Clin Exp Immunol 2004; **137**:166–172 doi:10.1111/j.1365-2249.2004.02503.x

Increased Fas-mediated apoptosis in polymorphonuclear cells from HIV-infected patients

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(Accepted for publication 8 April 2004)

SUMMARY

Neutrophils represent an important line of innate host defence against invading microorganisms and their functional detriment during HIV infection, including accelerated spontaneous cell death, has been shown to contribute to AIDS development. Neutrophils are susceptible to apoptosis via Fas and an interaction between Fas and FasL was suggested originally as a mechanism to explain constitutive neutrophil apoptosis. We have explored some intracellular pathways leading to PMN apoptosis from 28 HIV-infected patients and 24 healthy volunteers. As previously reported, accelerated spontaneous apoptosis was observed in HIV+ patients, but this did not correlate with viral load. Furthermore, an increase in the level of spontaneous apoptosis was detected in neutrophils from HIV-infected patients following inhibition of ERK, suggesting an impairment of this kinase pathway during the early stages of infection which may contribute to PMN dysfunction. An elevated susceptibility to undergo apoptosis was observed following cross-linking of Fas, which correlated both with viral load and co-expression of Fas/FasL surface molecules. Different mechanisms for spontaneous and Fas-induced apoptosis are proposed which together contribute to the neutropenia and secondary infections observed during the progression to AIDS.

Keywords apoptosis ERK Fas/ FasL HIV neutrophils polymorphonuclear cells

INTRODUCTION

During the immune response against viral infections, both specific and innate defence mechanisms are engaged sequentially in order to prevent disease progression [1]. Human neutrophils constitute an important line of innate host defence against invading microorganisms and the functional detriment of neutrophils during HIV infection, including their accelerated spontaneous cell death, has been demonstrated to contribute to AIDS development [2–5].

Neutrophils in the circulation have a life span of several hours, after which time they enter the spleen and liver and eventually die by apoptosis, or programmed cell death. Once neutrophils are recruited to this process, they are non-functional and lose the ability to move by chemotaxis, generate a respiratory burst or degranulate [6,7]. Conversely, during inflammation, neutrophils released from the bone marrow migrate rapidly from the blood to areas of acute inflammation, where they function as phagocytic cells and have the potential to cause tissue damage as a consequence of their high oxidative and proteolytic activity. In order to resolve inflammation, these cells should then be removed from the site of injury by apoptosis, a process to which neutrophils are constitutively programmed, so limiting their proinflammatory potential [8,9].

A number of *in vitro* studies have demonstrated that several factors can accelerate or suppress neutrophil apoptosis, including inflammatory cytokines which delay apoptosis and the tumour necrosis factor (TNF) family of pro-apoptotic proteins [10–12]. The CD95/Fas/Apo1 receptor is a 45 kDa transmembrane protein member of the TNF/nerve growth factor receptor superfamily [13]. The receptor mediates apoptosis when triggered by agonistic antibodies or its cognate oligomerizing ligand, Fas ligand (FasL), a type II transmembrane protein with a mass of 40 kDa, expressed on cell membranes or in soluble form. FasL also belongs to the TNF family and its related cytokines [12,14].

Neutrophils are susceptible to Fas-induced apoptosis [15–17] and an interaction between Fas and FasL was suggested originally to represent a mechanism to explain constitutive neutrophil apoptosis [16], although this theory has been opposed by others [18]. Neutrophils express significant levels of Fas [16,17] and there are some reports about expression of FasL on their surface [16,19].

The intracellular signalling pathway leading to granulocyte apoptosis, engaged through Fas/FasL, is not understood clearly. The mechanism of apoptosis induction is related closely to the

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cascade of cysteine proteases known as caspases, which represent a group of enzymes responsible for the initiation and execution of apoptosis [20], and the Bcl-2 family of proteins, which are internal key regulators of cell fate [21,22].

Another remarkable component to be considered in this study is the MAPK pathway, important mediators of survival and cell death signals. MAPKs are extracellular signal-regulated protein kinases that are influenced by many types of cell surface receptors. A common feature of all MAPK isoforms is the phosphorylation of both threonine (Thr) and tyrosine (Tyr) residues. Once activated, MAPKs are phosphorylated and activate other kinases or nuclear proteins, such as transcription factors, in either the cytoplasm or the nucleus. Phosphorylation of proteins, including activation of MAPK, may thus be important in controlling the activation of the various neutrophil processes required for their function in host defence but it may also be important in the pathways regulating neutrophil cell death and survival. Supporting evidence (generally via detection of the active forms of these kinases, using antibodies that detect only the phosphorylated proteins in Western blots) for the involvment of p42/44 extracellular signal-related protein kinase (ERK), p38 MAPK and c-Jun N-terminal kinase/stress-activated MAPK (JNK) in neutrophil apoptosis has been reported [6]. The functional role of these enzymes can be studied by using inhibitors; for instance, PD98059 inhibits MEK, a kinase whose substrate is ERK, thereby preventing the phosphorylation and activation of the ERK kinase [23], and SB203580 which is used to inhibit p38MAPK [24].

We have explored some intracellular pathways leading to constitutive and Fas-mediated apoptosis in neutrophils from HIV-infected patients. As reported previously, accelerated spontaneous apoptosis was observed in PMNs from HIV+ patients, but this did not correlate with viral load. A further increase in spontaneous apoptosis was detected in neutrophils from HIV-infected patients following inhibition of ERK activity, suggesting an impairment of this pathway during the early stages of infection which may contribute to PMN dysfunction and disease progression. Additionally, an elevated susceptibility to undergo apoptosis was observed in PMNs from these patients following cross-linking of Fas with a specific anti-Fas antibody; this Fas-induced increased level of apoptosis correlated with viral load. The mechanisms of spontaneous and Fas-induced apoptosis during HIV infection seem to be different, although a simultaneous occurrence cannot be excluded, and together they may contribute to the neutropenia and secondary infections observed during progression to AIDS.

MATERIALS AND METHODS

Media and reagents

Complete media consisted of RPMI-1640 supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin; all reagents were purchased from GIBCO Life Technologies, Inc (Paisley, UK). Mouse antihuman Fas MoAb (Clone DX2) FITC-conjugated, mouse antihuman FasL MoAb (NOK-1) biotin-conjugated, propidium iodide (PI), were purchased from PharMingen, San Diego, CA, USA. Phorbol 12-myristate 13-acetate (PMA), streptavidin-PE, PD98059 (ERK MAPK inhibitor) and SB202190 (p38 MAPK inhibitor), were purchased from Calbiochem, San Diego, CA, USA. Anti-p38, anti-ERK, antiphospho-p38, antiphospho-ERK and annexin V kit, were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Mouse IgG1 FITC-conjugated, PE and PerCp and TriTest (anti-CD4, anti-CD8, anti-CD3), were purchased from Becton Dickinson, San Jose, CA, USA. The monoclonal antibody IgM Anti-Fas (CH-11) was purchased from Upstate Biotechnology Inc., Lake Placid, NY, USA.

Human blood samples

Peripheral blood samples were obtained from 24 controls and 28 HIV-positive patients. The diagnosis of HIV infection was established by enzyme-linked immunosorbent assay (ELISA) and Western blot (Organon Teknika, Durham, NC, USA). The viral load was established by Amplicor Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). Disease classification was according to the Centers for Disease Control (CDC) criteria. All patients had 200 or more $CD4^+$ cells/mm^3 (840 cells/$mm^3 \pm 366$ s.d.), with a viraemia of 133 801 ± 201 695 s.d. HIV-1 RNA copies/ml. The mean ages in years (± s.d.) of the study subjects were as follows: 35 ± 10 for the control group and 32 ± 9 for the HIV+ patients. The experimental protocol was approved by the ethics committee of the University of Los Andes, and written informed consent was obtained from all the subjects.

Isolation of PMNs

Citrated venous blood was mixed with 6% dextran solution (mol wt 500 000) and incubated at room temperature for 30 min. The leukocyte-enriched supernant was collected, diluted with RPMI-1640 and layered onto Ficoll-Hypaque (1077, Sigma, St Louis, MO, USA). After density gradient centrifugation at 400 ***g*** for 30 min, PMNs were obtained from the bottom. The red blood cells contained in PMN pellets were lysed by hypotonic lysis using cold distilled water. This procedure resulted consistently in a highly purified polymorphonuclear cell population (98%), as visualized with acridine orange. Purified polymorphonuclear cells (98% viable by trypan blue exclusion) were resuspended at 2×10^6 cells/ml in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin.

Flow cytometry analysis of surface molecules

To analyse Fas expression, isolated PMNs were incubated with or without 100 ng/ml of PMA at 37°C for a time–course experiment, starting from 15 min up to 60 min. A steady expression of the marker was observed at 60 min. After incubation, cells were washed twice with cold phosphaye buffered saline (PBS) and stained with a monoclonal anti-Fas FITC-conjugated antibody for 30 min at 4°C, washed twice with cold PBS–EDTA and fixed in a PBS solution containing 1% paraformaldehyde for 10 min at room temperature. Cell surface expression of FasL was assessed by indirect immunofluorescense flow cytometry. In brief, primary staining was performed by incubating PMA-treated or untreated cells with a monoclonal anti-FasL biotin-conjugated antibody, in a solution containing 1% bovine serum albumin (BSA) for 30 min at 4°C. Cells were washed twice with cold PBS–EDTA and incubated with streptavidin–PE for 30 min at 4°C, then washed twice with PBS–EDTA and fixed with 1% paraformaldehyde in PBS.

PMNs were analysed by three-colour flow cytometry using a FACSort (Beckton Dickinson, San José, CA, USA), following the manufacturer's instructions. Gating criteria used to identify the cell population were based on the forward scatter and Fas- or FasL-positive fluorescence parameters. Evaluation of expression was assessed by looking at the relative number of positive cells and the mean fluorescence intensity (MFI), estimated from the

mean channel numbers of Fas and FasL. Isotype control antibodies were used to separate positive and negative cells on FITC and PE channels. A minimum of 10 000 events were collected for each analysis.

Analysis of spontaneous and induced cell death

Apoptosis was measured in cells cultured in complete media for different times. To induce apoptosis via Fas, neutrophils were resuspended at 2×10^6 cells/ml in complete media and plated on a 48-well tissue culture plate, to which 200 ng/ml of anti-Fas antibody was added. Cells were incubated at 37°C and placed in a 5% CO_2 incubator for 4 h [25]. Both untreated and anti-Fas treated cells were cultured with or without ERK or p38 MAPK inhibitors during the total incubation time.

DNA cytometric analysis

DNA analysis was performed by using flow cytometry. Briefly, after culture cells were harvested, washed in PBS containing 1% BSA, fixed with 1% paraformaldehyde for 10 min at room temperature and permeabilized for 30 min with 0·05% of saponin in PBS/0·1% BSA. After centrifugation cells were resuspended in PBS–EDTA containing 10 μg/ml bovine RNAse and incubated for 15 min at 37°C. Finally, 10 μg/ml of propidium iodide was added to the mixture. The percentage of the hypodiploid DNA (% apoptosis) in the pre-G_0/G_1 phase of the cell cycle was measured by FACSort flow cytometry (Becton-Dickinson Immunocytometry Systems, Mountain View, CA, USA)

Determination of apoptosis using annexin V

The annexin V-FITC staining procedure was conducted according to the manufacturer's instructions. Briefly, treated and untreated cells were collected by low-speed centrifugation, washed twice with cold PBS and resuspended in assay buffer at a concentration of 1×10^6 cells/ml. From these suspensions, 100 μl aliquots were incubated with 1 μg of annexin V-FITC and 10 μg of propidium iodide for 15 min at room temperature and analysed immediately by flow cytometry.

Protein immunoblotting

PMNs (30×10^6 cells/ml) were lysed in buffer A containing 50 mM TrisCl, pH 8, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin/aprotinin and 1 mM sodium orthovanadate and incubated on ice for 15 min; lysates were clarified by centrifugation at 14 000 ***g*** for 10 min at 4°C. Supernatants containing equivalent amounts of protein (Bradford, Bio-Rad), were mixed in sample buffer, heated in a boiling water bath for 3 min, separated by electrophoresis on 10% sodium dodecyl sulphate (SDS) polyacrylamide gels, transferred to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA, USA) and probed with different antibodies: anti-ERK, antiphospho-ERK, antip38 and antiphospho-p38. The antibody-labelled protein bands were detected by enhanced chemiluminescense (SuperSignal, Pierce, Rockford, IL, USA).

Statistical analysis

The significance of differences between spontaneous or induced apoptosis and Fas/FasL expression in PMNs from healthy controls and HIV-infected patients was calculated by Student's *t*-test. Correlations between spontaneous or Fas-induced apoptosis and $CD4^+/CD8^+$ counts, viral load and Fas/FasL expression were analysed by linear regression. The effects of anti-Fas and MAPK inhibitors on neutrophil apoptosis from controls and HIV-infected patients were analysed by the paired *t*-test. In all cases, $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Fas/FasL expression in PMNs from controls and HIV+ patients

It has been reported that Fas molecules are expressed constitutively on the surface of many cells, including PMNs [16,26], but that its ligand is expressed at very low levels [27,28]. To determine whether the previously observed increase in apoptosis was secondary to engagement of the Fas/FasL death receptor pathway, we first evaluated expression of both markers in resting and PMA-stimulated neutrophils. High constitutive levels of Fas were detected on PMNs from all studied individuals (patients and controls). A remarkable down-regulation of Fas molecule expression was observed on PMNs from both controls and HIV+ patients after 15 min incubation with PMA, but levels partially recovered by 60 min incubation time. No differences were observed in both Fas percentage and density expression when comparing HIV+ patients with healthy donors (Fig. 1a).

In contrast, FasL was expressed at low concentrations in unstimulated PMNs from both groups of subjects (Fig. 1b). A noticeable PMA-induced increase in FasL expression was observed in PMNs from HIV+ patients, demonstrating a statistically significant difference in comparison with the healthy donors ($P < 0{\cdot}05$) (Fig. 1b). Analysis of co-expression of Fas and FasL showed that both molecules were simultaneously present on neutrophils from HIV+ patients ($P < 0{\cdot}05$) (Fig. 1c).

Detection of spontaneous and Fas-induced apoptosis in neutrophils from HIV+ patients

Propidium iodide and annexin V staining were used to determine DNA fragmentation and phosphatidylserine exposure, respectively, both characteristics of apoptosis. It has been published that apoptosis of PMNs can be induced through the interaction of Fas with its receptor FasL, or by the activation of Fas after cross-linking with an anti-Fas antibody [16,29]. To determine whether such an event might be influenced by HIV infection, neutrophil suspensions, isolated from controls and HIV+ patients, were cultured in complete media (RPMI-1640, 10% FCS) and left either unstimulated or stimulated with anti-Fas IgM (CH-11) at 200 ng/ml final concentration. Following a time–course experiment, apoptosis values were detected at 1, 4 and 6 h. Apoptosis levels detected at 6 h were higher than those observed at 4 h but the differences in apoptosis levels between patients and controls were equivalent at the different time-points, as an increase in apoptosis in cells from the control group was also detected (data not shown). As reported previously, neutrophils from HIV-positive patients showed increased constitutive DNA fragmentation, evidenced by propidium iodide staining (Fig. 2a) as well as by annexin V labelling (data not shown), compared with control individuals ($P < 0{\cdot}05$) [5,30], which did not correlate with viral load ($r = 0{\cdot}05$; $P = 0{\cdot}5$).

As illustrated in Fig. 2a,b, an increase in the level of apoptotic neutrophils from HIV+ patients, stimulated by cross-linking of Fas, was observed ($P < 0{\cdot}05$). A statistically significant correlation was observed between Fas-induced apoptosis and viral load ($r = 0{\cdot}99$; $P < 0{\cdot}03$) in this group of patients. A positive correlation between Fas-induced apoptosis and co-expression of Fas/FasL molecules was also demonstrated ($r = 0{\cdot}78$; $P < 0{\cdot}01$)

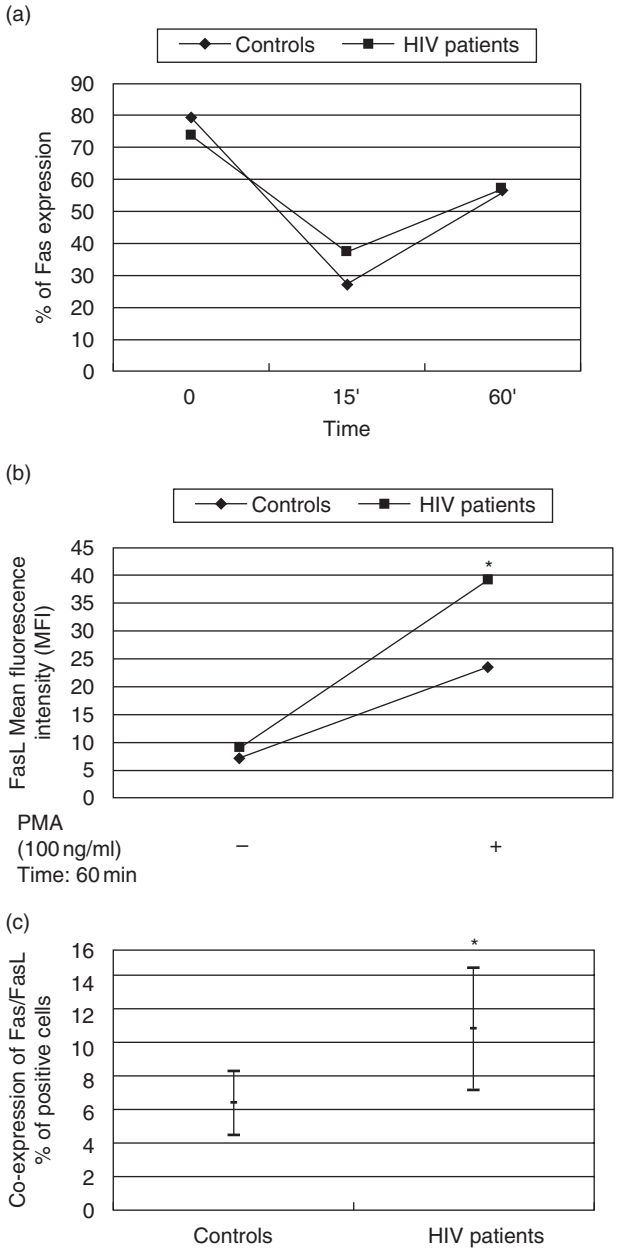

Fig. 1. Expression of Fas/FasL molecules on the surface of PMNs from HIV-positive patients and a control group. (a) Fas expression at two different times following incubation with PMA. Fas expression had dropped at 15 min and partially recovered by 60 min. No differences in expression were apparent between the two groups studied. (b) Mean fluorescence intensity of FasL expression on resting and PMA-stimulated PMNs from the control group and HIV+ patients ($7 \pm 0{\cdot}6$ *versus* $9{\cdot}1 \pm 2{\cdot}1$ resting; $23 \pm 3{\cdot}3$ *versus* $40 \pm 15{\cdot}3$ stimulated, confidence intervals, respectively). *Significant differences between the mean values of FasL fluorescence intensity on PMNs from HIV-positive patients compared with the control group. (c) Confidence intervals of Fas/FasL co-expression on resting PMNs from the control group and HIV+ patients. *Significant differences between Fas/FasL expression in HIV positive patients compared with the control group, analysed by Student's *t*-test.

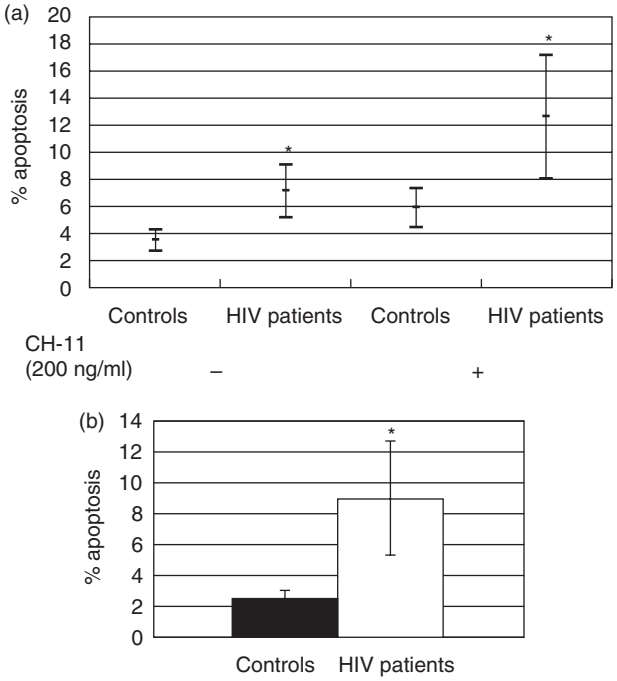

Fig. 2. Spontaneous and Fas-induced apoptosis in PMNs from HIV positive patients and controls. Apoptosis was determined by propidium iodide staining of hypodiploid DNA and analysed by flow cytometry, cell death was measured at 4 h. (a) Confidence intervals for the levels of constitutive and Fas-induced apoptosis, expressed as a percentage, are shown. *Significant differences between the mean values of apoptosis in the control group compared to the mean values in the HIV-positive patients. (b) Confidence intervals for the differences in apoptosis before and after incubation with CH11 antibody. A paired *t*-test was used to analyse the ability of Fas ligation to induce apoptosis, both in HIV-infected patients and the control group.

Role of the MAP kinase pathway in spontaneous and Fas-induced apoptosis in PMNs from HIV+ patients

There has been much interest in the role of ERK and p38 MAPK in the regulation of neutrophil survival and apoptosis, as MAPKs play a significant role in controlling the cell death machinery [11]. In order to explore the possible involvement of this pathway in the above described results, PMNs from healthy donors ($n = 8$) and asymptomatic HIV+ patients ($n = 10$) were treated with specific kinase inhibitors to block the activity of p38 (SB203580) and ERK (PD98059) in a time–course experiment. We observed that blocking ERK activity for 4 h, with PD98059 at a concentration of 50 μM, induced a considerable increase in the level of spontaneous apoptosis in PMNs within asymptomatic HIV+ patients ($P < 0{\cdot}01$) (Fig. 3). Inactivation of ERK was confirmed by immunoblotting experiments (Fig. 4). On the other hand, blocking p38 activity (10 μM 8EB203580) for 4 h did not result in any significant change in the level of neutrophil death (data not shown). The effects of inhibiting ERK and p38 activity were also tested following cross-linking with anti-Fas antibody, and no significant changes were observed (data not shown).

DISCUSSION

During HIV infection a gradual depletion and impairment of CD4 T lymphocytes [31], monocytes–macrophages [32], as well as

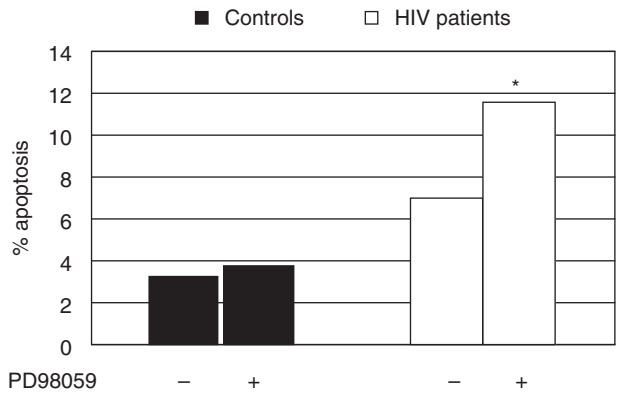

Fig. 3. Effects of an ERK inhibitor on constitutive apoptosis in PMNs from HIV-positive patients and controls. Mean values of apoptosis in untreated and treated neutrophils are shown. Cells were incubated with 50 μM of PD98059, a specific ERK inhibitor, for 4 h. A paired *t*-test was used to analyse the effect of inhibiting ERK activity on apoptosis in neutrophils from asymptomatic infected HIV+ patients and healthy donors. *Significant differences within the studied groups.

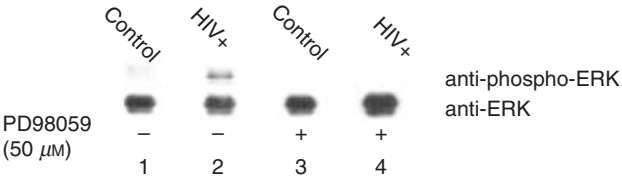

Fig. 4. Inactivation of ERK by the PD98059 inhibitor. Cell lysates from treated and untreated PMNs were separated in 10% SDS-polyacrylamide gel, transferred to poly(vinylidene difluoride) and probed with an antiphospho-ERK antibody (top). Membranes were stripped and reproved with an anti-ERK antibody (bottom) to show the amount of ERK protein. Lanes 1 and 3 are lysates from untreated and treated control neutrophils, respectively. Lanes 2 and 4 are cell lysates from untreated and treated neutrophils from HIV infected patients, respectively.

polymorphonuclear leucocytes [4], has been broadly demonstrated. One of the most important mechanisms to account for this cellular dysfunction and detriment during HIV infection is apoptosis [33,34], a process of non-inflammatory cell death, in which many elements such as death receptors and their ligands are involved [35].

We have explored both spontaneous and Fas-induced apoptosis in neutrophils from asymptomatic HIV-infected patients. As published previously [5,30], an increase in spontaneous apoptosis, starting 4 h after incubation, was demonstrated in neutrophils from HIV-infected patients, and this increase was not correlated with the viral load [30]. The underlying mechanism of this spontaneous accelerated apoptosis remains unknown.

Given the fact that neutrophils must be removed to resolve inflammatory reactions, it is probable that these cells undergo a process of self-suicide [16]. A mechanism that could explain such an event may be the simultaneous expression of Fas and FasL on their surface. In contrast to the wide tissue distribution of Fas molecules, constitutive expression of FasL is relatively limited. There are controversial reports about FasL expression on the surface of neutrophils; meanwhile, it is widely accepted that the Fas molecule is expressed constitutively on this cell type [16,27], suggesting a physiological role for this pathway in the neutrophil's life cycle.

Accelerated cell death in HIV-infected patients may be a consequence of elevated or deregulated expression of death receptors such as Fas/FasL. To explore this possibility we measured the surface expression of these molecules on resting and PMA-stimulated neutrophils. We did not find differences in either Fas receptor density or numbers of Fas-positive cells when comparing controls and asymptomatic HIV+ patients; however, elevated expression levels of FasL were demonstrated in PMA-stimulated neutrophils from HIV+ patients ($P < 0{\cdot}05$). Both molecules (Fas and FasL) were shown to be co-expressed in resting neutrophils from HIV+ patients ($P < 0{\cdot}05$). Taken together with the fact that the immune system of HIV+ patients is in a state of chronic activation, the co-expression of Fas and FasL in stimulated neutrophils from HIV+ patients suggests a mechanism whereby ensuing engagement of Fas and FasL on the cell surface initiates an autocrine accelerated cell death pathway.

To investigate further the significance of Fas/FasL expression, we used a specific monoclonal anti-Fas IgM antibody (CH-11) to induce apoptosis by Fas cross-linking. Purified neutrophils from asymptomatic HIV-positive patients showed an increase in Fas-induced-apoptosis after 4 h of incubation (Fig. 2a,b) which correlated with viral load. A positive correlation between Fas-induced apoptosis and co-expression of Fas/FasL molecules on resting PMNs was also demonstrated ($r = 0{\cdot}78$; $P < 0{\cdot}01$).

T cells from HIV-infected patients exhibit both increased Fas receptor expression and enhanced susceptibility to Fas-mediated cell death [36–38]. Importantly, only a minor fraction of apoptotic lymphocytes are physically infected by HIV, indicating that the enhanced apoptosis of lymphocytes seen in infected people could result from mechanisms other than direct infection [34]. Therefore, inflammatory non-HIV-infected cells such as neutrophils, which are recruited immediately to the site of inflammation, may be affected indirectly by viral proteins [39,40] and undergo apoptosis, thereby contributing to the risk of secondary infections. It is not clear why neutrophils from HIV-infected patients may have an increased susceptibility to undergo apoptosis via Fas. Neutrophils are not known to be infected by HIV but these, as other bystander cells, may be the target of HIV proteins, which are secreted by infected cells and could enhance their susceptibility to Fas-induced apoptosis [41,42]. Our data about a positive correlation between Fas-induced apoptosis and viral load suggest that the virus may manipulate the apoptotic machinery in these cells, which could explain the neutropenia observed during AIDS. Furthermore, it has been reported that incubation of the cell line HL60 (neutrophil, promyelocytic leukaemia) with purified Nef induces apoptosis [40]. A further study to explore the Fas death receptor pathway, including apoptotic and anti-apoptotic substrates (PI3K, ROS, Bcl-2 family, caspase activity and MAPK signal transduction) may help to understand the mechanism of neutrophil death during HIV infection.

Previously, sustained phosphorylation and activation of p38-MAPK has been observed during the programme of spontaneous apoptosis in normal neutrophils [11,43]. Signalling throughout the MAPK cascade has been studied, in part, by incubating living cells with specific kinase inhibitors. Using this approach, we showed that constitutive apoptosis is increased following inhibition of ERK activity in PMNs from HIV-infected patients, suggesting that the ERK pathway may be affected indirectly in PMNs from HIV-infected patients, thereby resulting in an imbalance of

apoptosis/survival signals in these cells. Our results also showed that ERK activity does not seem to be involved in Fas-induced apoptosis during HIV infection.

There are some contradictions about the role of MAPK in neutrophil apoptosis, thus a role for p38MAPK in constitutive apoptosis has been shown by using kinase inhibitors and antisense technology [43]; however, this finding has not been confirmed in other reports, which suggests instead that p38MAPK contributes to neutrophil survival [44]. We have failed to show any involvement of p38MAPK, although this result may be difficult to explain and one could expect an increase in the activity of this kinase to explain the elevated apoptosis levels seen in HIV-infected patients; however, the role of p38, ERK and JNK kinases in neutrophil apoptosis and survival is complex and not completely understood. Further studies to investigate the significance of these observations are required.

It is important to mention that regulation of the MAPK pathway also involves the dynamic interplay between kinases and phosphatases. Because kinase-mediated protein phosphorylation is utilized in every aspect of cellular function, the requirement that the extent and amplitude of this action be tightly controlled is essential. Recent studies have demonstrated the importance of phosphatase participation in such essential regulatory processes [45].

Another important survival signal to be considered within the hallmark of neutrophil cell death is that dependent on survival factors, the participation of which may also modulate apoptosis in granulocytes. It has been demonstrated that granulocyte colony stimulation factor (G-CSF) suppresses apoptosis by promoting cell survival and that incubation of neutrophils with G-CSF can reverse the accelerated constitutive apoptosis observed during HIV infection [5]. Additionally, nerve growth factor (NGF), a neurotrophic polypeptide, has been shown to enhance survival, phagocytosis and superoxide production of murine neutrophils [46]. Therefore, lack or deficiency of any of these survival factors as a consequence of HIV infection may contribute to accelerated apoptosis in this and other cell types, such as B cells, where low levels of NGF may be associated with the death of memory B lymphocytes in HIV-1 infection [47].

Finally, the mechanisms of spontaneous and Fas-induced apoptosis seem to be different but a simultaneous occurrence cannot be excluded, and together they may contribute to the neutropenia and neutrophil dysfunction observed during progression to AIDS.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from FONACIT, code S1-2000000522 and from the CDCHT-ULA, code M-655-99-07-B. We would also like to thank FUNDESI for referring their patients to our institution.

REFERENCES

1 Levy JA. The importance of innate immune system in controlling HIV infection and disease. Trends Immunol 2001; **22**:312–6.
2 Munoz JF, Salmen S, Berrueta LR *et al.* Effect of human immunodeficiency virus type 1 on intracellular activation and superoxide production by neutrophils. J Infect Dis 1999; **180**:206–10.
3 Ellis M, Gupta S, Galant S *et al.* Impaired neutrophil function in patients with AIDS or AIDS-related complex: a comprehensive evaluation. J Infect Dis 1988; **158**:1268–76.
4 Pitrak DL, Bak PM, DeMarais P, Novak RM, Andersen BR. Depressed neutrophil superoxide production in human immunodeficiency virus infection. J Infect Dis 1993; **167**:1406–10.
5 Pitrak DL, Tsai HC, Mullane MK, Sutton SH, Stevens P. Accelerated neutrophil apoptosis in the acquired immunodeficiency syndrome. J Clin Invest 1996; **98**:2714–9.
6 Akgul C, Moulding DA, Edwards SW. Molecular control of neutrophil apoptosis. FEBS Lett 2001; **487**:318–22.
7 Kobayashi SD, Voyich JM, Braughton KR, DeLeo FR. Down-regulation of proinflammatory capacity during apoptosis in human polymorphonuclear leukocytes. J Immunol 2003; **170**:3357–68.
8 Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 2000; **80**:617–53.
9 Savill J. Apoptosis in resolution of inflammation. J Leukoc Biol 1997; **61**:375–80.
10 Keel M, Ungethum U, Steckholzer U *et al.* Interleukin-10 counterregulates proinflammatory cytokine-induced inhibition of neutrophil apoptosis during severe sepsis. Blood 1997; **90**:3356–63.
11 Klein JB, Rane MJ, Scherzer JA *et al.* Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. J Immunol 2000; **164**:4286–91.
12 Liles WC, Klebanoff SJ. Regulation of apoptosis in neutrophils – Fas track to death? J Immunol 1995; **155**:3289–91.
13 Nagata S, Golstein P. The Fas death factor. Science 1995; **267**:1449–56.
14 Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 1999; **17**:331–67.
15 Watson RW, O'Neill A, Brannigen AE *et al.* Regulation of Fas antibody induced neutrophil apoptosis is both caspase and mitochondrial dependent. FEBS Lett 1999; **453**:67–71.
16 Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ. Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. J Exp Med 1996; **184**:429–40.
17 Ohashi M, Iwase M, Nagumo M. Changes in susceptibility to Fas-mediated apoptosis during differentiation of HL-60 cells. J Leukoc Biol 2000; **67**:374–80.
18 Fadeel B, Åhlin A, Henter JI, Orrenius S, Hampton MB. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. Blood 1998; **92**:4808–18.
19 Hsieh SC, Huang MH, Tsai CY *et al.* The expression of genes modulating programmed cell death in normal human polymorphonuclear neutrophils. Biochem Biophys Res Commun 1997; **233**:700–6.
20 Reed JC. Mechanisms of apoptosis. Am J Pathol 2000; **157**:1415–30.
21 Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998; **281**:1322–6.
22 Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998; **281**:1312–6.
23 Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J Biol Chem 1995; **270**:27489–94.
24 Cuenda AJ, Rouse J, Doza YN *et al.* SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 1995; **364**:229–33.
25 Frasch SC, Nick JA, Fadok VA, Bratton DL, Worthen GS, Henson PM. p38 mitogen-activated protein kinase-dependent and – independent intracellular signal transduction pathways leading to apoptosis in human neutrophils. J Biol Chem 1998; **273**:8389–97.
26 Jaber BL, Perianayagam MC, Balakrishnan VS, King AJ, Pereira BJ. Mechanisms of neutrophil apoptosis in uremia and relevance of the Fas (APO-1, CD95)/Fas ligand system. J Leukoc Biol 2001; **69**:1006–12.
27 Santos-Beneit AM, Mollinedo F. Expression of genes involved in initiation, regulation, and execution of apoptosis in human neutrophils and during neutrophil differentiation of HL-60 cells. J Leukoc Biol 2000; **67**:712–24.

28 Brown SB, Savill J. Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. J Immunol 1999; **162**:480–5.
29 Iwai K, Miyawaki T, Takizawa T *et al.* Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. Blood 1994; **84**:1201–8.
30 Baldelli F, Preziosi R, Francisci D, Tascini C, Bistoni F, Nicoletti I. Programmed granulocyte neutrophil death in patients at different stages of HIV infection. AIDS 2000; **14**:1067–9.
31 Kaplan D, Sieg S. Role of the Fas/Fas ligand apoptotic pathway in human immunodeficiency virus type 1 disease. J Virol 1998; **72**:6279–82.
32 Dockrell DH, Badley AD, Villacian JS *et al.* The expression of fas ligand by macrophages and its upregulation by human immunodeficiency virus infection. J Clin Invest 1998; **101**:2394–405.
33 Herbein G, Van Lint C, Lovett JL, Verdin E. Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. J Virol 1998; **72**:660–70.
34 Badley AD, Pilon AA, Landay A, Lynch DH. Mechanisms of HIV-associated lymphocyte apoptosis. Blood 2000; **96**:2951–64.
35 Nagata S. Fas ligand-induced apoptosis. Annu Rev Genet 1999; **33**:29–55.
36 Sloand EM, Young NS, Kumar P, Weichold FF, Sato T, Maciejewski JP. Role of Fas ligand and receptor in the mechanism of T-cell depletion in acquired immunodeficiency syndrome: effect on $CD4^+$ lymphocyte depletion and human immunodeficiency virus replication. Blood 1997; **89**:1357–63.
37 Estaquier J, Tanaka M, Suda T, Nagata S, Golstein P, Ameisen JC. Fas-mediated apoptosis of $CD4^+$ and $CD8^+$ T cells from human immunodeficiency virus-infected persons: differential *in vitro* preventive effect of cytokines and protease antagonists. Blood 1996; **87**:4959–66.
38 Badley AD, Dockrell D, Simpson M *et al.* Macrophage-dependent apoptosis of $CD4^+$ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor. J Exp Med 1997; **185**:55–64.
39 Okada H, Takei R, Tashiro M. Inhibition of HIV-1 Nef-induced apoptosis of uninfected human blood cells by serine/threonine protein kinase inhibitors, fasudil hydrochloride and M3. FEBS Lett 1998; **422**:363–7.
40 Okada H, Takei R, Tashiro M. HIV-1 Nef protein-induced apoptotic cytolysis of a broad spectrum of uninfected human blood cells independently of CD95 (Fas). FEBS Lett 1997; **414**:603–6.
41 Gougeon M. Apoptosis as an HIV strategy to escape immune attack. Nat Rev Immunol 2003; **3**:392–404.
42 Westendorp MO, Frank R, Ochsenbauer C *et al.* Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature 1995; **375**:497–500.
43 Aoshiba K, Yasui S, Hayashi M, Tamaoki J, Nagai A. Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils. J Immunol 1999; **162**:1692–700.
44 Alvarado-Kristensson M, Pörn-Ares MI, Grethe S, Smith D, Zheng L, Andersson T. p38 Mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities have opposite effects on human neutrophil apoptosis. FASEB J 2001; **16**:129–31.
45 Keyse SM. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr Opin Cell Biol 2000; **12**:186–92.
46 Kannan Y, Ushio H, Koyama H *et al.* 2.5S nerve growth factor enhances survival, phagocytosis, and superoxide production of murine neutrophils. Blood 1991; **77**:1320–5.
47 Titanji K, Nilsson A, Morch C *et al.* Low frequency of plasma nerve-growth factor detection is associated with death of memory B lymphocytes in HIV-1 infection. Clin Exp Immunol 2003; **132**:297–303.