ELSEVIER

European Journal of Pharmacology 317 (1996) 407–411

ejp

Vitamin C and E prevent lipopolysaccharide-induced apoptosis in human endothelial cells by modulation of Bcl-2 and Bax

Judith Haendeler, Andreas M. Zeiher, Stefanie Dimmeler *

Molecular Cardiology Group, Department of Internal Medicine IV, Division of Cardiology, Johann Wolfgang Goethe-University Frankfurt, Theodor Stern-Kai 7, 60590 Frankfurt, Germany

Received 10 June 1996; revised 4 September 1996; accepted 13 September 1996

Abstract

Lipopolysaccharide induced apoptosis and necrosis of human umbilicial venous endothelial cells in a time-dependent manner. Lipopolysaccharide (1 μg/ml)-induced apoptosis was maximal after 18 h, whereas necrosis occurred after prolonged incubation for more than 24 h. The increase in apoptosis correlated with a reduction in Bcl-2, a potent cell death inhibitor. Furthermore, lipopolysaccharide treatment upregulated Bax, which heterodimerizes with and thereby inhibits Bcl-2. Both the antioxidant *N*-acetylcysteine and the combination of vitamin C and E (10 μM) completely inhibited lipopolysaccharide-induced apoptosis, whereas vitamin C or E alone was less effective. The reduction of lipopolysaccharide-induced apoptosis by vitamin C and E was paralleled by an increase in Bcl-2 and a decrease in Bax protein levels. Thus, vitamin C and E seem to interfere with the Bcl-2 family of apoptosis regulators in human umbilicial venous endothelial cells.

Keywords: Endotoxin; Antioxidant; DNA fragmentation

1. Introduction

Apoptosis and necrosis are two different forms of cell death. They are involved in various physiological and pathophysiological processes (Wyllie, 1980; Cohen, 1993). Oxidants or other stimulators induce apoptosis, including tumor necrosis factor (Robaye et al., 1991), ionizing and ultraviolet radiation, heat shock (Sikora et al., 1993) and lipopolysaccharide with heat shock (Buchman et al., 1993). In contrast, several inhibitors of apoptosis have anti-oxidant activities. Bcl-2, a member of the expanding family of apoptosis regulators, is one of the protective proteins with supposed antioxidative function, which plays a role in preventing apoptosis (Hockenbery et al., 1993; Jacobson and Raff, 1995), whereas Bax heterodimerizes with Bcl-2 and thereby promotes apoptosis (Oltvai et al., 1993). A similar anti-apoptotic effect is mediated by exogenous antioxidants such as dimethyl sulfoxide (DMSO) or *N*-acetylcysteine (Abello et al., 1994; Chang et al., 1992).

Bacterial endotoxin is a proinflammatory mediator that induces the production of significant amounts of endogenous tumor necrosis factor α (TNFα), interleukins and prostaglandin E_2 (Moelvig et al., 1988). As previously published (Dimmeler et al., 1995), endothelial cell viability is decreased by lipopolysaccharide. Furthermore, this effect of lipopolysaccharide was inhibited by treatment with various antioxidants. Therefore, we speculated that the effect of lipopolysaccharide on cell viability might be mediated by apoptosis of endothelial cells. Since the gene products of Bcl-2 and Bax have been shown to play an important role in apoptotic cell death (Hockenbery et al., 1993; Jacobson and Raff, 1995; Sedlak et al., 1995; Boise et al., 1993; Jacobson et al., 1994), we studied the involvement of Bcl-2 and Bax in lipopolysaccharide-mediated apoptosis and the effects of the antioxidants vitamin C and E, or their combination, on Bcl-2 and Bax levels in human umbilicial venous endothelial cells.

2. Materials and methods

2.1. Reagents

Human umbilicial venous endothelial cells and endothelial basal medium were purchased from Cell

* Corresponding author. Tel.: (49-69) 6301-5988; Fax: (49-69) 6301-6374.

Systems/Clonetics (Solingen, Germany). Lipopolysaccharide was purchased from Sigma (Deisenhofen, Germany) and fetal calf serum was purchased from Gibco (Eggenstein, Germany). The antibody against Bcl-2 was from Boehringer (Mannheim, Germany) and against Bax from Santa Cruz (Heidelberg, Germany). All other chemicals were of ultrapure grade.

2.2. Cell culture and viability assays

Human umbilicial venous endothelial cells were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml) and epidermal growth factor (10 ng/ml) and 10% fetal calf serum at 37°C in 5% carbon dioxide and 95% air until the third passage. After detachment with trypsin, cells were grown for 12 h before starting the experiments as described below. Viability was assessed with the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Dimmeler et al., 1995) and lactate dehydrogenase was measured photometrically.

2.3. DNA fragmentation

For analysis of DNA fragmentation cells were grown on 12-well plates (Becton & Dickinson, Heidelberg, Germany) at a density of 10^5 cells/ml and apoptosis was detected by an enzyme-linked immunoassay (ELISA) specific for histone-associated DNA fragments (Boehringer, Mannheim, Germany). Cells were removed and were centrifugated at $700 \times g$ for 10 min, washed with PBS and resuspended in incubation buffer. The histone-associated DNA fragments were linked to the anti-histone antibody from mouse and the DNA part of the nucleosome to the anti-DNA peroxidase. The amount of peroxidase retained in the immunocomplex was photometrically determined with 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] as a substrate.

2.4. DNA isolation and Klenow labeling

DNA was extracted from 5×10^6 cells. The DNA samples were treated with 5 U of Klenow polymerase using 0.5 μCi of ^{32}P-labeled dCTP in the presence of 10 mM Tris/HCl pH 7.5 and 5 mM $MgCl_2$. The reaction was terminated after the addition of 10 mM EDTA, the unincorporated nucleotides were removed by Sephadex G-50 columns (Pharmacia, Freiburg, Germany). The labeled DNA was loaded on a 1.0% agarose gel, electrophoresed for 2 h at 90 V, transferred to nitrocellulose Hybond N (Amersham, Braunschweig, Germany) and exposed to X-ray film.

2.5. Western blot analysis

For Western blotting 1×10^6 cells were removed from culture flasks and incubated in lysis buffer (10 mM Tris/HCl pH = 8, 0.32 M sucrose, 5 mM EDTA, 1% Triton X-100, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The protein content of the samples was estimated according to Bradford (1976). Protein (50 μg) was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on membranes. Membranes were blocked with 2% bovine serum albumin overnight and were incubated with anti-Bcl-2 or anti-Bax for 2 h. After three washing steps anti-horseradish peroxidase was used as the second antibody and the membranes were stained with enhanced chemiluminescence. The autoradiographies were scanned and semiquantitatively quantified.

2.6. Nothern blot analysis

For Northern blotting RNA was prepared from endothelial cells (5×10^6 cells) after incubation with lipopolysaccharide and vitamins C and E for 6 h or 18 h. RNA was loaded on 1.0% agarose/formamid gel and electrophoresed at 20 V overnight. After the transfer on nitrocellulose Hybond N, the membranes were hybridized with a labeled Bcl-2 cDNA probe for 18 h and exposed to X-ray film.

3. Results

3.1. Induction of apoptosis and necrosis by lipopolysaccharide

Exposure of human umbilicial venous endothelial cells to lipopolysaccharide (1 μg/ml) induced DNA fragmentation in a time-dependent manner (Fig. 1). Maximal values were obtained after 18 h of incubation. Necrosis occurred after prolonged incubation for more than 24 h as demonstrated by increased lactate dehydrogenase levels (Fig. 1). The time course of apoptosis correlated with the reduction of cell viability as measured by the MTT assay. After 18 h incubation, 1 μg/ml lipopolysaccharide reduced cell viabilty to 78% compared to control cells. Furthermore, enhanced DNA fragmentation was detected after incubation with lipopolysaccharide by agarose-gel electrophoresis after radioactive labeling of the fragments with the Klenow enzyme (Fig. 2, lane b). The pro-apoptotic effects of lipopolysaccharide were not mediated by induction of TNFα, because simultaneous incubation with anti-TNFα (1 μg/ml) did not prevent lipopolysaccharide-induced apoptosis (data not shown).

3.2. Effect of lipopolysaccharide on Bcl-2 and Bax

Incubation of cells for 18 h with lipopolysaccharide dramatically reduced Bcl-2 protein levels to $51 \pm 5\%$ com-

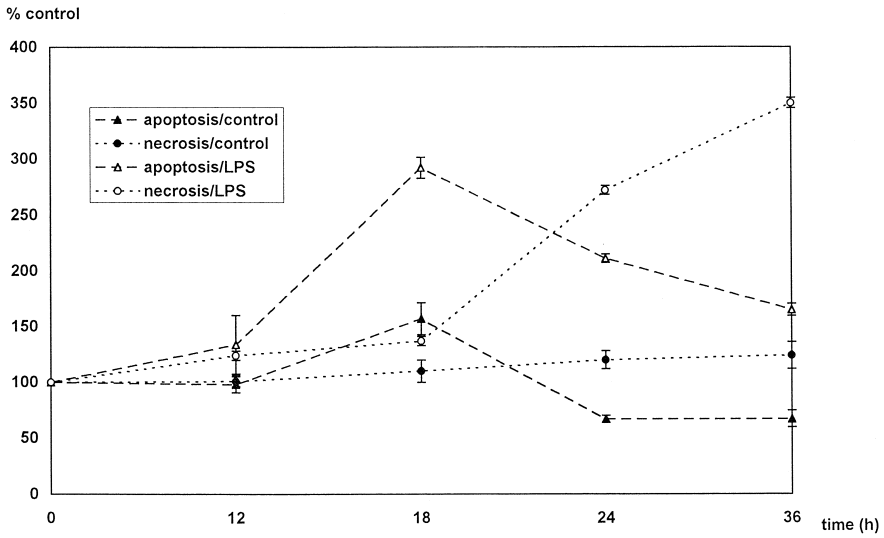

Fig. 1. Time-course of lipopolysaccharide-induced apoptosis and necrosis. 1×10^5 cells/ml were incubated with 1 μg/ml lipopolysaccharide at indicated time points and DNA fragmentation was measured by ELISA. Lactate dehydrogenase levels were determined in cell culture supernatants. Data are means ± S.E.M., $n = 3$.

pared to controls as measured by Western blot (Fig. 3A). However, Northern blot analysis of Bcl-2 mRNA levels with lipopolysaccharide revealed no significant reduction after 6 h or 18 h incubation (data not shown). The lipopolysaccharide-induced decrease in Bcl-2 was paralleled by an increase in Bax, an endogenous inhibitor of Bcl-2

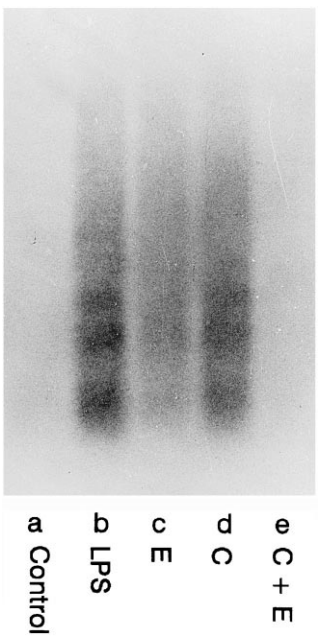

Fig. 2. Agarose gelelectrophoresis of DNA fragments. DNA (2 μg) was radioactively labeled as outlined in Materials and methods. A representative result out of three independent experiments illustrates the effect of lipopolysaccharide (1 μg/ml) and vitamins E and C (10 μM). Lane a, control; lane b, 1 μg/ml lipopolysaccharide; lane c, lipopolysaccharide + 10 μM vitamin E; lane d, lippolysaccharide + 10 μM vitamin C; lane e, lipopolysaccharide + vitamins E and C.

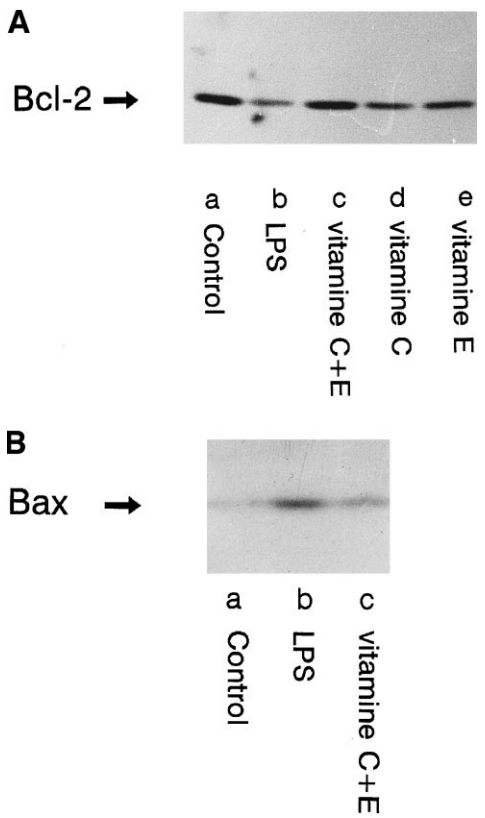

Fig. 3. Western blot analysis of Bcl-2 (A) and Bax (B). 50 μg protein of each sample was resolved on 10% SDS-PAGE and Western blot was performed as described in Materials and methods. Cells were treated for 18 h with lipopolysaccharide (1 μg/ml), vitamin E (10 μM), or vitamin C (10 μM) as indicated. A typical result out of three independent experiments is shown.

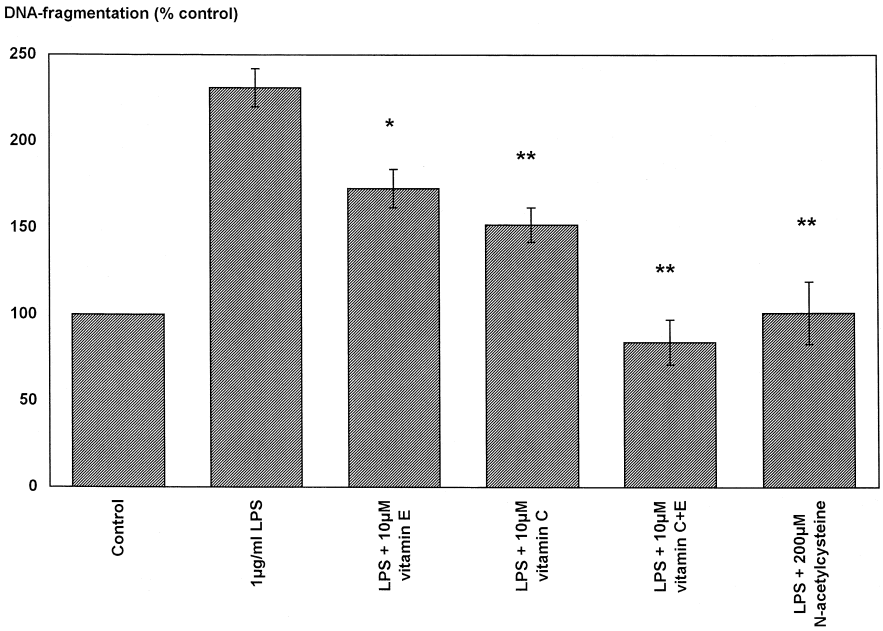

Fig. 4. Influence of the antioxidants *N*-acetylcysteine and vitamins C and E on lipopolysaccharide (LPS)-induced apoptosis. 1×10^5 cells/ml were incubated with lipopolysaccharide (1 μg/ml), *N*-acetylcysteine (200 μM), vitamin C (10 μM), and vitamin E (10 μM) as indicated for 18 h. DNA fragmentation was measured with the ELISA. Data are means ± S.E.M. ($n = 3$). * $P < 0.05$ versus LPS; ** $P < 0.01$ versus LPS.

(Fig. 3B). The protein level was thereby upregulated to 140 ± 8.6% in lipopolysaccharide-treated cells (controls are set as 100%).

3.3. Effect of antioxidants on lipopolysaccharide-induced apoptosis

As demonstrated in Fig. 4, both 200 μM *N*-acetylcysteine and the combination of 10 μM vitamin C and 10 μM vitamin E completely prevented lipopolysaccharide-induced apoptosis, whereas the vitamin C or E alone showed only minor inhibitory effects. These results were confirmed by detection of DNA fragmentation by agarose-gel electrophoresis as shown in Fig. 2. Furthermore, delayed necrosis induced by lipopolysaccharide (270%) was reduced by vitamin C and E addition to 110 ± 15% compared to controls.

Abrogation of lipopolysaccharide-induced apoptosis by the combination of vitamin C and E was paralleled by preserved levels of Bcl-2 (Fig. 3A). The decrease in the Bcl-2 content of about 50% by lipopolysaccharide treatment was thereby prevented by the combination of 10 μM vitamin C and 10 μM vitamin E (99 ± 12% of control values), whereas vitamin C and vitamin E alone showed only minor inhibitory effects (63 ± 17% and 65 ± 11% of controls, respectively). Furthermore, as shown in Fig. 3B, the enhanced levels of Bax induced by lipopolysaccharide incubation were reduced by vitamin E and C to basal levels (116 ± 11.2%), whereas the influence of vitamin C or E alone on Bax protein levels was less pronounced (127 ± 6% and 127 ± 11% compared to controls, respectively).

4. Discussion

Lipopolysaccharide induces apoptosis and necrosis in human umbilicial venous endothelial cells. The development of apoptosis starts before necrosis and inhibition of apoptosis further prevented necrosis, suggesting an important role of apoptosis in the development of endothelial cell dysfunction and organ injury in septic/endotoxic shock. The lipopolysaccharide effect was not due to induction of inflammatory cytokines, because glucocorticoids did not prevent the lipopolysaccharide effect (Dimmeler et al., 1995) and furthermore neutralizing antibodies against TNFα did not ameliorate the lipopolysaccharide response.

The family of Bcl-2 apoptosis regulators has gained interest, because of the protective effect of Bcl-2, which has been shown to enhance cell survival by inhibiting apoptosis induced under various circumstances (White, 1996). Bax is homologous to Bcl-2 and suppresses the ability of Bcl-2 to block apoptosis (Oltvai et al., 1993). Our results demonstrated a dramatical influence of lipopolysaccharide on both Bcl-2 and Bax. The protein level of the protective Bcl-2 was decreased, whereas Bax was increased, indicating that the protective activity of Bcl-2 might have been further reduced by up-regulation of the endogenous inhibitor Bax. These results suggest that lipopolysaccharide disarranges the ratio of Bcl-2 and Bax and therefore leads to apoptosis.

The role of Bcl-2 in the mechanism of programmed cell death is not yet known. Hockenbery et al. (1993) suggested that Bcl-2 protects against apoptosis by inhibiting the generation or action of reactive oxygen species. In contrast, Jacobson and Raff (1995) showed that reactive oxygen species are not required for programmed cell death, and that Bcl-2 protects against apoptosis in ways that do not depend on inhibition of oxygen radical production or activity. Our results now demonstrated a regulation of the Bcl-2 protein family by oxidative processes, because the antioxidants *N*-acetylcysteine and the combination of vitamin C and E completely prevented the dysregulation of Bcl-2 and Bax by lipopolysaccharide. The regulation by oxidants more likely involves protein degradation than transcriptional effects, because no influence on RNA levels were detectable in Northern blot analysis. Recent findings demonstrate the activation of several proteases such as cysteine proteases (Enari et al., 1996) and aspartic proteases (Deiss et al., 1996) in the apoptotic signal transduction pathway. The activation of one of these proteases possibly may also account for the decreased Bcl-2 protein levels. However, further studies should evaluate the underlying mechanism.

In summary, this study suggests that lipopolysaccharide-induced apoptosis is mediated by oxidative stress and leads to a reduction of the endogenous protection potential of the cells by disturbation of the balance between Bcl-2 and Bax.

References

Abello, A.P., S.A. Fidler, G.B. Bulkley, G. Timothy and T.B. Buchman, 1994, Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin, Arch. Surg. 129, 134.

Boise, L.H., M. Gonzalez-Garcia, C.E. Postema, L.E. Ding, T. Lindsten, C.A. Turka, X. Mao, G. Nunez and C.B. Thompson, 1993, Bcl-x, a bcl-2 related gene that functions as a dominant regulator of apoptotic cell death, Cell 74, 597.

Bradford, M.M., 1976, A rapid and sensitive method for the quantification of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Buchman, T.B., P.A. Abello, E. Smith and G.B. Bulkley, 1993, Induction of heat shock response leads to apoptosis in endothelial cells previously exposed to endotoxin, Am. J. Physiol. 265, H165.

Chang, D.J., G.M. Ringold and R.A. Heller, 1992, Cell killing and induction of manganous superoxide dismutase by tumor necrosis factor-alpha is mediated by lipoxygenase metabolites of arachidonic acid, Biochem. Biophys. Res. Commun. 188, 538.

Cohen, J.J., 1993, Apoptosis, Immunol. Today 14, 126.

Deiss, L.P., H. Galinka, H. Berissi, O. Cohen and A. Kimichi, 1996, Cathepsin D proteases mediates programmed cell death induced by interferon-γ, Fas/APO-1 and TNFα, EMBO J. 15, 3861.

Dimmeler, S., S. Brinkmann and E. Neugebauer, 1995, Endotoxin-induced changes of endothelial cell viability and permeability: protective effect of a 21-aminosteroid, Eur. J. Pharmacol. 287, 257.

Enari, M., R.V. Talanian, W.W. Wong and F. Nagata, 1996, Sequential activation of ICE-like- and CPP-32-like-proteases during Fas-mediated apoptosis, Nature 380, 723.

Hockenbery, D.M., Z.N. Oltvai, X.-M. Yin, C.L. Millman and S.J. Korsmeyer, 1993, Bcl-2 functions in an antioxidant pathway to prevent apoptosis, Cell 75, 241.

Jacobson, M.D. and M.C. Raff, 1995, Programmed cell death and Bcl-2 protection in very low oxygen, Nature 374, 814.

Jacobson, M.D., J.F. Burne and M.C. Raff, 1994, Programmed cell death and Bcl-2 protection in the absence of a nucleus, EMBO J. 13, 1899.

Moelvig, J., L. Baek and P. Christensen, 1988, Endotoxin stimulated stimulated human monocyte secretion of interleukin-1, tumor necrosis factor alpha and prostaglandin E_2 show interindividual differences, Scand. J. Immunol. 27, 705.

Oltvai, Z.N., C.L. Milliman and S.J. Korsmeyer, 1993, Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death, Cell 74, 609.

Robaye, B., R. Mosselmans, W. Fiers, J.E. Dumont and P. Galand, 1991, Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro, Am. J. Pathol. 138, 447.

Sedlak, T.W., Z.N. Oltavai, E. Yang, K. Wang, L.H. Boise, C.B. Thompson and S.J. Korsmeyer, 1995, Multiple Bcl-2 family members demonstrate selective dimerization with Bax, Proc. Natl. Acad. Sci. USA 92, 7834.

Sikora, E., E. Grassilli, E. Radziszewska, E. Bellesia, D. Barbieri and C. Franceschi, 1993, Transcription factors undergoing apoptosis after heat-shock or dexamethasone treatment, Biochem. Biophys. Res. Commun. 197, 709.

White, E., 1996, Life, death, and the pursuit of apoptosis, Genes Dev. 10, 1.

Wyllie, A.H., 1980, Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation, Nature 284, 555.