Effect of recombinant human activated protein C on apoptosis-related proteins

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The recombinant human activated protein C (rhAPC) has been reported to reduce mortality in patients with severe sepsis. An anti-apoptotic effect of rhAPC in sepsis is known, but the mechanism through which it acts on the apoptotic pathway is still unclear. Therefore, immunopositivity of the apoptosis-related proteins Bcl-2, an anti-apoptotic protein, c-myc, a proliferative protein, p-21 and p-53, two apoptotic proteins, was determined after rhAPC treatment in a mouse sepsis model. Sepsis was induced by *Escherichia coli* endotoxin injection. Increased neutrophil infiltration and immunoreactivity to p53 and p21 were observed in the group with sepsis and these immunoreactivities were decreased by rhAPC treatment. In the sepstic group; immunopositivity of Bcl-2 and c-myc was mild and moderate, respectively. In conclusion; p21- and p53-mediated apoptosis was increased in the sepsis model, and for the first time it has been shown that rhAPC decreases sepsis-induced apoptosis resulting from increased p21 and p53 proteins.

Key words: apoptosis, Bcl-2, c-myc, recombinant human activated protein C, p21, p53, sepsis.

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Apoptosis is programmed cell death characterized by nuclear degeneration, condensation, and nuclear DNA degradation; phagocytosis of cell residua (Cummings, 1997; Bowen, 1993). An increased rate of apoptosis in lymphoid cells and organ parenchyma is seen in sepsis syndromes. Therefore, the blockade of cell signaling pathways leading to apoptosis may represent a new therapeutic target for the critically ill patient with sepsis (Oberholzer, 2001).

Various genes are involved in the regulation of apoptosis with either positive or negative regulatory effects. Withdrawal of essential growth factors or hormones or engagement of various receptor and ligands including Fas, Fas ligand and tumour necrosis factor (TNF), TNF receptor are some of the triggering factors in apoptosis (Nagata, 1995; Abraham, 2005). Moreover, apoptosis is controlled and regulated by several genes which potentiate p53, Bax, c-myc, others, or inhibit B cell lymphoma 2 (Bcl-2), Bcl xL, sentrin, others (Okura, 1996; Sattler, 1997).

The proto-oncogene Bcl-2 family can have anti-apoptotic or pro-apoptotic functions and is one of the most studied inhibitors of apoptosis (Borner, 2003). The tumour suppressor protein p53 mediates activation of programmed cell death by up-regulation of Bax immunopositivity; by contrast, Bcl-2 can block p53-mediated apoptosis (Oltvai, 1993; Korsmeyer, 1992; Selvakumaran, 1994).

The role of apoptosis in sepsis has not been adequately explored, although there is rapidly developing evidence to suggest that increased apoptotic processes may play a determining role in the outcome of sepsis syndromes (Oberholzer, 2001).

In sepsis syndromes, lymphocyte apoptosis can be triggered by the absence of IL-2 or by the release of glucocorticoids, granzymes, or the so-called *death* cytokines: TNF α or Fas ligand. Apoptosis proceeds via auto-activation of cytosolic and/or mitochondrial caspases, which can be

influenced by the pro- and anti-apoptotic members of the Bcl-2 family (Oberholzer, 2001). Activated protein C (APC) is an important regulator of hemostasis. APC can interact at multiple points during the systemic response to infection. APC acts as an antithrombotic factor by inactivating Factors Va and VIIIa of the coagulation cascade. As a result of decreased levels of thrombin, the procoagulant, proinflammatory and antifibrinolytic effects of thrombin are reduced. Moreover, APC restores the endogenous fibrinolytic system by inhibiting plasmin activator inhibitor-1 and by preventing the activation of thrombin activated fibrinolysis inhibitor (Brueckmann, 2003; Hughes, 2006). Recombinant human APC (rhAPC) or drotrecogin alfa (activated) is homologous to plasma-derived human APC, differing only in the carbohydrate portion of the molecule (Brueckmann, 2003). Treatment with rhAPC significantly reduces morbidity and mortality in patients with severe sepsis (Rice, 2004; Nguyen, 2006). The aim of this study was to investigate immunopositivity of apoptosis-related proteins (p21, c-myc, Bcl-2 and p53) in a sepsis model of mouse lung after treatment with rhAPC.

Materials and Methods

Animals

All experiments were performed according to the institutional guidelines for animal experimentation at Celal Bayar University Faculty of Medicine. We obtained approval from the Research Animal Ethics Board of Celal Bayar University for the study.

Thirty C57BL6 F1 mice, weighing 30 g and 8-10 weeks of age, were used. These mice were maintained in animal quarters for at least 3 weeks before the experiments under conditions of controlled temperature and were fed a standard diet.

The mice were divided into 3 groups (n=10):

1. *Normal group* did not receive any medication;
2. *Sepsis control group* given 20 mg/kg lipopolysaccharide (LPS; from Escherichia coli O111:B4), i.p. for induction of sepsis;
3. *rhAPC treated group* given 48 mg/kg rhAPC, i.p.,1 h after LPS.

The mice were sacrificed 24 h after LPS by cervical dislocation and the lungs removed.

Tissue collection

Samples were washed with tap water and soaked in a series of 50%, 60%, 70%, 80% and 90% ethanol for 30 min and then in 95% and 100% ethanol for one hour. Then they were placed in a solution of 100% ethanol and xylene in a 1:1 ratio for 30 min, embedded in paraffin and kept at 60°C for 60 min to form paraffin blocks. Transverse sections (5 μ) were taken from the blocks and prepared for both histochemical and immunohistochemical labeling.

Histochemical observations

Sections dewaxed at 60°C overnight were immersed in xylene for one hour and then rehydrated through a graded series of ethanol (100%, 95%, 80%, 70%, and 60%) for 2 min at each concentration and were then washed in tap water. Sections were stained by hematoxyline-eosin (H-E) according to the routine protocols. Slides were mounted using entellan and covered with glass cover slips prior to viewing, and photographed under an Olympus BX-40 (Tokyo, Japan) light microscope.

Immunohistochemistry

5 μ sections were dewaxed with xylene for 30 min and, after rehydrating, the sections were incubated with 3% H_2O_2, 30 min for inactivation of endogenous peroxidase at room temperature. They were then washed with phosphate buffered saline (PBS, P-4417, Sigma, St. Louis, USA) and stained with primary antibodies; anti-p21 (1:100, MS-230-P, Neomarkers, Fremont, CA), anti-c-myc (1:100, MS-139-R7, Neomarkers, Fremont, CA), anti-Bcl-2 (1:100, Oncogene Research Products, Boston, USA) and anti-p53 (1:100, Oncogene Research Products, Boston, USA) for 18 h. After washing with PBS, the secondary antibody biotinylated goat IgG anti-rabbit/mouse IgG (supplied ready to use by Zymed, Histostain-Plus kit, 85-9043 San Francisco, USA) was applied for 30 min, followed by three washes in PBS. The streptavidin–peroxidase complex (supplied ready to use by Zymed) was added for 30 min and washed 3 times with PBS.

Samples were stained with diaminobenzidine for 10 min (Zymed, San Francisco, USA) for the detection of immunoreactivities. Slides were washed twice with deionized water for 10 min each, and counter-stained with Mayer's hema-

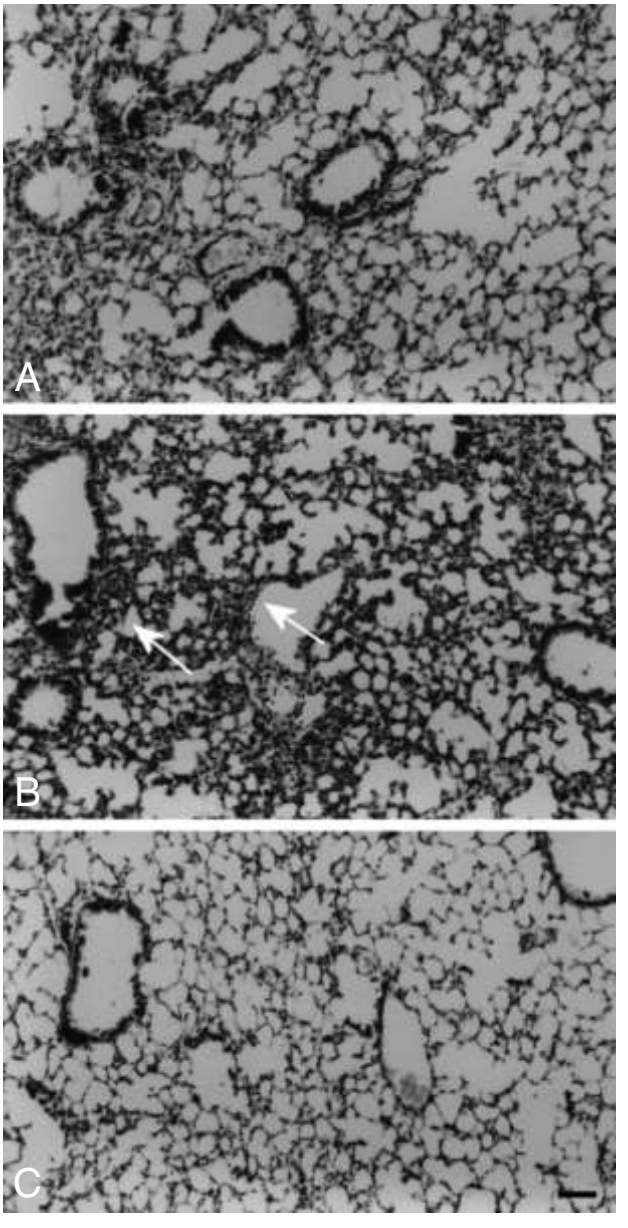

Figure 1. Histology of normal (A), sepsis control (B) and rhAPC-treated (C) groups. The lung specimens in normal and rhAPC treated groups are seen to be normal, leukocyte infiltration (arrows) and thickened alveolar walls are observed in the sepsis control group. Hematoxylin-eosin staining. Original magnification 100X, Scale bar: 10,000 µm.

toxylin for 3 min, dehydrated, and cleared, after which a cover-slip was applied. The slides were examined under a BX 40 microscope (Olympus, Tokyo, Japan). The presence of a brown precipitate indicated positivity for the primary antibody. The negative immunolabelling controls received the same treatment, with rabbit IgG or mouse IgG instead of the primary antibody. Seriall sections were examined and immunolabelling patterns were compared. Two observers who were blinded to the clinical information of the lung samples independently evaluated the immunolabelling scores. Immunolabelling intensity was graded as negative (-), mild (+), moderate (++) and strong (+++),

Statistical analysis

Results are expressed as the mean ± SD. SPSS 10.0 software for Windows (SPSS, Chicago, IL, USA) was used for statistical evaluation. Differences among groups were statistically analysed by one-way ANOVA where appropriate. A $p<0.05$ was assumed to indicate statistically significant differences.

Results

In the histochemical evaluation of the specimens of the normal group, thin alveolar walls, columnar epithelial cells of terminal and respiratory bronchioles were observed. However, neither intraalveolar edema fluid nor leukocyte infiltration was found which are considered as normal tissue findings. In the sepsis control group, leukocyte infiltration and thickened alveolar walls were observed; however, there were no pathologic changes in either the alveolar or epithelial cells of the bronchioles. Withdrawal of leukocyte infiltration was detected in the intraalveolar space after treatment with rhAPC (rhAPC treated group) (Figure 1).

Immunohistochemical findings showed that the immunoreactivity of Bcl-2 in alveolar cells was high in the normal group and mild in both the sepsis control group and rhAPC-treated group (Figure 2). While immunoreactivity of c-myc was moderate in the normal and sepsis control groups, the immunopositivity of c-myc was absent,in the rhAPC-treated group (Figure 3). Immunolabelling of p21 in the sepsis control and in the rhAPC-treated group was moderate and mild, respectively (Figure 4). Negative immunolabelling of p21 was also detected in the normal group. Strong immunoreactivity of p53 was detected in the intraalveolar walls of lungs from the sepsis control group. This immunoreactivity was absent in the normal group. However, mild immunoreactivity of p53 was also present in the rhAPC-treated group (Figure 5). The epithelial cells of terminal and respiratory bronchioles were negative for Bcl-2, c-myc, p21 and p53 immunolabelling in all groups.

The immunopositivity intensity of Bcl-2 in the normal group, of c-myc in the rhAPC-treated

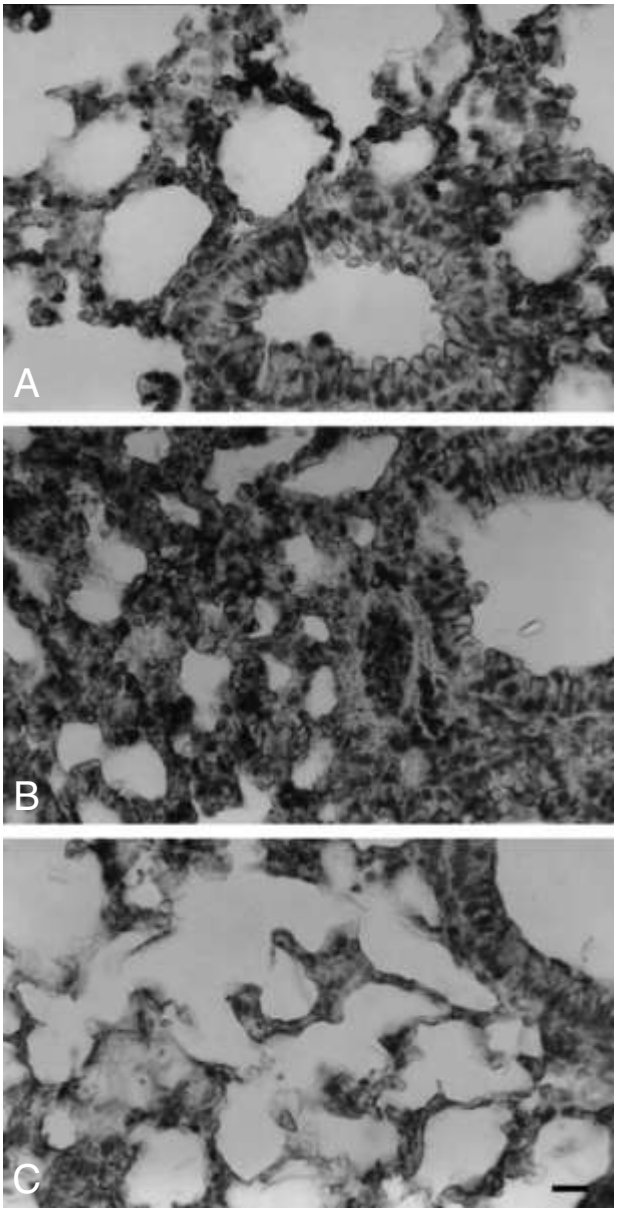

Figure 2. Immunolabelling of Bcl-2 in normal (A), sepsis control (B) and rhAPC-treated (C) groups. Immunoreactivities of Bcl-2 in alveolar cells are detected as strong in normal, and mild in the sepsis control and rhAPC-treated groups (immunopositivities shown by arrows) Original magnification 400X, Scale bar: 250 µm.

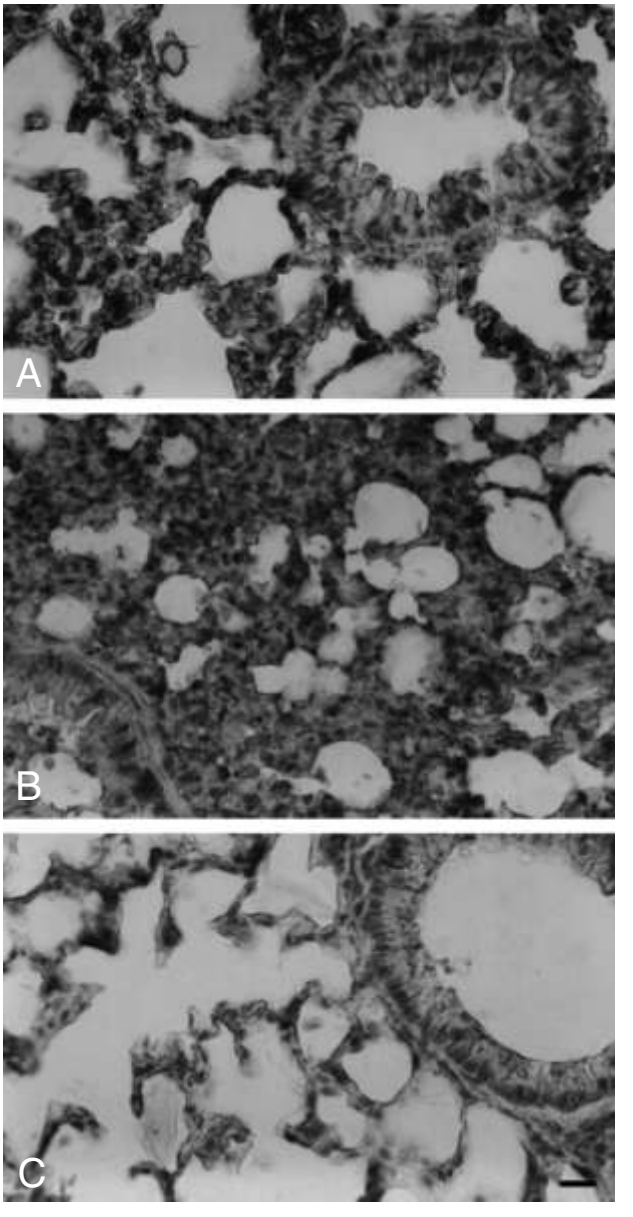

Figure 3. Immunolabelling of c-myc in normal (A), sepsis control (B) and rhAPC-treated (C) groups. While immunoreactivity of c-myc is moderate in the normal and sepsis control groups, the immunopositivity of c-myc is absent in the rhAPC-treated group (immunopositivities shown by arrows). Original magnification 400X; Scale bar: 250 µm.

group and the intensities of p21 and p53 in the sepsis control group were significantly different from the other groups ($p<0.001$)(Table 1).

Discussion

We found increased leukocyte infiltration in the sepsis control group when compared with the normal and rhAPC-treated groups. Immunoreactivities of p53 and p21 were increased in the sepsis control

Table 1. Immunolabelling intensities of Bcl-2, c-myc, p21 and p53 in lung from normal, sepsis control and rhAPC-treated mice.

Group	Bcl-2	c-myc	p21	p53
Normal	2.75±0.46*	1.62±0.52	0.12±0.35	0.25±0.46
Sepsis control	0.63±0.52	2.0±0.53	2.0±0.54*	2.88±0.35*
rhAPC-treated	1.0±0.0	0.25±0.46*	1.0±0.53	1.0±0.53

**p<0.001 vs the other groups*

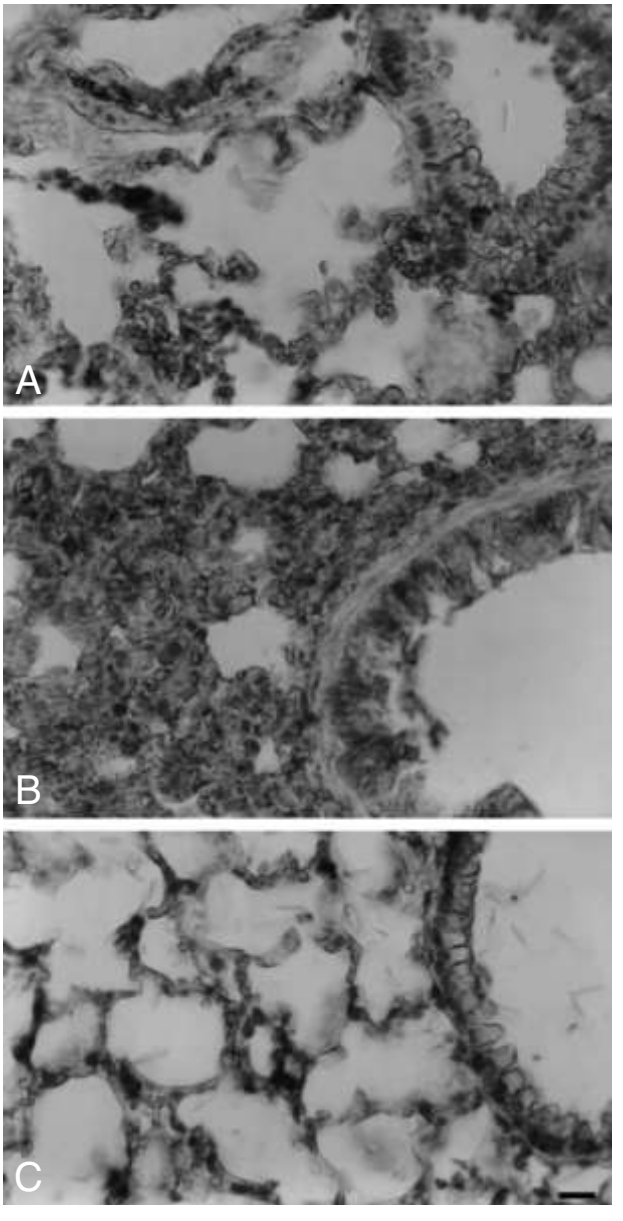

Figure 4. Immunolabelling of p21 in normal (A), sepsis control (B) and rhAPC-treated (C) groups. Moderate, mild and negative labelling of p21 in the sepsis control, rhAPC treated and normal groups are detected, respectively (immunopositivities shown by arrows) Original magnification 400X, Scale bar: 250 µm.

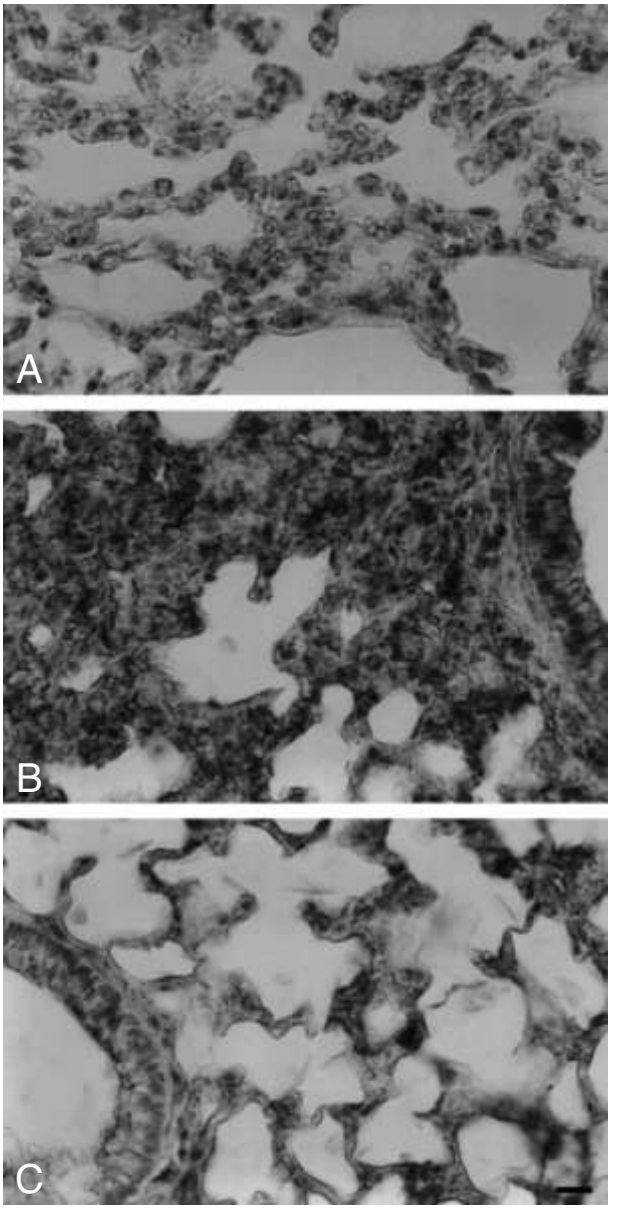

Figure 5. Immunolabelling of p53 in normal (A), sepsis control (B) and rhAPC-treated (C) groups. Strong, mild and negative labelling of p53 in sepsis control, rhAPC-treated and normal groups are detected, respectively (immunopositivities shown by arrows) Original magnification 400X, Scale bar: 250 µm.

group when compared with other groups, and these immunoreactivities were decreased after rhAPC treatment. In the sepsis control group, immunopositivities of Bcl-2 and c-myc were mild and moderate, respectively.

Members of the TNF receptor family and the Bcl-2 gene family having pro-apoptotic or anti-apoptotic functions are the two major pathways regulating apoptosis. The ratio of immunopositivity of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 determines the regulation for cell survival following an apoptotic stimulus. Drugs assumed to influence the Bcl-2/Bax ratio are now available, and the Bcl-2/Bax rheostat could, therefore, become a new target for therapeutic interventions (Cory, 2002; Bilbault, 2004).

Previous studies have shown that the overexpression of the Bcl-2 gene in transgenic septic mice significantly improves the survival of these animals and that Bcl-2 protects against cell death in sepsis.

The role of the Bcl-2 gene in the host defense system and in the final outcome of septic patients is important (Hotchkiss, 1994; Liacos, 2001; Coopersmith, 2002; Iwata, 2003; Wellmer, 2004). In the present study, the immunopositivity of Bcl-2 was decreased in both sepsis control and rhAPC treated groups when compared with the normal group. These results suggest that apoptosis was triggered in the sepsis control group. However, the immunopositivity of Bcl-2 in the rhAPC-treated group did not differ from the sepsis control group which could be explained by the control of the apoptotic pathway by other anti-apoptotic agents, such as Bak or Bax, in the rhAPC-treated group. Liacos *et al.* (2001) noticed that immunopositivity of the Bcl-2 gene was higher in the controls, whereas in septic patients, the immunopositivity of the Bcl-2 gene was significantly lower in human bronchoalveolar lavage (BAL) samples. This finding suggests that Bcl-2 is not the key pathway responsible for the decrease of apoptosis in the BAL cells of septic patients.

Another study suggested that the level of Bcl-2 in circulating mononuclear cells was decreased in sepsis-induced immune deficiency; this might determine the survival of patients (Bilbault, 2004).

A previous study by Coopersmith *et al.* (2002) has shown that the overexpression of the Bcl-2 gene in transgenic septic mice significantly improved the survival of these animals. The overexpression of Bcl-2 decreased in the next 12-24 hours of sepsis. Therefore, in the present study, the finding of a decreased immunoreactivity of Bcl-2 in sepsis may depend on the fact that Bcl-2 was measured 24 hours after giving LPS. The determination of Bcl-2 immunoreactivity in the late periods of sepsis may not be helpful for the evaluation of apoptosis.

The protooncogene myc gene family, which includes c-myc, N-myc and L-myc, plays a key role in cell proliferation, differentiation, and apoptosis (Zajac-Kaye, 2001; Cole, 1986). The cellular myc gene (c-myc) has been found to induce apoptotic cell death under certain conditions. Impaired apoptosis is a crucial step in tumorigenesis; indeed, neoplastic progression reflects loss of normal apoptotic mechanisms. Immunopositivity of c-myc can initiate proliferation and increase sensitivity to apoptosis under low serum conditions when antiapoptotic mechanisms are not activated (Dang, 1999; Pastorino, 2003). p53-dependent and -independent mechanisms underlying c-myc-mediated apoptosis have been reported, and c-myc-induced apoptosis can be prevented by overexpression of the Bcl-2 oncoprotein (Cory, 2002).

The intrinsic apoptotic pathway can be activated by oncoproteins, which are one of the intrinsic stresses. As a sensor of cellular stress, p53 is a critical initiator of this pathway by transcriptionally repressing antiapoptotic Bcl-2 proteins (Pastorino, 2003).

It is known that Bcl-2 can specifically block the apoptotic function of c-myc, but preserves its proliferative activity. Bcl-2 suppression of c-myc-induced apoptosis exposes multiple oncogenic properties of c-myc and triggers carcinogenic progression (Jin, 2004). However, the mechanism of the functional synergism of Bcl-2 and c-myc is not clear. Our results suggest that detection of moderate immunoreactivity of c-myc and mild immunoreactivity of Bcl-2 may explain the cell proliferation and induction of apoptosis during repair of lung after sepsis.

p53 expression is induced following DNA damage leading to a late G1 block and plays considerable roles in cell cycle control, differentiation, genomic stability, angiogenesis and apoptosis. It has been proposed that functional disability of p53 might cause a decrease in the ability of these cells to undergo apoptosis. The Waf1/Cip1 gene is also induced by p53 and leads to growth suppressor functions of p53. The p21 protein, which inhibits the activities of G1 cyclin-dependent protein kinases and arrest cells in G1, is encoded by the Waf1/Cip1 gene. Two genes that regulate apoptosis are targets for p53. The Bax gene has been shown to be activated by p53 and the promoter for the Bcl-2 gene has a negative effect on p53 (Maxwell, 1997).

In our study, while the immunoreactivities of the pro-apoptotic factors p53 and p21 were not observed in the normal group, these immunoreactivities were increased in the sepsis control group and decreased after treatment with rhAPC. In addition, strong and moderate immunoreactivities of p53 and p21, respectively, were detected in the sepsis control group.

Cheng *et al.* (2003) reported that APC directly prevents apoptosis in hypoxic human brain endothelium through transcriptionally-dependent inhibition of tumor suppressor protein p53, normalization of the pro-apoptotic Bax/Bcl-2 ratio and reduction of caspase-3 signaling. In our study, we found that

rhAPC inhibited p21 and p53 mediated apoptosis and c-myc mediated cell proliferation.

In conclusion, the immunoreactivities of p21 and p53 may be used as a marker for detection of apoptosis in sepsis, because their immunoreactivities were increased in the sepsis control group when compared with the other groups. In addition, c-myc may be used as a marker for controlling of cell proliferation in sepsis. This is the first time that rhAPC has been shown to decrease sepsis-induced apoptosis resulting from increased p21 and p53 proteins. Understanding the apoptotic mechanisms during sepsis may open the door to new therapeutic approaches and prolong the survival of septic patients.

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