of EC apoptosis has recently attracted considerable interest as a potential target for anti-tumor therapy [10–12] and for treatment of childhood hemangiomas [13–15]. Four anti-apoptotic members of the bcl-2 family, i.e. Bcl-2, Bcl-x, A1 and Mc1-1 as well as the pro-apoptotic protein Bax have been shown to be expressed in EC [16–20] and to be involved in regulating cell death in these cells [18, 21–23]. Since the pro- and anti-apoptotic effects of the respective bcl-2 family members depends on their relative abundance, modulation of their expression represents an important target for angiostatic as well as angiogenic therapy. Recently, inflammatory cytokines have been shown to be involved in the regulation of the expression of bcl-2 family proteins in EC. bFGF [21] and VEGF [24] upregulate Bcl-2, whereas TGF-β downregulates it [25], IFN-γ induces Bcl-x [16] and TNF-α, IL-1β [17] as well as VEGF [24] upregulate A1. Thereby these cytokines may occupy a central role in the regulation of EC survival and death [16, 26, 27].

When investigating the expression of regulators of programmed cell death during tissue inflammation we detected the presence of Bak, a recently described pro-apoptotic member of the bcl-2 family [28], in EC. For the present report we investigated the expression of Bcl-2 and Bak in vessels of regular and inflamed tissues and studied their regulation in EC by IFN-γ *in vitro.*

METHODS

Tissue samples. Formalin-fixed, paraffin-embedded tissue blocks were obtained from the files of the Institute of Clinical Pathology at the University of Vienna. Samples consisted of regular skin (n = 3), regular large (n = 2) and small (n = 3) bowel, fistulas of the small bowel of patients with Crohn's disease (n = 6), fistulas of the colon of patients with diverticulitis (n = 3) and fistulas of the skin (n = 5).

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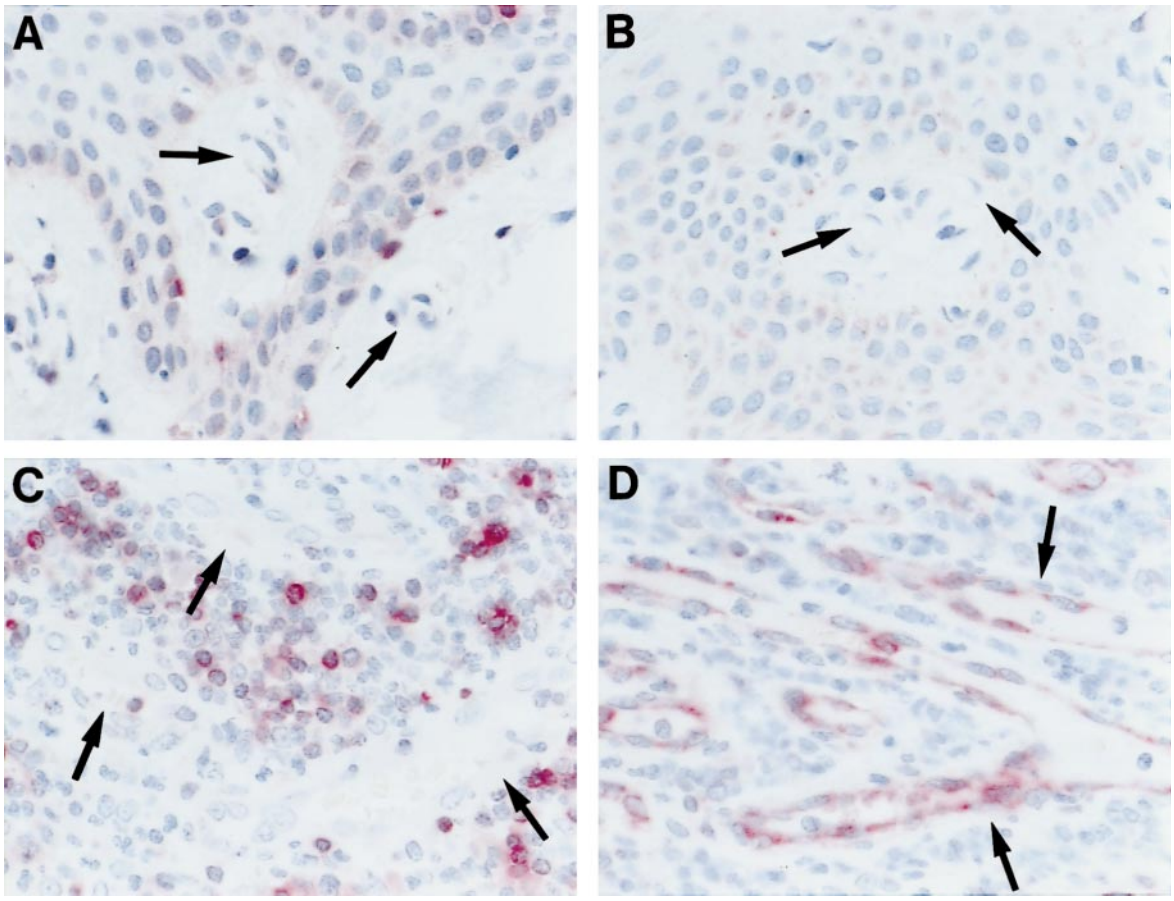

FIG. 1. Expression of Bcl-2 and Bak in endothelial *in situ*. EC of vessels (arrows) in regular tissue of the skin were mostly negative for Bcl-2 (A) and Bak (B). EC of vessels within the walls of fistulas were negative for Bcl-2 (C), but stained distinctly for Bak (D).

Immunohistochemistry. All samples were immunostained for Bcl-2 and Bak expression using monoclonal antibodies (moabs) against Bcl-2 (bcl-2-124, Dako, Glostrup, Denmark) and Bak (TC-100, Calbiochem, Cambridge, MA) as described previously [29]. Briefly, antigen retrieval for Bcl-2 and Bak staining was performed by boiling sections in 0.01 mol/L citrate buffer (pH6) at 450W for 20 min in a microwave oven. Primary antibody was applied at 1/50 dilution (Bcl-2) or 1/40 (Bak) overnight. Bound antibodies were detected by an APAAP kit (Dako). The enzyme reaction was developed with an alkaline phosphatase substrate kit (Vector, Burlingame, CA). Negative controls were carried out on consecutive tissue sections with an isotype-matched control reagents (IgG1 and IgG2a, Coulter, Hialeah, FL).

Apoptotic cells were detected by TdT-mediated dUTP-biotin nick end labeling (TUNEL) using an *in situ* apoptosis detection kit according to the instructions of the manufacturer (ApopTag, Oncor, Gaithersburg, MD) as described previously [29].

Cell culture. HDMEC and HUVEC were prepared and serially cultured on gelatin-coated plastic culture dishes in endothelial cell growth medium (PromoCell, Heidelberg, Germany) or Medium 199 supplemented with 20% fetal calf serum and EC growth supplement as described previously [30]. Cultured EC were used for experiments between passages 4 and 11. Twenty four and 48 hours after the addition of IFN-γ or PMA (see below), EC cultures were monitored by light microscopy for the presence of dead cells.

Western blotting. Confluent endothelial cell monolayers were incubated with 200, 500 or 1000 U/ml IFN-γ (Imukin, Bender Med, Vienna, Austria) or 100 nM PMA (phorbol 12-myristate 13-acetate, Sigma Aldrich, Vienna, Austria). For blocking experiments a moab against IFN-γ (1 μg/ml, IgG2a, Genzyme, Cambridge, MA) was preincubated with the lymphokine for 1 hour. After 48 hours incubation, cells were lysed in 1% NP-40/ PBS supplemented with 1 mM PMSF. Protein concentrations were measured by a Micro BCA Protein Assay Reagent (Pierce, Rockford, IL). Lysates were subjected to SDS-PAGE in 12% gels under reducing conditions. Subsequently the proteins were electroblotted onto a nitrocellulose membrane (Schleicher & Schüll, Germany) at 0.8 mA/cm^2 for 2 hours. The membranes were dried and incubated in blocking buffer (5% non-fat dry milk in PBS) followed by immune overlay with anti-Bcl-2 (0.5 μg/ml) and anti-Bak (1.0 μg/ml) moabs. After washing, bound moabs were detected with HRP-labeled sheep anti mouse-IgG (Amersham Life Science, UK). The immunoreactions were visualized by chemiluminescence using the ECL reagent (Amersham) according to the instructions provided by the manufacturers. After rinsing membranes were exposed for 5 and 60 minutes to a X-OMAT-AR film (Eastman Kodak, NY).

RESULTS

Bak but Not Bcl-2 Expression Is Upregulated in EC within the Walls of Fistulas of Skin and Bowel

By immunohistochemistry EC of blood vessels were mostly negative for Bcl-2 and Bak expression in regular skin (n = 3) (Figs. 1A and 1B, arrows) and bowel

TABLE 1

Expression of Bcl-2 and Bak in Endothelia *in Situ*

	Regular tissue without inflammatory changes		Perinecrotic tissue within the walls of fistulas	
	Bcl-2	Bak	Bcl-2	Bak
bowel	5 neg.	5 neg.	9 neg.	focally endothelia of all 14 sections positive (distinct to strong)
skin	3 neg.	3 neg.	3 neg., 2 focally pos.* (weak)	

* Little inflamed tissue adjacent to the fistulas and abscesses.

tissue (n = 5) (Table 1). EC of vessels within the walls of fistulas and abscesses limiting to necrosis remained negative for Bcl-2 (Fig. 1C, arrows, Table 1) but stained distinctly for Bak (Fig. 1D, arrows, Table 1). Strong Bcl-2 positive cells in figure 1 panel C represent infiltrating leukocytes. EC of vessels in the surrounding inconspicuous tissue again were negative for both proteins (not shown). TUNEL staining did not reveal apoptotic EC within the lumina of capillaries whereas abundant apoptotic cells could be identified within the lumina of the fistulas (data not shown).

Bcl-2 and Bak Are Constitutively Expressed by HUVEC and HDMEC in Vitro and Are Oppositely Regulated by IFN-γ

When studying EC in tissue culture, we found that both unstimulated HUVECs and HDMECs expressed Bcl-2 (Fig. 2A, lanes 1 and 4) and Bak (Fig. 2A, lanes 7 and 10). Incubation of EC with 1000 IU IFN-γ downregulated Bcl-2 (Fig. 2A, lanes 2 and 5) and distinctly upregulated Bak expression (Fig. 2A, lanes 8 and 11). Incubation with PMA distinctly upregulated Bcl-2 (Fig. 2A, lane 3 and 6) in all of 6 experiments (Table 2), whereas Bak was only weakly upregulated (Fig. 2A, lanes 9 and 12) in 4 experiments and no regulation at all was observed in additional 4 experiments (Table 2). The constitutive expression of Bak and Bcl-2 as well as their regulation by IFN-γ was independent of the culture medium used and was observed with both ECGM and M199 medium supplemented with 20% FCS (Table 2).

The effect of IFN-γ on Bcl-2 and Bak expression was completely abrogated when the cytokine was inactivated by microwave treatment before addition to the medium (Fig. 2B, lane 6). To further confirm the specificity of the regulation of Bcl-2 and Bak by IFN-γ, we preincubated the lymphokine with a neutralizing anti-IFN-γ moab for 1 hour prior to the addition to the culture medium. As shown in Fig. 2, both the downregulation of Bcl-2 as well as the induction of Bak could be completely blocked by this moab (Fig. 2B, lane 5). Addition of the neutralizing moab alone had no effect on the expression of Bcl-2 and Bak (Fig. 2B, lane 7).

Monitoring of the EC cultures after addition of IFN-γ or PMA by light microscopy did not show any alterations of the EC monolayers. When compared to nontreated cultures no increase of dead cells was found with any of the stimulation protocols (data not shown).

DISCUSSION

Bcl-2 and Bak influence cell survival with opposing effects-whereas Bcl-2 is able to block apoptotic cell death triggered by different stimuli [31], Bak has been shown to accelerate the rate of apoptosis and antagonize the death suppressing activity of Bcl-2 [32]. The expression of Bcl-2, as well as several other members of the bcl-2 family, has been amply documented in human EC previously [21, 24]. To the best of our knowledge we show in the present report for the first time that Bak

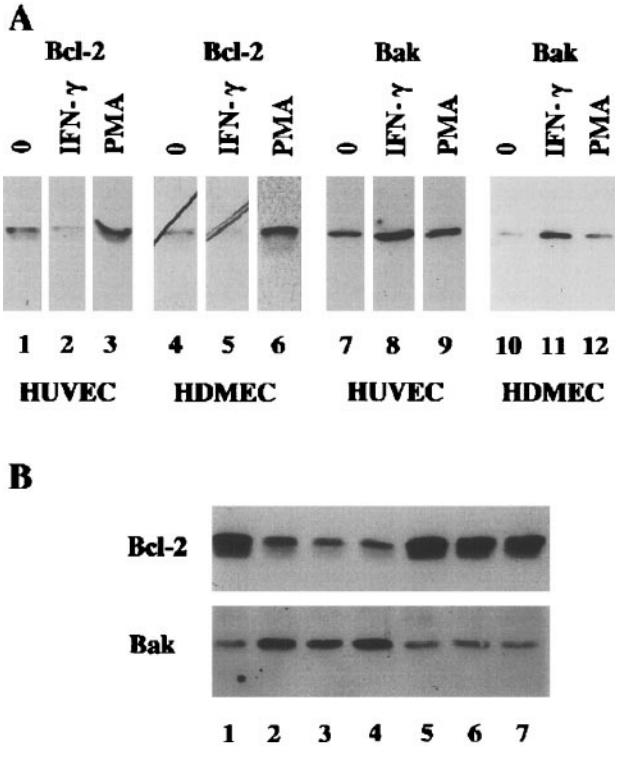

FIG. 2. Bcl-2 and Bak are expressed by HUVEC and HDMEC *in vitro* and are regulated by IFN-γ and PMA. (A) Bcl-2 (lanes 1 and 4) and Bak (lanes 7 and 10) were constitutively expressed by HUVECs and HDMECs. Whereas IFN-γ (1000 U/ml) downregulated Bcl-2 (lanes 2 and 5) and upregulated Bak (lanes 8 and 11), PMA distinctly upregulated Bcl-2 (lanes 3 and 6), but only weakly upregulated Bak (lanes 9 and 12). (B) Both the downregulation of Bcl-2 (upper panel) as well as the induction of Bak (lower panel) by IFN-γ (lanes 2–4, 200–1000 U/ml) were completely blocked by an anti- IFN-γ moab (lane 5). The effect of IFN-γ was also blocked by inactivation of the cytokine by microwave treatment (lane 6). The neutralizing Ab itself did not influence the expression of Bcl-2 or Bak (lane 7).

TABLE 2

Regulation of Bcl-2 and Bak by IFN-γ and PMA in EC *in Vitro*

Cells	Experiment number	Medium	IFN-γ		PMA	
			Bcl-2	Bak	Bcl-2	Bak
HUVEC-1	1	EGM	−−	++	+	+
HUVEC-1	1	EGM	−−	++	+	n.c.
HDMEC-1	1	M199	n.c.	++	++	n.c.
HUVEC-2	2	M199	−	++	++	+
HDMEC-2	2	EGM	−	+	++	+
HUVEC-2	2	EGM	−	++	++	+
HDMEC-3	3	EGM	n.d.	++	n.d.	n.c.
HDMEC-4	4	EGM	n.d.	++	n.d.	n.c.

Note. +, −: weak up- and downregulation: ++, −−: distinct up- and downregulation; n.c.: no change, n.d.: not done.

protein is expressed in these cells *in situ* and *in vitro*. In addition we demonstrate that Bcl-2 and Bak are differentially regulated by IFN-γ and PMA in tissue culture.

Except for very rare vessels, Bcl-2 and Bak were virtually absent from EC in regular tissue whereas in EC of vessels in granulation tissue adjacent to tissue necrosis, Bak but not Bcl-2 was strongly present. The expression of Bak in EC adjacent to necrotic areas indicates that it plays a role in the regulation of EC survival *in vivo*. Although we expected the strong expression of this pro-apoptotic protein to be paralleled by enhanced cell death, we were unable to identify apoptotic cells within vessel lumina by TUNEL staining. However, we cannot exclude that the absence of apoptotic EC is due to their detachment into the vessel lumen. Therefore to address the question as to possible effects of Bak on EC survival more directly we investigated the expression of this pro-apoptotic protein by EC in tissue culture. In contrast to the *in vivo* situation and in analogy to what has been reported for other members of the bcl-2 family including Bcl-2 itself [19, 21], Bak was constitutively expressed by EC growing in tissue culture. Very likely under these conditions its potential pro-apoptotic effect on EC is counterbalanced by the various anti-apoptotic bcl-2 family members coexpressed.

The finding that Bak expression *in situ* was confined to areas of strong inflammation, suggested that cytokines derived from the inflammatory cells play a role in its regulation. Recently, one such proinflammatory cytokine i.e. IFN-γ has been reported both to inhibit the proliferation of EC *in vitro* [33] and to induce apoptosis in a dose dependent manner [12, 34]. Further evidence for a critical role of IFN-γ in the induction of EC apoptosis comes from work by Koide *et al.* who showed that simultaneous administration of anti-IFN-γ antibody in the preparative injection of lipopolysaccharide for induction of a generalized Shwartzman reaction completely blocked apoptosis of vascular EC *in vivo* [35]. These findings prompted us to investigate the effect of IFN-γ on the regulation of Bak and Bcl-2 in EC. Addition of this lymphokine distinctly downregulated Bcl-2 in both HUVEC and HDMEC over a dose range of 200–1000 IU/ml, whereas Bak was strongly upregulated. These data suggest that the apoptotic effects of IFN-γ on EC [34] might be due to the counterregulatory effects of this lymphokine on Bcl-2 and Bak expression. Since as opposed to IFN-γ phorbol esters are able to inhibit EC death [36, 37] we compared its effect on Bak and Bcl-2 expression to that of IFN-γ. In contrast to the latter, PMA strongly upregulated Bcl-2 but had only little effect on Bak expression. Thus Bcl-2 might, together with A1 [17], be involved in the anti-apoptotic effect of phorbol esters in EC.

In conclusion, we have shown that both unstimulated HUVEC and HDMEC expressed Bak *in vitro* and that this pro-apoptotic protein could be distinctly upregulated by IFN-γ. Our data suggest that the counterregulatory effects of IFN-γ and PMA on Bcl-2 and Bak expression might be involved in the pro- and antiapoptotic effect of these two substances.

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