HIV-1 Nef protein-induced apoptotic cytolysis of a broad spectrum of uninfected human blood cells independently of CD95(Fas)

Harue Okada[a], Ryoichi Takei[a,b], Masato Tashiro[a,*]

[a]*Department of Virology 1, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162, Japan*
[b]*Asahi Chemical Industry, Ohito-cho, Shizuoka-ken, Japan*

Received 28 July 1997; revised version received 15 August 1997

Abstract The Nef protein of HIV-1 binds to uninfected CD4$^+$T lymphocytes and induces apoptotic cytolysis of the cells. We examined several human blood cell lines and peripheral blood mononuclear cells (PBMCs) for Nef-induced apoptotic cell death. Soluble Nef protein was shown to bind to the cell surface of not only CD4$^+$T cells but also CD8$^+$T lymphocytes, B lymphocytes, macrophages and neutrophils. PBMCs from normal subjects resisted Nef binding, and activation of the cells with phytohemagglutinin or concanavalin A converted the cells to be susceptible to the binding. Cross-linking of the Nef proteins bound to the cell surfaces with anti-Nef antibody-induced apoptotic cytolysis of the cells. The Nef-mediated apoptosis occurred independently of CD95(Fas). These results suggest that soluble Nef protein, which is found in sera of HIV-1 infected patients, is involved in the destruction of a broad spectrum of uninfected blood cells leading to immune suppression.

Key words: AIDS; HIV-1; Nef; Cytotoxicity; Apoptosis

1. Introduction

HIV-1 infects and kills CD4$^+$T lymphocytes and macrophages. In addition to the viral infected cells, a variety of uninfected cells including CD4$^+$T and CD8$^+$T cells, and neutrophils are also extensively destroyed during the course of HIV-1 infection [1–3]. This is considered to result in the development to immune suppression leading to AIDS [4]. To prevent the death of uninfected cells, it is important to elucidate the mechanisms involved in the cytolysis. We have shown that the Nef protein of HIV-1 is present in a soluble form in sera of HIV-1 infected individuals and that it binds to the cell surface of uninfected CD4$^+$T cells and induces apoptotic cytolysis of the cells [5–7]. The Nef-mediated cytolysis may be responsible, at least in part, for the depletion of CD4$^+$T cells. In this communication, we report that a variety of human blood cells were also susceptible to Nef binding and Nef-induced apoptotic cytolysis, which occurred independently of CD95(Fas) and required cellular activation.

2. Materials and methods

2.1. Soluble Nef protein

Non-myristoylated soluble forms of recombinant Nef proteins derived from the Eri and the III-B strains of HIV-1 were obtained from Intracel, Cambridge, USA, and Immuno Diagnostics, Inc., New York, USA, respectively.

2.2. Antibodies

Mouse IgG monoclonal antibodies (mAbs) to the N-terminus, aa 21 to 41, of Nef protein (BRU strain) were obtained from Advanced Biotechnologies, Inc., Columbia, USA. Anti-CD95(Fas) mAbs (CH-11) were obtained from Medical and Biochemical Laboratories, Co., Ltd. Japan.

2.3. Cells

Human CD4$^+$T cell lineage derived from cutaneous T cell lymphoma (H9), lymphoblastoma (CEM-5) and T cell leukemia (Jurkat), CD4$^+$CD8$^+$T cell lineage derived from lymphosarcoma (Tall-1), CD8$^+$T cell lineage derived from normal peripheral blood (5B5), B cell lineage derived from Burkitt lymphoma (Ramos), monocyte lineage derived from histiocytic lymphoma (U937), and neutrophil lineage derived from myelogenous leukemia (K562) and promyelocytic leukemia (HL60) were used. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml of penicillin. For 5B5 cells, human recombinant IL-2 (50 U/ml) was added to the medium. Peripheral blood mononuclear cells (PBMCs) from healthy adults were obtained by centrifugation through Lymphosepal (Immuno-Biological laboratories, Japan). Cells were incubated for 3 days in RPMI 1640 medium supplemented with 10% FCS in the presence of 1 μg/ml phytohemagglutinin (PHA) or 5 μg/ml concanavalin A (ConA).

2.4. Assay of soluble Nef protein bound to cell surfaces

Cell suspension was incubated for 40 min at 4°C with 1 μg/ml HIV-1 Nef protein. After washing with cold phosphate buffered saline (pH 7.4, PBS), cells were stained with anti-Nef (N-terminus) mAb and FITC-conjugated secondary antibody, and analyzed by a flow cytometer (FACScan, Becton Dickinson, San Jose, USA).

2.5. Analysis of apoptotic cytolysis induced by Nef protein

A 96-well ELISA plate (ImmunoPlate Maxsorp, Nunc, Glostrup, Denmark) was coated with anti-Nef mAb overnight at 4°C, and washed with cold PBS. Cell suspension (1×10^5/tube) in RPMI 1640 medium supplemented with 10% FCS was incubated with various concentrations of Nef protein for 2 h on ice. The cells were then incubated in the same medium in the anti-Nef mAb-coated plate for 48 h at 37°C. Cultured cells were harvested and then incubated with lysis solution (0.1% Triton X-100 and 50 μg/ml propidium iodite (PI) in 0.1% sodium citrate) overnight at 4°C [8]. The nuclear fraction was analyzed for fragmentation of chromosomal DNA by both flow cytometry and pulse field gel electrophoresis (PFGE) using Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries Ltd., Japan).

2.6. Analysis of CD95(Fas)-mediated apoptosis

Cells (2×10^5/well) were incubated with 1 μg/ml anti-human CD95 mAb (CH-11) for 6 h at 37°C in a CO_2 incubator. Cultured cells were harvested and then incubated with lysis solution and analyzed by flow cytometry.

3. Results

3.1. Binding of HIV-1 Nef protein to uninfected human blood cells

Various human blood cells were incubated with the soluble Nef protein and the amount of Nef protein bound to the cell surface was measured by flowcytometry (Fig. 1, Table 1). The

*Corresponding author. Fax: +81 (3) 5285-1169.
E-mail: mtashiro@ nih.go.jp

PII S0014-5793(97)01080-6

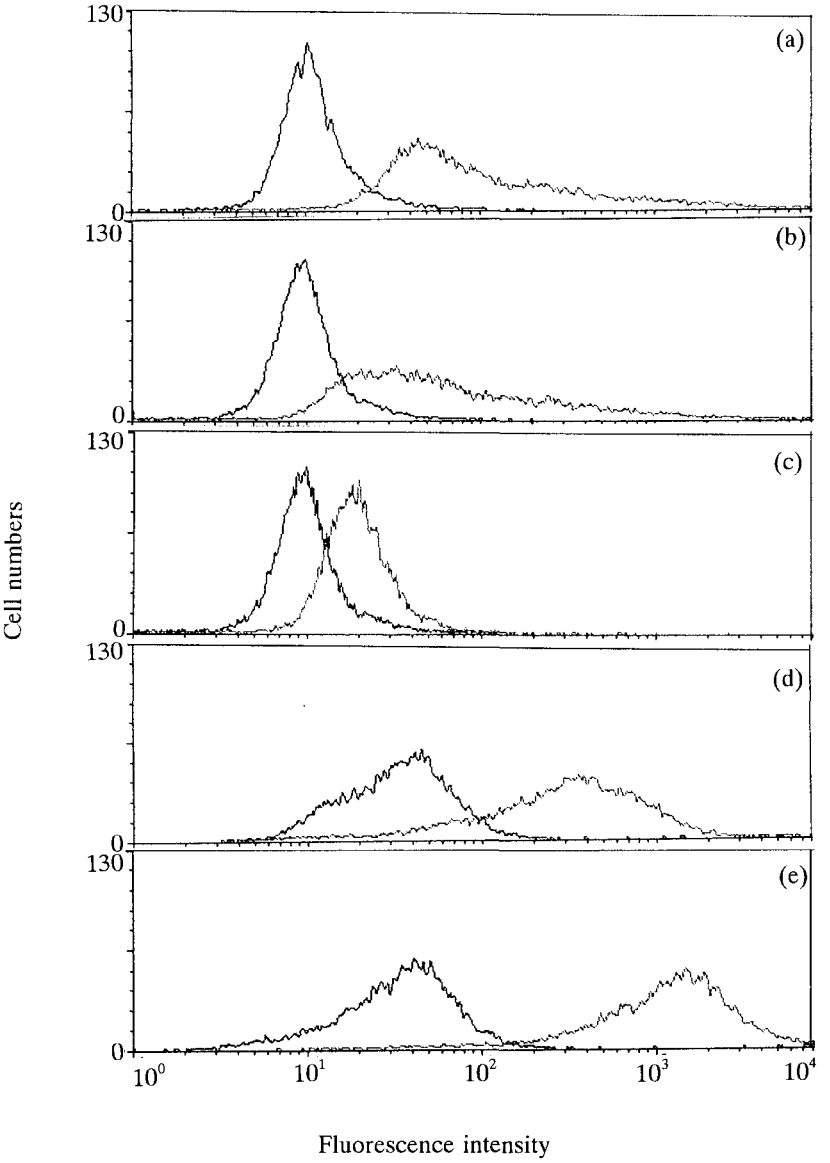

Fig. 1. Binding of soluble Nef protein to human blood cells. Cell suspensions were incubated without (bold lines) or with (thin lines) 1 μg/ml of soluble Nef protein (HIV-1 III-B strain) for 1 h at 4°C and assayed for Nef binding by FACS analysis with anti-Nef mAb and FITC-conjugated anti-mouse IgG Ab. (a) CEM-5, (b) H9, (c) Tall-1, (d) K562, and (e) U937 cell lines.

Table 1
Nef binding, Nef-induced apoptosis and Fas-induced apoptosis of human blood cells

Cells	Nature (origin)	Nef binding[a]	Nef-induced apoptosis (%)[b]	Fas-induced apoptosis (%)[c]
CEM-5	$CD4^+$T lymphocyte (lymphoblastoma)	+	90	22
H9	$CD4^+$T lymphocyte (cutaneous T cell lymphoma)	+	92	86
Jurkat	$CD4^+$T lymphocyte (T cell leukemia)	+	85	70
Tall-1	$CD4^+8^+$T lymphocyte (lymphosarcoma)	+	75	1
5B5	$CD8^+$ T lymphocyte (normal peripheral blood)	+	61	23
Ramos	B lymphocyte (Burkitt lymphoma)	+	73	3
K562	Neutrophil (myelogenous leukemia)	+	77	1
HL60	Neutrophil (promyelocytic leukemia)	+	80	3
U937	Macrophage (histiocytic lymphoma)	+	93	8
PBMC	Control (normal peripheral blood)	−	<5	2
PBMC	PHA stimulation	+	67	37
	ConA stimulation	+	65	39

[a]Nef binding was determined by FACS analysis. +, more than 10-fold increase in fluorescence intensity. −, no shift in fluorescence intensity.
[b]DNA fragmentation after 48 h incubation with Nef protein was quantified against total DNA by FACS analysis.
[c]DNA fragmentation after 6 h incubation with anti-CD95(Fas) mAb (CH-11) was quantified against total DNA by FACS analysis.

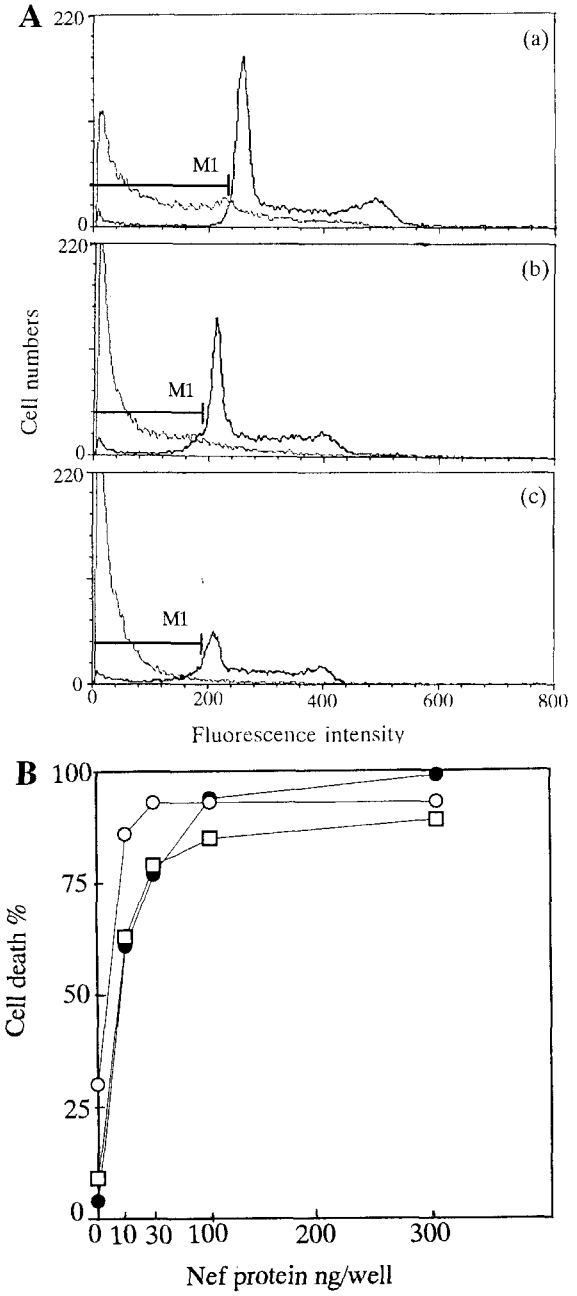

Fig. 2. Nef-induced apoptotic cytolysis of human blood cells. A: Cell suspensions were incubated with or without 30 ng/ml of Nef protein (HIV-1 III-B strain) for 2 h at 4°C. The cells were then cultured for 48 h at 37°C in a 96-well ELISA plate coated with anti-Nef mAb and analyzed for DNA fragmentation by FACS. Bold lines, control cells; thin lines, Nef-treated cells. (a) CEM-5, (b) H9 and (c) K562 cells. B: Cell suspension was incubated with various concentrations of the Nef protein and apoptotic cytolysis was measured by FACS analysis. The percentage of apoptotic cells (M1 region in A) was calculated against total cells. (●) CEM-5, (□) K562, (○) H9 cells.

Nef protein of both Eri and III-B strains was shown to bind to all cell lines tested, not only CD4$^+$T cells (Jurkat, CEM-5 and H9) but also CD4$^+$CD8$^+$T cells (Tall-1), CD8$^+$T cells (5B5), macrophages (U937) and neutrophils (K562 and HL60). On the other hand, PBMCs from healthy controls were negative for Nef binding. However, after activation with PHA or ConA treatment for 2 days, PBMCs became positive for binding by the Nef protein.

3.2. *Apoptotic cytolysis of human blood cells by HIV-1 Nef protein*

Cross-linking of the HIV-1 Nef protein bound to the surface of uninfected CD4$^+$T cells induces apoptotic cytolysis [5,6]. Since the Nef proteins were shown to bind to various cell types in addition to CD4$^+$T cells, Nef-induced apoptotic cytolysis was examined for these cells. Results of representative cells are shown in Fig. 2, and those obtained for all cell types tested are summarized in Table 1. When cells were incubated for 48 h in the absence of Nef, DNA of each cell line was in diploid, tetraploid and intermediate forms, indicating that each cell suspension was a mixture of cells in G_0/G_1, M and S phases, respectively, of cell cycle. No DNA fragmentation was found. On the other hand, when cells were incubated with the Nef protein at 37°C, DNA fragmentation was evident in FACS analysis; the amount of polyploid full-length DNA was decreased and smaller DNA fragments (M1 region) appeared reciprocally (Fig. 2A). Typical DNA ladder formation was also seen in PFGE analysis (Fig. 3). Analysis of the cells with Hoechst 33258 staining also indicated DNA fragmentation and electron microscopic study showed typical blebbing of the nucleus (data not shown). Incubation of the cells with Nef at 4°C showed no effect. These results indicated that apoptosis had occurred. The apoptotic cytolysis was dependent on the amount of Nef protein and incubation period (Fig. 2B). Nef protein at 30 ng/well was enough to kill 1×10^5 cells of all cell lines tested within 48 h.

It should be noted that not only CD4$^+$T cell lines (Jurkat, CEM-5 and H9), but also CD4$^+$CD8$^+$T cell line (Tall-1), CD8$^+$T cell line (5B5), macrophage line (U937) and neutrophil lines (K562 and HL60) were killed by the Nef protein of both HIV-1 Eri and III-B strains. PBMCs from healthy controls resisted the Nef-induced apoptosis, but they became sensitive after activation with PHA or ConA (Table 1). The spectrum of cell types susceptible to the Nef-mediated cytolysis was identical to that of Nef binding.

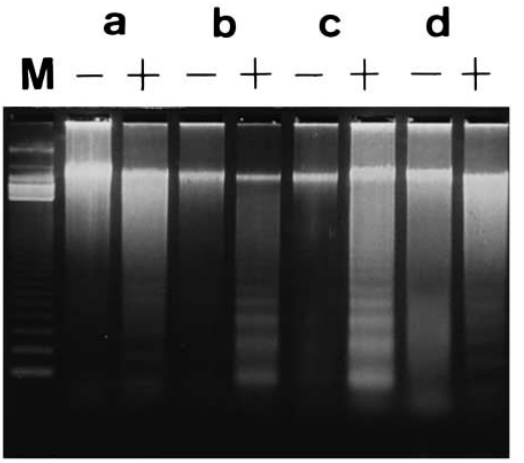

Fig. 3. PFGE analysis of cellular DNA. Cells were treated with the Nef protein as described in the legend for Fig. 2, and cellular DNA was analyzed for ladder formation by PFGE. Lane M, a standard 123 bp DNA ladder. a: Jurkat, b: H9, c: Ramos and d: K562 cells. (−) Control cells; (+) Nef-treated cells.

3.3. Nef-induced apoptotic cytolysis independent of CD95(Fas)

Apoptosis induced by gp120 and Tat protein of HIV-1 has been shown to be mediated by CD95(Fas) [9]. To clarify whether the Nef-induced apoptotic cytolysis is dependent on CD95(Fas), cell surface expression of the CD95(Fas) antigen was examined for each cell line. Whereas all cell types tested underwent Nef-induced apoptosis, only part of the cells expressed CD95(Fas) and were susceptible to apoptosis by anti-CD95(Fas) antibody (Table 1). These results indicated that the Nef-induced apoptosis occurred independently of CD95(Fas).

4. Discussion

The present study confirmed our previous observation that the HIV-1 Nef protein possesses binding affinity to the cell surface of uninfected $CD4^+$T lymphocytes and the cell bound Nef, upon cross-linking, induces apoptotic cytolysis of the cells [5–7]. We provide evidence that a wide range of human blood cell lines including $CD4^+$T, $CD8^+$T, and B lymphocytes, macrophages, and neutrophils could also be the target of Nef binding and Nef-induced apoptotic cytolysis. The results suggest the presence of a putative Nef receptor on the surface of these uninfected cells. Although such Nef receptor was not expressed on the surface of PBMCs from normal controls, its expression was induced by activation of the cells. Therefore, the Nef binding and Nef-induced apoptotic cytolysis required cellular activation. Cell line cells were also, more or less, activated. PBMCs of HIV-1 infected patients may be activated to some extent by cytokines induced by HIV-1 and other opportunistic infections [10]. Soluble Nef protein is found in sera of patients at a high concentration (5–10 ng/ml), which is sufficient for inducing the cytotoxicity in vitro [5]. Anti-Nef antibodies are also present in the sera [11]. It is, therefore, likely that in the patients, the soluble Nef protein binds to a broad spectrum of PBMCs and, upon cross-linking by the antibodies, it induces apoptotic cell death to these cells. This may explain, in part, the mechanism of loss of various blood cells such as $CD4^+$T and $CD8^+$T lymphocytes and neutrophils, in the course of HIV-1 infections [1–3]. It should be noted that uninfected B cells and macrophages were also susceptible in vitro to the Nef-induced cytolysis.

The Nef-induced apoptotic cytolysis occurred independently of cell surface expression of CD95(Fas). The spectrum of cell types susceptible to the Nef-induced apoptosis was much broader than that of CD95(Fas)-dependent apoptosis. The Nef-mediated apoptosis was induced with all cell types tested, whereas only Jurkat and H9 cells expressed CD95(Fas) and underwent apoptosis by anti-CD95(Fas) mAb. Similar to the CD95(Fas)-mediated apoptosis, however, cellular activation was required for the expression of putative Nef receptor and Nef-induced apoptosis. Molecular characterization of the putative Nef receptor, identification of the natural ligands and the mechanism of the apoptosis remain to be studied.

Acknowledgements: We are grateful to Ms. M. Takizawa for technical assistance of FACS analysis, and Dr. A. Kojima for valuable discussions. This work was supported in part by Scientific Grants from the Ministry of Health and Welfare of Japan.

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