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Caspase-3-dependent pathway mediates apoptosis of human mononuclear cells induced by cellulosic haemodialysis membranes

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Abstract

Background. Mononuclear cells from patients dialysed with cellulosic membranes undergo rapid apoptosis *in vitro*. The aim of the present study was to determine whether the apoptosis associated with cellulosic haemodialysis membrane shares similar features with the spontaneous apoptosis described in normal monocytes. Thus, we determined whether apoptosis is dependent on caspase-3 activity and is inhibited by lipopolysaccharide (LPS), which are two features of spontaneous apoptosis in normal monocytes.

Methods. We examined mononuclear cells from healthy subjects and from 14 end-stage renal failure patients on haemodialysis with cellulosic membranes ($n=7$) and non-cellulosic membranes ($n=7$). Isolated mononuclear cells were cultured for 48 h. To determine the effect of haemodialysis membrane exposure on caspase-3 activity, on mononuclear apoptosis, or both, cells from healthy subjects were cultured in mini-dialysers with the same membrane types that were used in the haemodialysis patients. Caspase-3 active form was determined by flow cytometric analysis using anti-human-active caspase-3 antibodies. The effect of LPS and Ac-DEVD-CHO, a specific inhibitor of active caspase-3, was also evaluated. Cell apoptosis was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling method.

Results. After 48 h of culture, the percentage of mononuclear cells expressing the active form of caspase-3 was greater in patients dialysed with cellulosic membranes than in patients using non-cellulosic membranes and in healthy subjects. This increase in caspase-3 activity was associated with a high rate of apoptosis, which was prevented by Ac-DEVD-CHO, an inhibitor of caspase-3 activity. LPS decreased both apoptosis and caspase-3 activity in mononuclear cells from patients dialysed with cellulosic membranes. Finally, in cells from healthy subjects, both caspase-3 activation and apoptosis were induced after incubation with cellulosic membranes. In contrast, the active form of caspase-3 was not increased in cells cultured with non-cellulosic membranes and was significantly lower than with cellulosic membranes.

Conclusion. These findings suggest that the apoptosis of mononuclear cells induced by cellulosic haemodialysis membranes occurs through a pathway that is similar to the spontaneous apoptosis of normal monocytes. They additionally suggest that LPS regulates the proteolytic activation of caspase-3.

Keywords: apoptosis; caspase-3; cellulosic haemodialysis membranes; human mononuclear cells; lipopolysaccharide

Introduction

Clinical studies have shown that haemodialysis with cellulosic membranes is associated to mononuclear cell activation [1]. Incubation of normal mononuclear cells with cellulosic haemodialysis membranes results in phosphorylation of cell surface proteins, release of pro-inflammatory cytokines, and increased expression of cell adhesion molecules [2–4]. In addition, we have shown that incubation of normal monocytes with cellulosic membranes produces an increase in apoptosis, an effect that may be secondary to the inflammatory responses induced by the cellulosic membrane [4,5].

Normally, monocytes undergo spontaneous apoptosis after circulating for 1–3 days in the blood. The term spontaneous apoptosis describes the spontaneous programmed cell death that occurs in the absence of an appropriate stimulus [6]. In fact, spontaneous apoptosis is a critical process that regulates monocyte removal and survival, allowing control of this cell

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population. The spontaneous apoptosis of monocytes is inhibited by pro-inflammatory cytokines and enhanced by anti-inflammatory cytokines [7–11]. Thus, during an inflammatory process, pro-inflammatory cytokines act on monocytes to prolong their life span up to 15 days. Moreover, monocyte spontaneous apoptosis can be prevented by addition of endotoxin [6–9].

The mononuclear cell apoptosis induced by cellulosic membranes observed *in vitro* resembles the spontaneous apoptosis described in normal monocytes *in vivo* [6]; however, the *in vitro* apoptosis induced by cellulosic membranes occurs after only 24 h of incubation [4]. In addition, it is unclear whether this represents spontaneous apoptosis since interactions between mononuclear cells and cellulosic membranes result in mononuclear cell activation [2].

Caspases belong to a specific family of cysteine proteases that are involved in the apoptotic cascade and lead to proteolysis of specific substrates and morphological changes associated with monocyte-programmed cell death [12,13]. Although spontaneous apoptosis in normal monocytes is mediated by a caspase-3-dependent pathway [6], virus-induced apoptosis of monocytes is independent of caspase-3 activation [14]. Caspase-3 activation performs a number of functions, including activation of a latent cytosolic endonuclease, caspase-activated deoxyribonuclease that cleaves DNA into oligonucleosomal fragments [15–17].

To our knowledge, there have been no studies examining the intracellular mechanisms involved in haemodialysis-membrane-induced mononuclear-cell apoptosis. Furthermore, it is not known whether this form of apoptosis is caspase 3-dependent. Therefore, the aim of the present study was to evaluate whether apoptosis associated with cellulosic haemodialysis membrane exposure is dependent on caspase-3 activity and inhibited by pro-inflammatory cytokines, two features that are characteristic of spontaneous apoptosis.

Subjects and methods

Subjects

The present study included 14 haemodialysis patients: seven were dialysed with cellulosic membranes (Hemophan, GFS 20 Plus-Alwall, Gambro, Germany), and seven with non-cellulosic membranes (polysulphone HF80S, Fresenius, Germany). The mean age was 48.3 ± 15.8 years (range 20–67) and 44.4 ± 15.7 years (23–68) for cellulosic and non-cellulosic groups respectively. All patients underwent bicarbonate haemodialysis three times per week through arteriovenous fistulae (12 native and two arteriovenous grafts). The blood flow was 300–400 ml/min and the duration of dialysis was individually adjusted to maintain a Kt/V above 1.3. The two groups were not different in gender, time on dialysis, erythropoietin therapy, or aetiology of chronic renal failure. The type of membrane had remained unchanged during at least 3 months prior the study. Criteria for patient selection included the absence of acute or chronic infection, autoimmune disease, liver disease, diabetes or malignancy. Patients were not taking anti-inflammatory medication, calcitriol, or immune-suppressive therapy. Seven healthy volunteers participated as controls. Informed consent was obtained from all subjects after institutional approval.

Isolation of human mononuclear cells

Human monocytes were obtained from 20 ml of heparinized whole blood. In haemodialysis patients, a blood sample was drawn immediately before the first haemodialysis session of the week. Buffy coat cells were separated by differential centrifugation gradient (Ficoll/Hypaque; Pharmacia LKB, Uppsala, Sweden); mononuclear cells were washed and seeded in 24-well culture plates (Falcon, Becton-Dickinson, Paramus, New Jersey, USA) with complete culture medium as described below. Mononuclear cells were isolated after adherence to plates; adherent cells were removed using a cell scraper system (Falcon, Becton-Dickinson). A purity of $>75\%$ of cells was demonstrated by positive staining with anti-CD14 monoclonal antibody (Mab) (M5E2, Pharmingen, CA, USA). Contamination with $CD3^+$ and $CD19^+$ (Leu-4 and Leu-12, Becton-Dickinson, California, USA) lymphocytes was less than 8%.

Because isolated monocytes may have been contaminated by other leukocytes adhering to plastic, representative control experiments were performed in cells isolated by flow cytometry and sorted using a Mab against the CD14 molecule. In these experiments, the forward and side light-scatter analysis in the flow cytometer identified a gate for grouping mononuclear cells; other leukocytes were gated out.

Cell culture

Mononuclear cells were cultured at 37°C in complete culture medium containing RPMI 1640 supplemented with L-glutamine (2 mmol/l), HEPES (20 mmol/l), sodium pyruvate (1 mmol/l), streptomycin (50 ng/ml), penicillin (100 UI/ml), and 10% human autologous serum (Bio-Whittaker, Walkersville, Maryland, USA). Serum was heated to 56°C during a 60 min period to inactivate complement. Cells were seeded in 24-well microtitre plates (Falcon, Becton-Dickinson) at 5×10^5 cells/well. In some experiments, lymphokines and/or lipopolysaccharide (LPS) (100 ng/ml) were added to the culture medium at the onset of culture. LPS was obtained from *Escherichia coli*, strain 0127 : B8 (Sigma Chemical Co. Poole, UK). In some experiments, an inhibitor of the caspase-3 family, Ac-DEVD-CHO (Pharmingen) was added in a concentration of 100 nmol/l. To demonstrate direct effects of LPS and TNFα instead of through induction of cytokine production, additional experiments were performed using paxilline (5 μmol/l) (an inhibitor of LPS activity) and anti-TNFα mAb (50 U/ml). Both compounds were purchased from Sigma (Sigma Chemical Co. Poole, UK).

To determine the effect of haemodialysis membranes on caspase-3 activation, on apoptosis, or both mononuclear cells (2×10^6) from healthy donors were cultured for 16 h in 1 : 50-scale minidialysers with the same cellulosic and non-cellulosic membranes used by haemodialysis patients. These minidialysers were kindly provided by Hospal, SA.

Determination of caspase activity

Caspase-3 pro-enzyme is cleaved into an active form that is preferentially recognized by a rabbit anti-active caspase-3 polyclonal antiserum (Pharmingen). The active form of caspase-3 was measured by flow cytometry using phycoerythrin (PE)-conjugated polyclonal anti-human-active caspase-3 antibodies (Pharmingen). Mononuclear cells (5×10^5) were washed, fixed, and permeabilized using the FIX & PERM cell permeabilization kit (Caltag Laboratories, California, USA). Briefly, a 100-µl sample of cells was incubated with the fixation medium; after 5 min of incubation at room temperature, 4 ml of pre-cooled absolute methanol was added and incubated for 10 additional minutes at 4°C. Thereafter, cells were washed (5 min at 1500 r.p.m.) in wash buffer (PBS + 0.1% NaN_3 + 10% autologous serum) and permeabilized using 100 µl of permeabilization reagent. The cells were then incubated with 10 µl of PE-conjugated polyclonal anti-active caspase-3 antibody (Pharmingen) or the isotype control antibody. After incubation, cells were washed and resuspended in 0.5 ml of 1% formaldehyde and stored at 4°C until flow-cytometer analysis.

Cell apoptosis

Apoptosis-associated DNA strand breaks were analysed after 48 h culture using a kit based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) method (Boehringer Mannheim). In accordance with the manufacturer's instructions, 10^6 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then washed and permeabilized for 2 min in ice with 0.1% Triton X-100. After washing, cells were decanted and resuspended in 50 µl TUNEL reaction mixture (5 µl TUNEL enzyme, containing TdT, mixed with 45 µl TUNEL label, containing PE-dUTP and dNTP nucleotides) or in 50 µl TUNEL Label as negative control. After 60 min of incubation at 37°C in a humidified atmosphere, cells were washed three times in wash buffer (PBS + 0.1% NaN_3 + 10% autologous serum) and analysed by flow cytometry. While changes in caspase-3 activation occur in 4 h, molecular changes in DNA characteristics during apoptosis require 48 h.

Statistical analysis

Results are presented as means ± SE. Non-parametric data were compared by Kruskal–Wallis test. Comparisons between two means were analysed by Mann-Whitney test for unpaired data and by Wilcoxon singed-rank test for paired data. Differences were considered significant when $P<0.05$.

Results

Expression of caspase-3 active form by incubated mononuclear cells from patients dialysed with cellulosic membranes

After a 48-h incubation period, the percentage of mononuclear cells expressing the active form of caspase 3 was greater in patients dialysed with cellulose membranes than in patients using non-cellulosic membranes or in healthy controls (Figure 1A). The increase in caspase 3 activity in mononuclear cells from patients dialysed with cellulosic membranes was associated with a high percentage of apoptosis. Mean percentages of apoptotic cells in mononuclear cells

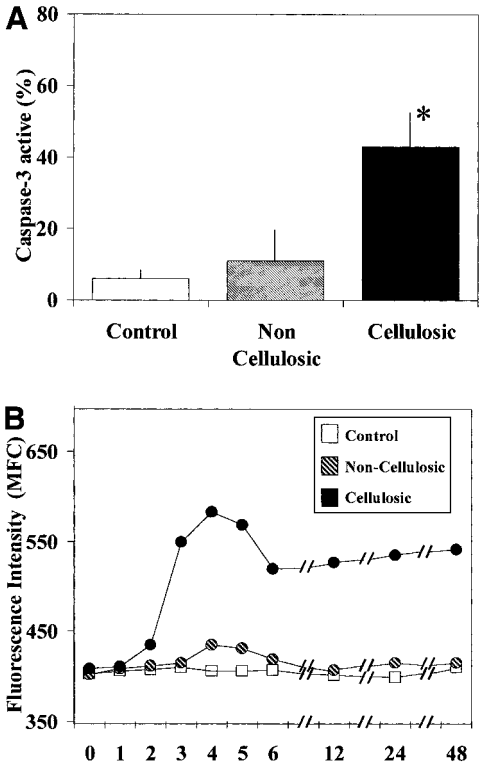

Fig. 1. Caspase-3 active form of mononuclear cells from control subjects (□), and dialysed patients treated with non-cellulosic membranes (▨) or cellulosic membranes (■). (**A**) Percentage of cells expressing the active form of caspase-3 in cells from controls ($n=7$) and from patients dialysed with non-cellulosic ($n=7$) and cellulosic ($n=7$) membranes (*$P<0.001$ *vs* control and non-cellulosic). (**B**) The time-course per cell of the caspase-3 active form throughout 48 h of cell culture ($n=3$). The level of expression per cell was measured in arbitrary units from the mean fluorescence channel (MFC ± SD).

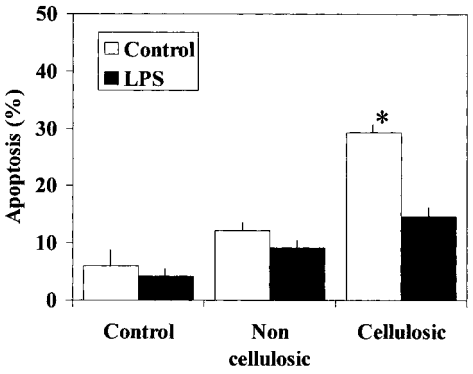

Fig. 2. Determination of apoptosis in cells cultured for a period of 48 h in complete medium alone ($n=7$) (□) or with LPS ($n=7$) (■). LPS significantly reduced the percentage of apoptosis in monocytes from cellulosic-membrane patients (*$P<0.001$).

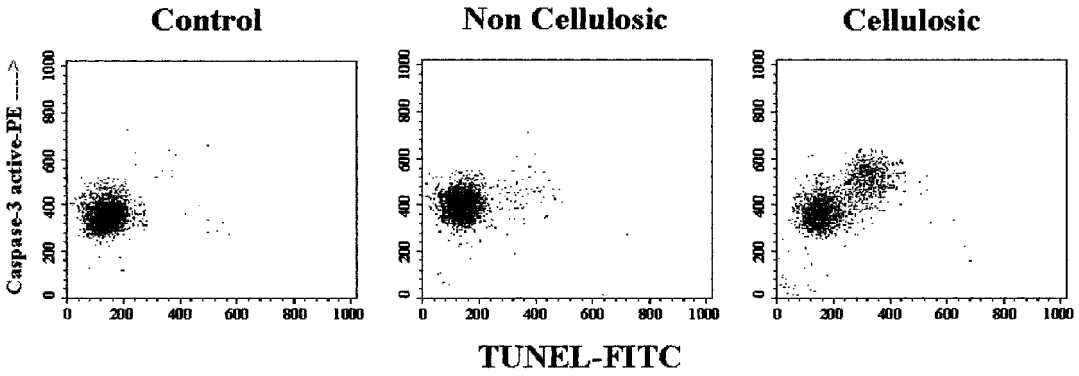

Fig. 3. Representative histograms of caspase-3 active form expression with simultaneous labelling of apoptosis.

Table 1. Effect of paxilline and anti-TNFα mAb on apoptosis and expression of caspase-3 active form induced by cellulosic membranes ($n=5$)

	Not-treated		Paxilline		Anti-TNFα	
	−LPS	+LPS	−LPS	+LPS	−LPS	+LPS
Apoptosis (%)	29 ± 5	14 ± 2*	32 ± 3	27 ± 5	36 ± 6	30 ± 2
Caspase-3 active (MFC)	582 ± 26	451 ± 18*	570 ± 15	534 ± 12	560 ± 7	503 ± 16

*$P<0.01$ *vs* not LPS treated.

from patients in cellulosic, non-cellulosic, and control groups were 29.31 ± 5.2, 12.1 ± 3.3, and 6.0 ± 4.1% respectively, and were significantly greater ($P<0.001$) in the cellulosic group than in the non-cellulosic and control groups.

A time course experiment in subjects dialysed with cellulosic membranes showed that average caspase-3 activity increased after 2 h in culture, peaked at 4 h, and remained elevated throughout the 48 h of culture (Figure 1B).

Apoptosis in mononuclear cells from patients dialysed with cellulosic membranes; effect of LPS

In an LPS-free medium, a high percentage of mononuclear cells from patients dialysed with cellulosic membranes underwent apoptosis after 48 h of culture (Figure 2). However, the addition of LPS (100 ng/ml), which is a survival stimulus, reduced the percentage of apoptosis from 29.31 ± 5.2 to 14.7 ± 2.1, $P<0.001$; this latter value was similar to the apoptosis percentage in mononuclear cells from patients using non-cellulosic membranes (12.1 ± 3.3%).

To clarify whether these effects on apoptosis were due to the direct effects of LPS and TNFα rather than through induction of cytokine production, additional experiments were performed using paxilline (5 μmol/l) (an inhibitor of LPS activity) and anti-TNFα mAb (50 U/ml). As shown in Table 1, both paxilline and anti-TNFα mAb prevented the inhibition of apoptosis by LPS in monocytes from cellulosic dialysed patients.

Role of caspase-3 activity in the apoptosis of mononuclear cells from patients dialysed with cellulosic membranes

Simultaneous labelling of caspase-3 activity and apoptosis showed that cells undergoing apoptosis had an increase in caspase 3 activity (Figure 3). To show a role of caspase-3 activity in mononuclear cell apoptosis induced by cellulosic membranes, caspase-3 activation was inhibited by the addition of Ac-DEVD-CHO, a caspase-3 family inhibitor. As shown in Figure 4, addition of Ac-DEVD-CHO at the beginning of the culture prevented not only caspase-3 activation but also apoptosis ($P<0.001$) (Figure 4).

In mononuclear cells from patients dialysed with cellulosic membranes, LPS prevented apoptosis through a caspase-3-dependent pathway

To determine whether the inhibition of apoptosis by LPS was mediated by a reduction in caspase-3 activity, the active form of caspase-3 was evaluated in mononuclear cells from cellulosic membrane patients during 48 h of incubation in the presence or absence of LPS. The active form of caspase-3 increased in cells cultured without LPS (from 468 ± 29 to 582 ± 26 at 4 h of culture, $P<0.01$). However, in LPS-treated cells, caspase-3 activity did not change during the 48-h culture (from 468 ± 29 to 451 ± 21) (Figure 5). This result suggests that the inhibition of apoptosis by LPS is due to a decrease in caspase-3 activity. The blocking

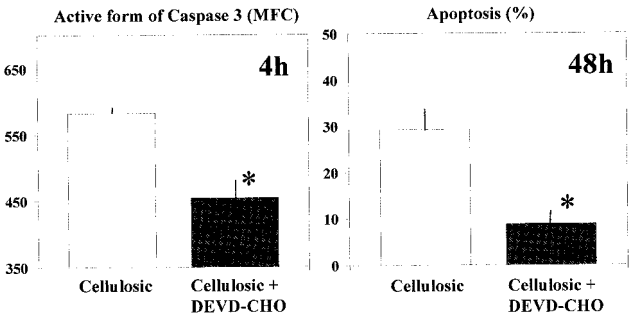

Fig. 4. The role of caspase-3 activity in the spontaneous apoptosis of monocytes from patients dialysed with cellulosic membranes ($n=7$). Monocytes were treated with the caspase-3 inhibitor DEVD-CHO (100 nmol/l). After 4 h of culture, the active form of caspase-3 (left panel) and the percentage of monocyte apoptosis (right panel) were reduced compared with control cells without DEVD-CHO. (*$P<0.001$) ($n=7$).

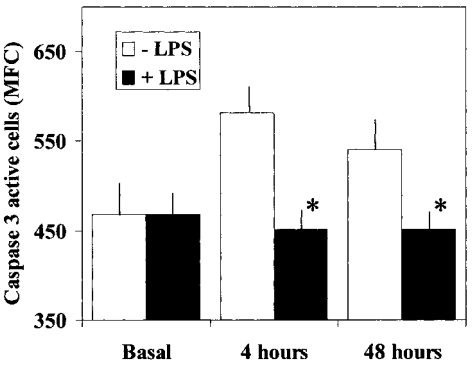

Fig. 5. Effect of LPS on caspase-3 activation. Mononuclear cells from patients dialysed with cellulosic membranes were cultured in complete medium alone (□) or with LPS (■)(100 ng/ml) ($n=7$). The active form of caspase-3, determined by flow cytometry using a specific PE-anti-caspase-3 antiserum, was decreased by LPS (*$P<0.05$ *vs* LPS untreated cells).

of soluble TNFα with anti-TNFα mAb resulted in a partial decrease in the caspase-3 activity induced by LPS. In addition, paxilline also induced a decrease in caspase-3 activity (Table 1).

In mononuclear cells from healthy subjects cultured with cellulosic membranes, there were increases in caspase-3 activation and apoptosis

To determine whether the cellulosic membrane was a direct cause of caspase-3 activation and the subsequent apoptosis, mononuclear cells (2×10^6) from healthy donors were cultured for 16 h in minidialysers made of cellulosic and non-cellulosic membranes (Figure 6). In cells cultured with cellulosic membranes, the active form of caspase-3 was increased as early as 8 h (537.3 ± 19.5, $P<0.05$) and remained elevated up to completion of the 16-h culture (532.1 ± 2.5, $P<0.05$) (Figure 6A). In contrast, increases in the active form of caspase-3 were not detected in cells cultured with non-cellulosic membranes at 8 h (433.2 ± 10.6) nor at 16 h (456.0 ± 6.5); these values were lower ($P<0.05$) than in cells cultured with cellulosic membranes. Increases in the active form of caspase-3 were accompanied by increases in the rate of apoptosis in cells cultured with cellulosic membranes at 8 h (31.0 ± 1) ($P<0.001$) and at 16 h (35.6 ± 3) ($P<0.001$) of culture. In contrast, only a moderate degree of apoptosis was observed in cells cultured with non-cellulosic membranes at 8 h (6.6 ± 2) (NS) and at 16 h (14.3 ± 2) ($P<0.05$) of culture, and these values were significantly lower ($P<0.05$) than in cellulosic membranes (Figure 6B).

Discussion

The characterisation of mechanisms involved in the different pathways protecting or inducing cell death may help us to understand the spontaneous apoptosis observed in mononuclear cells from chronic renal failure patients undergoing haemodialysis with cellulosic membranes [18,19]. In the present study, we investigated whether mononuclear cell apoptosis induced by cellulosic haemodialysis membranes is caspase-3 dependent and regulated by LPS, two characteristic features of spontaneous mononuclear-cell apoptosis [6,7,9–11,13]. As in previous observations, we found that mononuclear cells from patients dialysed with cellulosic membranes or from healthy controls cultured in the presence of cellulosic membranes underwent apoptosis when cultured in the absence of a survival stimulus [2,4,19]. In contrast, apoptosis was not observed in cultured mononuclear cells from patients dialysed with non-cellulosic membranes, nor in healthy controls cultured with or without the presence of non-cellulosic membranes.

Mononuclear cells from patients dialysed with cellulosic membranes showed time-dependent changes in activation of caspase-3 during culture. Similarly, cells from healthy controls cultured with cellulosic membranes also exhibited a progressive activation of caspase-3. However, caspase-3 activation was not observed in cells from patients dialysed with non-cellulosic membranes or in healthy controls cultured with or without the presence of non-cellulosic membranes. Although activation of caspase-3 occurs in spontaneous mononuclear-cell apoptosis [13], this contrasts with virus-induced apoptosis, which is independent of this caspase pathway [14]. The finding of increased apoptosis together with caspase-3 activation *in vitro* suggests that cellulosic membranes are associated with enhancement of spontaneous apoptosis. To confirm that apoptosis observed *in vitro* in mononuclear cells from patients dialysed with cellulosic membrane was caused by caspase-3 activity, cells were incubated with a PE-conjugated antibody anti-active human caspase-3. The addition of a caspase-3 family inhibitor, Ac-DEVD-CHO, to the culture inhibited *in vitro* apoptosis, suggesting that caspase-3 activation precedes the spontaneous mononuclear cell apoptosis that occurs with cellulosic membranes.

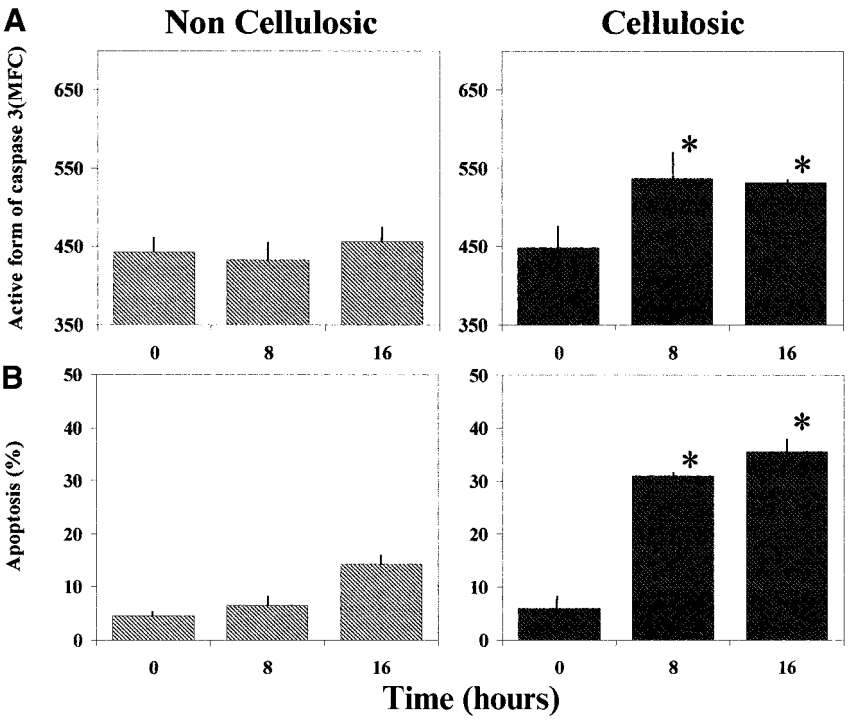

Fig. 6. (**A**) The active form of caspase-3 and (**B**) apoptosis in cells from healthy individuals cultured with cellulosic ($n=5$) and non-cellulosic minidialysers ($n=5$). The active form of caspase-3 and apoptosis were increased in cells cultured with cellulosic compared with non-cellulosic membranes ($^*P<0.05$).

Because Ac-DEVD-CHO also inhibited spontaneous apoptosis of mononuclear cells from healthy controls cultured with cellulosic membranes, this further supports the hypothesis that spontaneous monocyte apoptosis associated with cellulosic membranes utilizes a caspase-3-dependent pathway.

We had previously demonstrated that LPS prevents the spontaneous apoptosis observed in cultured mononuclear cells from haemodialysed patients using cellulosic membranes [20]. In agreement with this, other authors have shown that both bacterial products and pro-inflammatory stimuli increase the lifespan of normal mononuclear cells, and that apoptosis is favoured by anti-inflammatory stimuli [7,9–11]. In the present study we also demonstrated that LPS prevents increases in caspase-3 activation in cultured mononuclear cells from patients using cellulosic membranes. Similarly, Fahy *et al.* [6] have shown that spontaneous activation of caspase-3 in fresh blood monocytes is prevented by LPS. This effect of LPS on caspase-3 activation does not seem to be mediated by LPS-induced cytokine production, since the inhibition of caspase-3 activity by LPS was not altered by inhibition of LPS activity or by the addition of anti TNF antibodies (Table 1).

These findings provide new information about dialysis-membrane-induced monocyte apoptosis. The micro-inflammatory state of haemodialysis patients may be an important factor in the generation of cardiovascular damage. More precise knowledge of the mechanisms involved in apoptosis associated with haemodialysis may provide clues for the development of new technological approaches in haemodialysis therapy.

In conclusion, our results suggest that apoptosis in mononuclear cells induced by cellulosic haemodialysis membranes is mediated by the proteolytic activation of caspase-3 and is regulated by LPS. This pathway is thought to be similar to that observed during spontaneous apoptosis in normal monocytes.

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