Modulation of Bcl-2 Family Proteins in Primary Endothelial Cells during Apoptosis

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Key Words

Bcl-2 family proteins · Verotoxin · Cytokines · Apoptosis · Endothelial cells

Abstract

We studied the expression of Bcl-2 family proteins during cytokine- and verotoxin (VT)-induced apoptosis in primary human umbilical vein endothelial cells (HUVECs). Our experiments demonstrated that high initial expression of Bcl-2 protein was significantly downregulated in HUVECs treated with IFN- γ whereas TNF- α gave a less pronounced decrease in Bcl-2 level. Treatment with the combination of cytokines was more efficient in downregulating Bcl-2 protein. HUVECs pretreated with cytokines and incubated with VT gave a further significant decrease in Bcl-2 level. Simultaneous measurement of Bcl-xl level did not reveal any significant changes. Bax protein was upregulated in HUVECs stimulated with TNF- α alone or in combination with IFN- γ . However, addition of VT did not give any further increase in Bax level suggesting that Bax upregulation is more important for cytokine- rather than VT-mediated apoptosis. Total endothelial cell growth factor deprivation gave a significant increase in apoptosis accompanied by a decrease of Bcl-2 in apoptotic cells while Bcl-xl and Bax levels were

unaffected. Our data indicate that anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are reciprocally regulated during apoptosis, whilst Bcl-xl is essentially unaffected. This implies that Bcl-2/Bax ratio rather than Bcl-xl controls apoptosis in primary endothelial cells.

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Introduction

The Bcl-2 family consists of proteins that play a crucial role in controlling apoptosis induced by various stimuli [1]. These proteins can form both homo- and heterodimers and consequently they can function either independently or in concert to regulate apoptosis [2]. Dimerisation of Bcl-2 proteins requires interactions between conserved motifs known as Bcl-2 homology domains (BH1–BH4) [3]. Prosurvival members of the family such as Bcl-2 and Bcl-xl possess all four BH domains and both BH1 and BH2 seem to be important for their anti-apoptotic function [1]. Two mechanisms were proposed to explain their anti-apoptotic activity. First, they are thought to stabilise the mitochondrial membrane via interaction with Bax and prevent the release of cytochrome c and subsequent apoptosis [4]. On the other hand, it has been reported that heterodimerisation of Bcl-xl with Bax was

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not always essential for its prosurvival action [5]. Indeed, it was later shown that Bcl-xl can act downstream of Bax activation via inhibition of Apaf-1 and caspase-9 association and block apoptosis after the release of cytochrome c [6].

The pro-apoptotic members are divided into two sub-families – Bax and BH3-only proteins. They all possess BH3 domain that is thought to be essential for their killing function and is normally hidden inside the molecule. Possible mechanisms of Bax activation include Bax dimerisation and translocation to the mitochondria or Bax conversion to an active conformation [7, 8]. It has been shown that after apoptotic stimuli (treatment with staurosporine or TNF- α) Bax protein undergoes Bid-induced conformational change resulting in exposure of its BH3 domain and insertion into the outer mitochondrial membrane followed by cytochrome c release and apoptosis [1].

Apoptosis of endothelial cells is thought to be an underlying mechanism in the development of a number of systemic vascular disorders including vasculitis, diabetic microangiopathy, atherosclerosis and haemolytic uraemic syndrome [9–11]. Haemolytic uraemic syndrome is an age-dependent disease with the majority of cases occurring in children under 3 and in the elderly and is the primary cause of acute paediatric renal failure due to toxic microangiopathy of renal glomerular blood vessels [12]. Endothelial cell damage and inflammation result in the production of large amounts of TNF- α , IFN- γ and other cytokines which influence endothelial cell viability by altering the balance of regulatory molecules that either induce or suppress apoptosis [13].

Verotoxin (VT) produced by *Escherichia coli* O157:H7 are thought to play a decisive pathogenic role in *E. coli* infections although the precise molecular basis of their action is not fully understood [14]. VT has been shown to suppress protein synthesis and mitosis in various cell types eventually causing cell death [15]. The CD77 glycolipid antigen has been identified as the receptor for VT, and binding of CD77 leads to the internalisation and proteolytic cleavage of the toxin resulting in the inhibition of protein synthesis and apoptosis [16, 17]. In recent years, however, another mechanism of cell death has been reported – VT-induced cell death independent of protein synthesis inhibition [15]. Suzuki et al. [14] propose that such mechanism could involve the formation of mitochondrial VT-Bcl-2 complex.

It should be noted, however, that current literature contains very few data regarding the role of Bcl-2, Bcl-xl or Bax proteins in cytokine-mediated apoptosis of primary endothelial cells and no information concerning their

possible involvement in VT-induced endothelial cell death. Our research was aimed to investigate the role of Bcl-2 proteins in apoptosis of normal, primary endothelial cells induced by pathophysiological factors such as pro-inflammatory cytokines, ECGF deprivation and VT.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins by treatment with collagenase and maintained in medium 199 supplemented with 20% fetal calf serum, 20 U/ml heparin (Leo Laboratories), antibiotics and endothelial cell growth factor (ECGF; standard dilution 1:100, Sigma). The cells were seeded into 25-cm² flasks coated with 1% gelatin and grown under 5% CO₂ at 37 °C in medium renewed every 3 days. Confluent cells were detached by trypsin/EDTA and seeded into 6-well (10-cm²) gelatin-coated culture plates with a split ratio 1:3. HUVECs in the experiments were used between 2 and 4 passages.

Apoptosis Experiments

Confluent HUVECs were incubated with 500 U/ml TNF- α or 500 U/ml IFN- γ (Serotec, UK) or with a combination of 500 U/ml of each cytokine. Apoptosis was induced by treatment with 10 ng/ml VT or by ECGF withdrawal. Each experiment was carried out at least 3 times.

Apoptosis was detected using annexin V apoptosis detection kit (Calbiochem) according to the manufacturer's protocol. Labeled cells were immediately analysed by flow cytometry. Viable cells were double negative, early apoptotic cells were positive for annexin V staining and negative for PI staining while late apoptotic cells were double positive.

Bcl-2, Bcl-xl and Bax Staining

Endothelial cells were washed twice in PBS and 100 μ l of suspension containing at least 5×10^5 cells/ml were mixed with 1 ml of 4% paraformaldehyde for fixation. After incubation for 20 min at room temperature in the dark, the cells were washed in PBS and incubated for 1 h in 1 ml of absolute methanol on ice. Permeabilised cells were washed in PBS, stained for 30 min at 4 °C with 10 μ l of the FITC-conjugated anti-bcl-2 (F7053, DAKO), washed again and analysed by flow cytometry. For Bcl-xl and Bax staining we used primary rabbit polyclonal anti-Bcl-xl and anti-Bax (NT) (directed against amino terminal epitope) followed by incubation with FITC-labeled secondary antibody (all from Santa Cruz Biotechnology).

Flow Cytometry

Two-colour flow cytometry was performed using a FACStar flow cytometer with Consort 30 software (Becton Dickinson). Measurement was done at excitation with the 488-nm line of an argon laser. The emission was measured at the fluorescence line of 530 nm with the areas of green (FITC) fluorescence recorded in logarithmic scale. Data were acquired in a list mode with 5,000 cells analysed in each sample. Antigen density was expressed as the mean fluorescence intensity (MFI) compared with control taken for 100%.

For statistical analysis we used descriptive statistics, Pearson correlation, Student's *t* test and Mann-Whitney *U* test.

Fig. 1. Expression of Bcl-2 (a), Bcl-xl (b) and Bax (c) antigens in HUVECs treated with cytokines (500 U/ml) and VT (10 ng/ml) for 48 h. Data are presented as mean $\pm$ SD of four independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. control; + $p < 0.05$ vs. TNF alone; # $p < 0.05$ vs. TNF + IFN.

Results

Bcl-2 Expression

Our experiments showed that the initial high level of Bcl-2 protein was significantly downregulated after HUVECs were treated with IFN- γ for 24 h (76.2%, $p < 0.05$)

Fig. 2. Histograms showing Bcl-2 expression in HUVECs treated with cytokines. Cells were cultured with 500 U/ml of TNF- α or with the combination of TNF- α and IFN- γ (both 500 U/ml) for 48 h. HUVECs were stained with anti-Bcl-2-FITC and analysed by flow cytometry.

while incubation with TNF- α gave less pronounced decrease in Bcl-2 expression. The combination of cytokines was more efficient in downregulating Bcl-2 level (70.8%, $p < 0.01$). Incubation for 48 h produced a slight additional decrease in Bcl-2 expression (see fig. 1a with p values). Bcl-2 histograms in figure 2 taken from one of the representative experiments show a considerable downregulation of bcl-2 level in HUVECs treated with cytokines.

HUVECs pretreated with TNF- α alone or in combination with IFN- γ and incubated with VT showed a further significant decrease in Bcl-2 expression (down to 43.1%, $p < 0.01$; fig. 1a). Of note, HUVECs did not change their Bcl-2 level after incubation with VT alone.

Apoptosis was measured in parallel with Bcl-2 protein level. Earlier we reported that treatment with TNF- α or IFN- γ separately did not induce apoptosis in HUVECs, whereas the combination of cytokines was efficient enough to cause a significant increase in apoptotic cell death [18]. HUVECs pretreated with cytokines and incubated with VT gave the highest levels of apoptosis. Interestingly, we found a close negative correlation between the percentage of apoptotic cells and Bcl-2 expression (r varied in different experiment from -0.66 to -0.78 ; $n = 22$; $p < 0.01$; fig. 3). These data give additional evidence that Bcl-2 may participate in the regulation of cytokine- and VT-mediated apoptosis of endothelial cells.

Fig. 3. Correlation between Bcl-2 expression and the level of apoptosis in HUVECs treated with cytokines and VT. Data from one experiment representative of four independent experiments. $n = 22$; $r = -0.76$; $p < 0.01$.

Table 1. Bcl-2 expression in HUVEC treated with cytokines and VT in relation to ECGF concentration

	Bcl-2 MFI at 48 h % control
Bcl-2 expression in HUVEC treated with cytokines ¹	
Control (ECGF 1/100)	100
ECGF 1/800	77.5 ± 10.6*
TNF alone	83.7 ± 11.6
ECGF 1/800 + TNF	57.7 ± 10.5**, +
IFN alone	71.2 ± 10.7*
ECGF 1/800 + IFN	54.8 ± 6.8***, °
TNF + IFN	61.9 ± 7.4**
ECGF 1/800 + TNF + IFN	46.8 ± 9.2**, #
Bcl-2 expression in HUVEC treated with cytokines and VT ²	
TNF + VT	58.2 ± 9.1**
ECGF 1/800 + TNF + VT	46.1 ± 11.2**
TNF + IFN + VT	38.9 ± 9.7***
ECGF 1/800 + TNF + IFN + VT	28.8 ± 8.4***

Data are presented as the mean ± SD of four independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control; + $p < 0.05$ vs. TNF alone; ° $p < 0.05$ vs. IFN alone; # $p < 0.05$ vs. TNF + IFN.

¹ TNF- α and IFN- γ were added at final concentration of 500 U/ml.

² HUVECs were pre-incubated with cytokines for 24 h and then treated with 10 ng/ml VT for another 24 h.

Bcl-xl Expression

Staining for Bcl-xl showed that the levels of this anti-apoptotic protein in HUVECs treated with cytokines for 24 h were not different from control (data not shown). At 48 h we observed a significant upregulation of Bcl-xl expression in HUVECs treated with TNF- α (up to 127.8%, $p < 0.05$; fig. 1b) suggesting a possible cytoprotective effect exerted by this cytokine. Bcl-xl expression in HUVECs pre-incubated with cytokines and treated with VT tended to be lower (fig. 1b); however, the effect was not statistically significant.

Bax Expression

Measurement of Bax expression showed that treatment with TNF- α resulted in a moderate but significant upregulation of this antigen (123.5%, $p < 0.05$; fig. 1c); the effect of IFN- γ was less pronounced. Again, incubation with the combination of cytokines gave more pronounced upregulation of Bax level both at 24 and 48 h (up to 131.5%, $p < 0.05$).

HUVECs treated with VT alone had a Bax level similar to control (fig. 1c). Addition of VT to endothelial cells prestimulated with TNF- α alone or in combination with IFN- γ did not produce any additional increase in Bax expression as compared with cytokine-treated HUVECs.

Bcl-2 Family Protein Expression in Relation to ECGF Concentration

It is known that ECGF routinely used for cell culture is a potent mitogen for endothelial cells [19, 20]. However, studies of endothelial function in vivo showed a very low rate of cell proliferation and apoptosis suggesting that normally endothelial cells are in the resting state [21]. In order to replicate these in vivo conditions we carried out a series of experiments with different ECGF concentrations. They showed that a 1/800 dilution of ECGF preparation was sufficient to prevent apoptosis and maintain low stable levels of proliferation that were statistically not different from standard (1/100) ECGF dilution [18]. We also found that in HUVECs cultured at 1/800 ECGF concentration, Bcl-2 level was significantly reduced (down to 77.5%; table 1).

Comparison of Bcl-2 expression in HUVECs treated with cytokines and VT in standard and low ECGF culture conditions revealed significantly lower levels of Bcl-2 protein in endothelial cells cultured with ECGF at 1/800 concentration (table 1). However, no synergy was observed between cytokines, VT and partial ECGF deprivation and their mutual effect was rather additive.

Bax expression in HUVECs cultured with 1/800 ECGF concentration was not different from control culture (104%; $p = 0.42$). Comparison of Bax levels in cells treated with cytokines and VT in standard and experimental cultures did not reveal any significant differences either (data not shown). These results suggest that Bax does not seem to be involved in ECGF-mediated effects on endothelial cells.

Complete ECGF deprivation led to a progressive induction of apoptosis starting at 24 h after growth factor removal. This was accompanied by a significant downregulation of Bcl-2 protein in apoptotic endothelial cells (down to 55.3% at 48 h, $p < 0.01$; fig. 4). HUVECs histograms in figure 5 illustrate a considerable decrease in Bcl-2 level in ECGF-deprived cells.

Measurement of Bcl-xl and Bax expression did not reveal any significant changes in these protein levels (data not shown). Taken together, our data suggest that apoptosis caused by ECGF deprivation is associated with a decrease in Bcl-2 protein level.

Discussion

Bcl-2 and related cytoplasmic proteins are regarded as key regulators of apoptosis determining the fate of cells by forming homo- and heterodimers at different relative concentrations causing either pro- or anti-apoptotic effects [3]. However, the mechanisms by which these antigens stimulate or inhibit endothelial cell apoptosis caused by pro-inflammatory cytokines and VT are poorly understood. This present study focused on investigating the modulation of Bcl-2 family proteins during cytokine- and VT-induced apoptosis of endothelial cells.

Our initial experiments demonstrated that the expression of Bcl-2 protein was significantly decreased after HUVECs were treated with IFN- γ whereas incubation with TNF- α gave less pronounced downregulation of Bcl-2 level. Treatment with the combination of TNF- α and IFN- γ was more efficient in downregulating Bcl-2 protein than each cytokine separately, still their mutual effect was only additive. These findings are in line with the observations of Pammer et al. [22] who demonstrated that exposure of endothelial cells to 200–1,000 U/ml IFN- γ significantly downregulated Bcl-2 expression. Breitschopf et al. [23] revealed similar effects for TNF- α and showed that treatment of endothelial cells with TNF- α induced dephosphorylation and subsequent ubiquitin-dependent degradation of anti-apoptotic Bcl-2 protein.

Fig. 4. Bcl-2 expression in HUVECs during ECGF deprivation. Data are presented as mean $\pm$ SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. control.

Fig. 5. Histograms of HUVECs stained for Bcl-2 antigen after ECGF deprivation for 48 h. Note a significant decrease in Bcl-2 expression in cells cultured without ECGF compared to control HUVECs cultured with 1/100 ECGF.

The current literature has no data about the role of Bcl-2, Bcl-xl or Bax proteins in VT-mediated apoptosis in endothelial cells. That is why we were especially interested in this aspect of VT action. In our experiments addition of VT to HUVECs pretreated with TNF- α alone or in

combination with IFN- γ gave a further decrease in Bcl-2 level (down to 43%). These data support the hypothesis recently proposed by Suzuki et al. [14] who studied the effects of VT on human hepatoma cells and suggest that Bcl-2 involved in the regulation of VT-mediated apoptosis possibly through formation of mitochondrial Bcl-2-VT complex.

Analysis of Bcl-xl expression showed a moderate significant increase in Bcl-xl level after incubation with TNF- α . This was in contrast with the data of several previously published studies that demonstrated a more or less pronounced decrease in the level of Bcl-xl antigen in endothelial cells after TNF- α treatment [24, 25]. However, our results are not entirely surprising. It has been shown that in most cells TNF- α -mediated apoptosis is controlled by simultaneous upregulation of cytoprotective genes [26]. Such antideath activity requires *de novo* protein synthesis; that is why cells resistant to the cytotoxic effects of TNF- α , including endothelial cells, can be rendered sensitive by pretreatment with protein synthesis inhibitors [27].

Pretreatment of HUVECs with the combination of cytokines followed by VT did not induce any significant changes in Bcl-xl level indicating that this protein does not seem to be mediating VT-induced apoptosis.

For analysis of Bax expression we used Bax (NT) antibody which specifically recognises amino terminal fragment of this antigen exposed only after Bax activation [8]. Our experiments revealed upregulation of the active form of this pro-apoptotic protein in HUVECs stimulated with TNF- α alone or in combination with IFN- γ . Similar results were obtained by Messmer et al. [24] who showed that TNF- α apoptotic action was mediated via a 40% increase in Bax level in glomerular endothelial cells. Interestingly, Jones et al. [28] showed that in human laryngeal epithelial cells Stx-1-mediated apoptosis was associated with an enhanced expression of pro-apoptotic Bax protein. However, our experiments did not reveal any further increase in Bax protein level when HUVECs were co-treated with VT suggesting that Bax upregulation is more important for cytokine- rather than VT-mediated apoptosis of endothelial cells.

Comparative analysis of Bcl-2 expression in endothelial cells treated with cytokines and VT in different culture conditions revealed significantly lower levels of Bcl-2 protein in HUVECs cultured with 1/800 ECGF concentration compared to the standard culture. However, we did not observe any synergy between cytokines, VT and partial ECGF deprivation and their combined effect was rather additive indicating that they were using similar

competing mechanisms regulating the level of this anti-apoptotic protein.

Total ECGF deprivation gave a considerable increase in apoptosis and a decrease in cell proliferation starting at 24 h after ECGF removal. This was accompanied by a significant decrease of Bcl-2 protein in apoptotic cells. However, measurement of Bcl-xl and Bax expression did not reveal any significant changes. Our findings are consistent with the data of Nor et al. [20] and Liu et al. [29] who demonstrated an important role of Bcl-2 protein in mediating the survival effects of vascular endothelial growth factor in endothelial cell cultures. Interesting results were obtained by Yamamoto et al. [30]. They showed that Bcl-2, but not Bcl-xl and Bax, contribute to the anti-apoptotic action of hepatocyte growth factor in cultured endothelial cells. Analysis of these data indicates that apoptosis caused by ECGF deprivation is mediated via a decrease in Bcl-2 protein level independently of Bcl-xl and Bax expression.

In conclusion, cytokine-mediated apoptosis in HUVECs is associated with downregulation of Bcl-2 and upregulation of Bax proteins (decrease of Bcl-2/Bax ratio). Bcl-2 protein, but not Bcl-xl, is downregulated in HUVECs during VT-mediated apoptosis. Likewise, apoptosis caused by ECGF deprivation is associated with reduced Bcl-2 expression. Our data indicate that anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are reciprocally regulated during apoptosis, whilst Bcl-xl is essentially unaffected. This implies that Bcl-2/Bax ratio rather than Bcl-xl controls apoptosis in primary endothelial cells.

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