

Down-modulation of CD4 antigen during programmed cell death in U937 cells

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It has been hypothesized that programmed cell death (PCD), an active cell suicide process occurring in place of necrosis, can be associated with the pathogenesis of acquired immunodeficiency syndrome (AIDS). The entry of human immunodeficiency virus (HIV) into competent cells is mediated by the CD4 molecule present on the surface of certain lymphocyte subpopulations as well as on some cultured cell lines, e.g. U937 myelomonocytic cells. The present paper focuses on some specific aspects of PCD induced by the cytokine tumor necrosis factor (TNF). The results obtained indicate that the exposure of U937 cells to cycloheximide facilitates TNF-mediated PCD via a *short term* cell death program and modifies the expression of CD4 surface molecules. This change in surface antigen expression, manifested by internalization of the CD4 molecule, occurs in cells in which apoptosis has been triggered, but not in cells undergoing necrosis. These results indicate that the progression of cell death could be associated with specific alterations of certain surface molecules and could have a role in the entry of HIV into cells.

Down-modulation; CD4; Apoptosis

1. INTRODUCTION

Programmed cell death (PCD) is defined as a cell suicide process which is an alternative to necrosis and is characterized by a specific series of intracellular events leading ultimately to DNA fragmentation in multiples of 200 base pairs. It is postulated that PCD, also termed apoptosis, must be considered as an active process since it needs protein synthesis to occur [1] and, in contrast to necrosis, is accompanied by membrane and organelle integrity. The apoptotic process can be induced experimentally *in vitro* by using certain chemical agents, e.g. tumor necrosis factor (TNF) [2] and can be prevented, in most cases, by protein synthesis inhibitors such as cycloheximide [3]. More recently, it has been hypothesized that PCD may play a central role not only in embryonic development, but also in neoplastic proliferation [2] and in the pathogenesis of acquired immune deficiency syndrome (AIDS) [4].

The lymphocyte differentiation antigen CD4 is expressed mainly by a subset of T-lymphocytes restricted to the class II major histocompatibility complex and on some cell lines of macrophage-monocyte lineage [5]. CD4 is also involved in several cell processes such as thymocyte ontogeny and peripheral T-cell activation as well as being the receptor for human immunodeficiency virus (HIV) [6]. The mechanisms regulating CD4 molecule expression are not clearly understood. However, it is believed that this antigen is normally excluded from

the endocytic pathway in lymphoid cells, but that it can be constitutively internalized and recycled in non-lymphoid cells and can be down-modulated in certain experimental conditions [7].

The aim of the present work was to investigate the importance of active protein synthesis during TNF-mediated PCD of HIV-responsive U937 cells and the antigenic characteristic of the plasma membrane during this process.

2. MATERIALS AND METHODS

2.1. Cells and treatments

U937 cells, a monomyelocytic human cell line, were grown at 37°C as previously reported [8]. 24 h after seeding, cell cultures were treated, by direct addition to the culture medium, with cycloheximide (CHX, 4 µM), a tumor necrosis factor (α TNF, 50 U/ml). Menadione (MEN, 2-methyl-napthoquinone) was diluted in DMSO and a concentration of 200 µM was used. Corresponding amounts of DMSO alone were considered as controls. All chemicals were purchased by Sigma. In order to evaluate the effects of protein synthesis inhibitors during apoptosis, cells were either first exposed for two hours to CHX and then to TNF which was added to the medium, or to CHX and TNF together. Samples were collected after 4 and 6 h of TNF treatment. Cells treated with CHX or TNF alone for the same lengths of time were considered as controls. In order to test the effects of prolonged exposure of the TNF alone, cells were treated with TNF for 96 h and demonstrated to undergo apoptosis, as previously reported [2]. In order to evaluate differences between necrosis and apoptosis, the effects of MEN (up to 3 h), which is capable of inducing necrosis [9], were also examined.

2.2. DNA gel electrophoresis

Cells, washed in PBS, were lysed in 0.5 ml of lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, pH 8.0, 0.2% Triton X-100) with proteinase

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K (100 $\mu\text{g/ml}$) for 1 h at room temperature and were centrifuged at 13,000 r.p.m. for 15 min, in order to separate high molecular weight chromatin from the nucleosomal DNA fragments. DNA was extracted and then separated overnight by electrophoresis on 1% agarose gel as previously described [8].

2.3. Flow cytometry

Fluorescence measurements were performed on a FACScan flow cytometer (Becton Dickinson) and data were recorded by Hewlett Packard computer using a specific FACScan research software. **DNA labeling:** about 10^6 cells were pelleted and fixed in 2 ml ice-cold 70% ethanol for 30 min. The cells were then centrifuged, washed in PBS and resuspended in 1 ml PBS containing 100 $\mu\text{g/ml}$ propidium iodide (Sigma), 1 mg/ml RNase (Sigma) and incubated at 37°C for 30 min. Red fluorescence ($> 610 \text{ nm}$) was recorded. Pulse shape analysis (pulse width and area) was performed in order to eliminate the signal from cell clumps. **Surface antigens labeling:** the antibodies used were: anti CD4-FITC and anti CD3-FITC (DAKO), anti CD71 (Boehringer Mannheim) and goat-anti mouse-FITC (F(ab')₂ specific) (Sigma). About 10^6 cells for each sample were washed in ice-cold PBS, pelleted and incubated with antibodies diluted 1:5 (50 μl of final suspension) for 30 min in an ice bath. For anti CD71, a second incubation with GAM-FITC (diluted 1:50) was performed. Finally, cells were washed 3 times in ice-cold PBS and green fluorescence (535 nm) was recorded. Supravital staining of cells was performed by adding propidium iodide before surface antigen analysis. Only viable cells which excluded propidium iodide were considered.

2.4. Fluorescence microscopy

Control and treated cells were seeded on glass coverslips coated with polylysine. For CD4 staining, after adhesion to the glass surface, cells were fixed at 4°C with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, washed and directly prepared for fluorescence or confocal microscopy (Sarastro 2000 confocal laser scanning microscope) observation. For intracellular localization, after fixation and washing, cells were permeated with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells were then incubated with CD4-FITC (Dako) at 37°C for 30 min. In order to evaluate PCD, Hoechst 33258 dye analysis was used [10]. After washing, all the samples were mounted with glycerol-PBS (1:1) and observed with a Nikon Microphot fluorescence microscope.

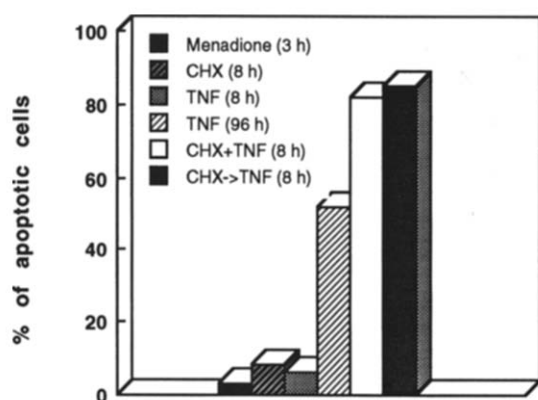


Fig. 1. Percent apoptosis in U937 cells after exposure to CHX (8 h), TNF (8 h), MEN (3 h), TNF (96 h), CHX together with TNF (CHX + TNF) (8 h), and CHX (2 h) followed by TNF (6 h) (CHX $\rightarrow$ TNF). Apoptosis was determined by counting the number of cells containing a nucleus with DNA fragmentation as determined by Hoechst staining with respect to the total number of cells examined (at least 200). Control cells are not shown since less than 2% of these cells are spontaneously apoptotic. The average value of each experiment, which was repeated at least three times, is shown.

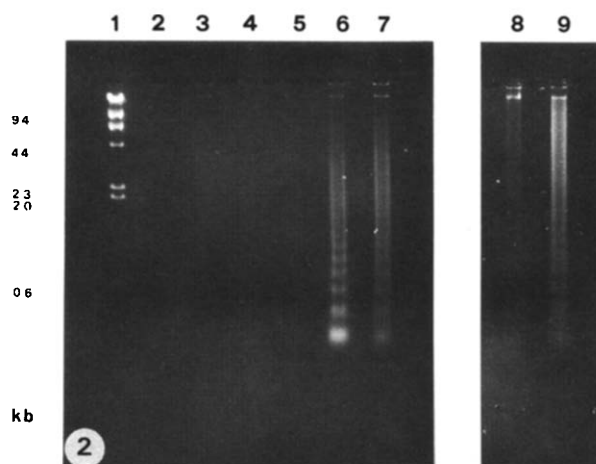


Fig. 2. DNA fragmentation in U937 cells. Untreated cells (lane 2 and lane 8), or cells treated with MEN 3 h (lane 3), CHX 8 h (lane 4), TNF 8 h (lane 5), CHX (2 h) $\rightarrow$ TNF (6 h) (lane 6), CHX together with TNF 8 h (lane 7), and TNF 96 h (lane 9). A HindIII digest of λ -DNA provided molecular weight standards (lane 1).

3. RESULTS

An analysis of cell death and accompanying surface antigen expression was carried out by using different methodological approaches. First, an evaluation of the temporal progression of TNF-induced PCD (ranging from 4 h to 96 h) with and without the presence of protein synthesis inhibitors (CHX) was conducted in U937 cells. Second, an analysis of CD4, CD3 and CD71 membrane antigenic expression as well as possible changes in the structural properties of the plasma membrane during apoptosis were carried out.

3.1. PCD analysis

Apoptosis induced by various experimental conditions was investigated in U937 cells by means of flow cytometry, gel electrophoresis and fluorescence microscopy. Neither CHX or TNF administered alone were capable of inducing significant PCD in the cultured cells after short time intervals (8 h) while TNF treatment could cause significant cell death after longer times (96 h). In fact, when either CHX or TNF alone were added to the cultures for 8 h, little PCD was observed (Fig. 1). As an additional control, oxidative stress-induced necrosis [9] was also evaluated in these cells by using the quinone menadione. Apoptotic cell death was not significant after 3 h of treatment with menadione (Fig. 1). In contrast, fluorescence analysis by Hoechst staining (not shown) displayed over 50% of apoptotic cells after 96 h of TNF treatment (Fig. 1). Base pair fragmentation was also confirmed by the results obtained by gel electrophoresis (Fig. 2). In addition, experiments in which CHX was either given together (8 h, CHX + TNF) or in sequence (2 h CHX followed by 6 h TNF, CHX $\rightarrow$ TNF) demonstrate that protein synthesis inhibition can increase the percentage of TNF-induced

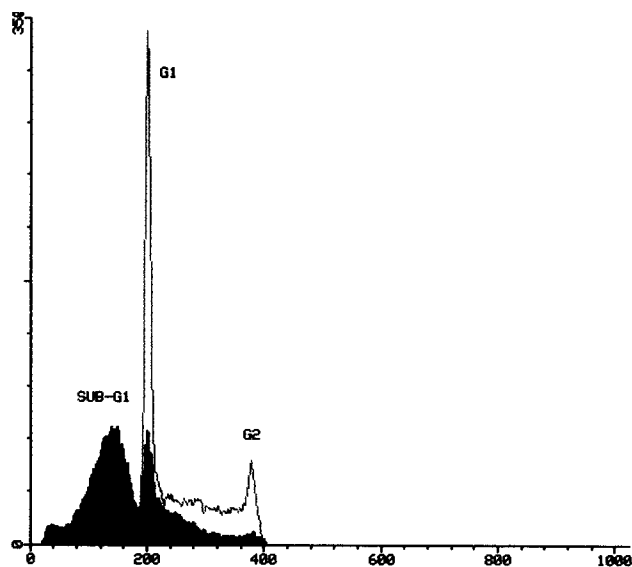


Fig. 3. DNA histograms obtained by flow cytometric analysis in U937 cells. Superimposed white profile of control cells and black profile of cells after treatment with CHX → TNF are shown. Sub G1 peak corresponds to cells undergoing PCD.

apoptosis in U937 cells (Figs. 1 and 2, Hoechst staining not shown). Apoptosis was also confirmed by flow cytometry analysis which showed the formation of the typical broad hypodiploid DNA peak (sub G₁ peak) typical of DNA base pair fragmentation (Fig. 3). Increasing the exposure times up to 12 h (2 h CHX → 10 h TNF) resulted in complete cellular disruption (not shown). Reversing the sequence (TNF followed by

CHX) was ineffective in inducing short-term (8 h) apoptosis.

3.2. Plasma membrane analyses

The expression of the CD3, CD4 and CD71 surface antigens was evaluated in U937 cells undergoing PCD induced by both protocols described above, as well as in the same cells undergoing necrosis. These antigens were chosen because they are important in normal cell function and because their expression follows different physiological pathways. Flow cytometry results demonstrate that these antigens are expressed in a different manner during the apoptotic process studied here. In fact, transferrin receptor (CD71) as well as CD3 expression remained unchanged in all apoptotic cells (Fig. 4A,B). In contrast, when the association CHX → TNF was used, the decrease of the CD4 molecule on the cell surface was much more significant ($P < 0.01$; Fig. 4C) with respect to short treatments with CHX or TNF alone (8 h and 8 h; Fig. 4D,E, respectively). In addition, in order to rule out the possibility that the redistribution of the CD4 receptors could merely be related to the cell injury process and in order to evaluate differences between PCD and necrosis, experimental analyses were also performed in cells undergoing menadione-induced necrosis [9]. In this case, no changes were detected in antigen expression by flow cytometry (Fig. 4F). Furthermore, as demonstrated by immunofluorescence confocal laser scanning microscopy (Fig. 5), a remarkable positivity for CD4 antigen was detected in the cell cytoplasm of cells exposed to CHX → TNF, with respect to control cells. In fact, fluorescent spots in the cell

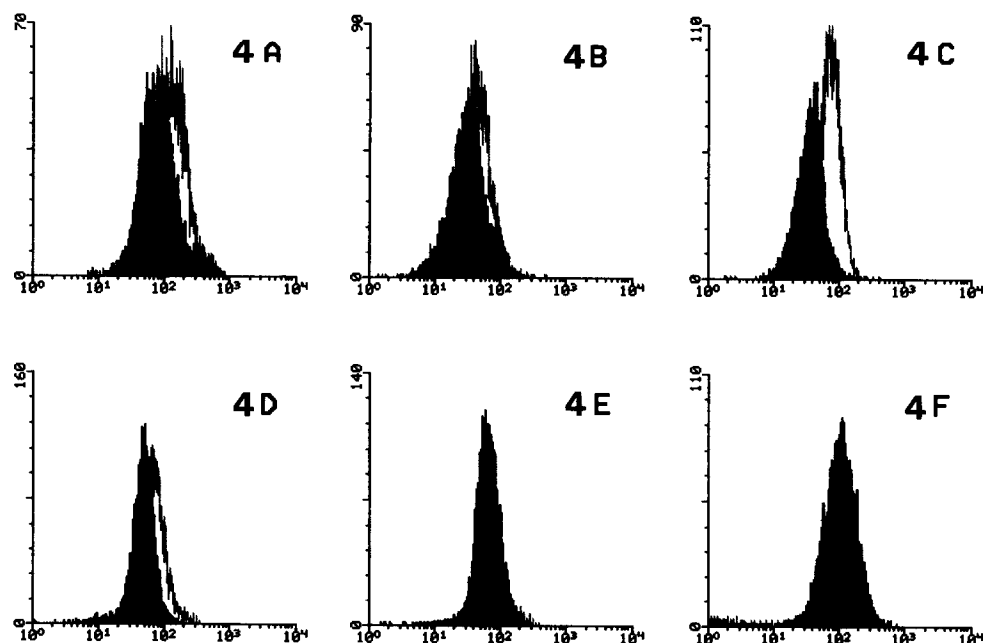


Fig. 4. Flow cytometric fluorescence histograms of U937 cells stained with anti-CD71 (A) anti-CD3 (B) and anti-CD4 (C, D, E and F). Overlay histograms (empty profiles) represent control cells while filled histograms represent cells treated with CHX → TNF (A, B and C), CHX (D), TNF (E) and menadione (F). In all the experiments 10,000 cells were analyzed using the same fluorescence gain.

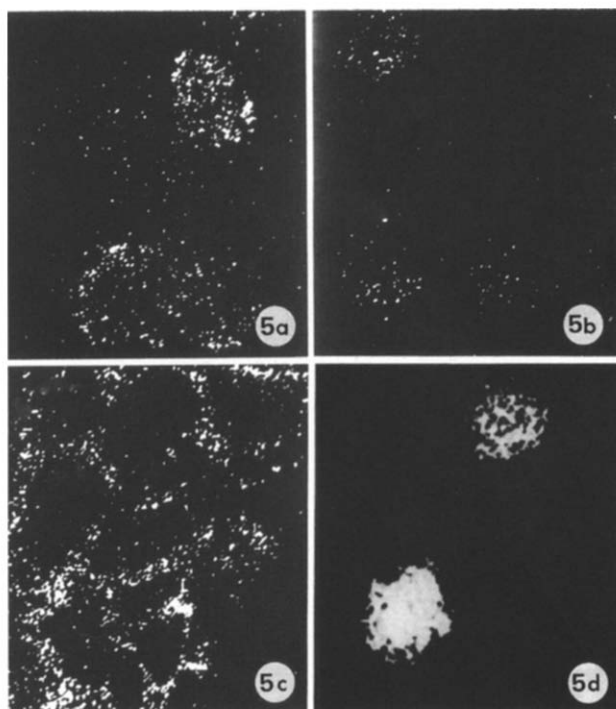


Fig. 5. Confocal laser scanning microscopy of unpermeated (a,c) and Triton-permeated (b,d) U937 cells. In control samples, a single full field optical section allowed visualization of scattered fluorescent spots representing CD4 antigen on either the surface of unpermeated cells (a) or marginalized in certain regions of the cytoplasm of permeated cells (b). In contrast, cells undergoing PCD after CHX → TNF treatment, displayed a marked decrease of CD4 fluorescence on the surface of unpermeated cells (c) while the intracellular presence of this antigen in permeated cells appeared notably increased (d).

cortical regions after both staining procedures, i.e. unpermeated (surface) and permeated (intracytoplasmic) cells (Fig. 5a,b) were visible, while fluorescent labeling of CD4 molecules in CHX → TNF-exposed cells undergoing PCD appeared completely modified, the antigen being easily observable intracellularly in permeated cells (Fig. 5c,d). This could signify that most of the CD4 molecules were internalized. Furthermore, in order to test the possibility that TNF-induced cell death could directly modify CD4 molecule regulation at different times, shorter exposure times with CHX and TNF

(CHX 2 h → TNF 4 h, Fig. 6A) and exposures of 48 and 96 h with TNF alone were conducted and CD4 as well as CD71 expression were evaluated. No changes were induced by this treatment on the CD4 molecule at 48 h (Fig. 6B) while at 96 h CD4 expression was modified (Fig. 6C). Finally, no variations of CD71 expression were noted at both time periods (not shown).

4. DISCUSSION

4.1. Apoptosis

The results reported in this paper suggest that a 'slow' PCD mechanism, induced by TNF alone, dependent on protein synthesis, and a 'fast' PCD process induced by CHX → TNF, independent of active protein synthesis, but still involving DNA fragmentation, can occur in U937 cells. In fact, various mechanisms of apoptotic cell death with different biochemical and structural features were previously hypothesized [10]. These cell death programs can be induced by certain compounds or experimental conditions and the possibility of 'fast' protein synthesis-independent PCDs as well as 'slow' death mechanisms were previously suggested [11,12]. Furthermore, the 'spontaneous' commitment to PCD occurring in some cell lines (e.g. in HL60 leukemic cells) must also be taken into account. The U937 line, widely used for in vitro maintenance of HIV since 1987 [5], displays a low number of cells undergoing spontaneous PCD. For this reason as well as for the fact that these cells are able to trigger *short term* apoptosis after CHX → TNF treatment, U937 cells can provide an interesting model for the study of the HIV-PCD association [13].

4.2. CD4 internalization

In contrast to human lymphocytes, T cell lines normally show very little endocytosis of CD4 molecule which in fact is not internalized efficiently [7]. However, as is demonstrated in this report, CD4 can be internalized quite efficiently by U937 cells. Thus, the study of U937 cells could demonstrate that CD4 internalization may be an important route for HIV entry into these cells. Furthermore, in view of the close relationship between HIV and PCD, it can be hypothesized that, in certain conditions, the commitment to PCD can play

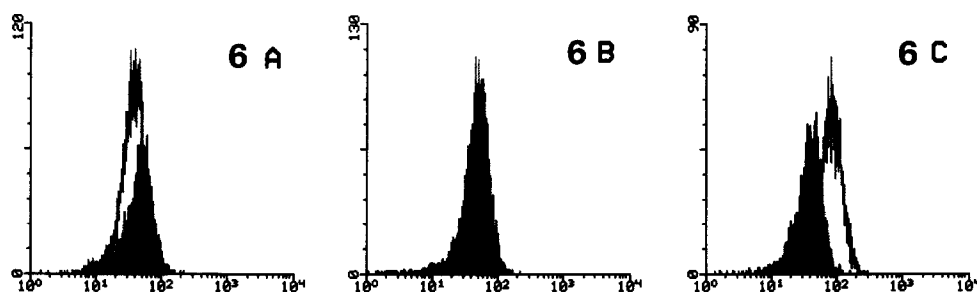


Fig. 6. Flow cytometric fluorescence histograms of U937 cells stained with anti-CD4. Overlay histograms (empty profiles) represent control cells. (a) Cells treated with the sequence CHX (2 h) → TNF (4 h). (b) Cells treated with TNF for 48 h. (c) Cells treated with TNF for 96 h.

an important role in HIV pathogenesis by a mechanism triggering CD4 internalization.

The CD4 molecule is a type I transmembrane protein which can interact with protein tyrosine kinase p56^{lck}, a member of the src family. It was previously observed that CD4 can be internalized via coated pits [7] and can be down-regulated in certain experimental conditions. In particular, modulation was demonstrated in human peripheral blood lymphocytes after stimulation with phorbol esters, anti-CD4 monoclonal antibodies, gangliosides or HIV [14,15]. The role of phosphorylation in causing the dissociation of p56^{lck} from the complex and determining the CD4 internalization still appears to be controversial [16].

The results reported here demonstrating a PCD-associated internalization of CD4 could be partially explained by considering relationships between reactive oxygen intermediates (ROI), PCD and HIV pathogenesis. In fact, it is known that TNF exerts some of its effects by stimulating production of ROI [17]. It can thus be hypothesized that TNF-induced ROI, as demonstrated previously for certain oxidizing agents [18], can contribute to CD4 internalization by activating p56^{lck}, or preventing its dephosphorylation. Accordingly, the role of protein kinase C (PKC) activity in the outcome of PCD has been considered [19], but has still to be elucidated. In addition, ROI are also capable of potently and rapidly activating the multisubunit transcription factor NFkB which is present in the cytoplasm in a non-DNA-binding form, thus releasing the inhibitory subunit Ikb from the other subunits (p65 and P50) [20]. The replication of HIV is under control of NFkB and the activation of this enhancer factor by TNF and protein synthesis inhibitors such as CHX could therefore lead to increased HIV expression [21].

In conclusion, the interrelationships between PCD and AIDS pathogenesis [4] as well as those between TNF exposure and viral progeny production [22] could partially be related to the *short term* apoptosis demonstrated here. In fact, specific apoptotic mechanisms could be permissive to HIV entry and infection. However, it must also be considered that other mechanisms could exist which render the PCD *abortive* and maintain the cells viability once HIV-CD4 internalization has occurred. This possibility of a block of the programmed cell death process can be plausible from a general point of view [3,23,24] and cannot be ruled out for HIV-related PCD. This block of PCD could be associated to the internalization of CD4 which, in turn, is due to a

protein tyrosine kinase activation or to a PKC activation, processes which can also be induced by TNF alone after longer treatment [18,25]. This could partially explain the role of CD4 in HIV entry and the chronic nature of HIV infection in competent cells.

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