第16卷4期 中国病毒学 16(4):304-308
2001年12月 VIROLOGICA SINICA December 2001

猴免疫缺陷病毒引起多形核嗜中性白细胞凋亡的可能机理*

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摘要:本实验目的是研究猴免疫缺陷病毒(SIV)引起多形核嗜中性白细胞(PMNs)凋亡的机理。实验用PCR技术扩增 *gag* 基因,用Western blot法测定p53和bcl-2基因的表达。结果显示PMNs在被SIV感染后随着保温时间的延长存活率下降,在感染后24h可以从PMNs中扩增出 *gag* 基因。PMNs中 *p53* 基因的的表达在感染后24h增加。同时 *bcl-2* 基因的表达在对照组和SIV感染组都增加,但在SIV感染组 *bcl-2* 蛋白的表达明显低于对照组。结果揭示SIV能够感染PMNs,*p53* 和 *bcl-2* 基因表达的改变可能是SIV感染PMNs引起细胞凋亡的机理。

关键词:多形核嗜中性白细胞;猴免疫缺陷病毒;基因表达;细胞凋亡
中图分类号:R392.11 **文献标识码**:A **文章编号**:1003-5125(2001)04-0304-05

A Possible Mechanism of Apoptosis Induced by Simian Immunodeficieny Virus in Polymorphonuclear Neutrophils*

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Abstract: The aim of this study is to investigate the mechanisms of apoptosis in polymorphonuclear neutrophils (PMNs) induced by simian immunodeficiency virus(SIV). In this experiment, *gag* gene was amplified by the polymerase chain reaction and the expression of *p53* and *bcl-2* gene were determined by Western blot assay. The results showed that the viability of PMNs after treated with SIV was declined as the incubation time prolonged. *gag* gene could be amplified from PMNs at 24 h postinfection. The expression of *p53* gene in PMNs was enhanced at 24 h postinfection. At the same time, the expression of *bcl-2* gene were elevated in both of control and SIV infected group, but the production of *bcl-2* protein in SIV infected group was obviously lower than control. Our data indicate that PMNs could be infected with SIV and the changed expression of *p53* and *bcl-2* gene might be one of the mechanisms of apoptosis occurred in PMN infected with SIV.

Key words: Polymorphonuclear neutrophils; Simian immunodeficiency virus; Gene expression; Apoptosis
CLC number: R392.11 **Document Code**: A **Article ID**: 1003-5125(2001)04-0304-05

Acquired immunodeficiency syndrome(AIDS) is due to infection of human immunodeficiency virus (HIV). The alteration of immune function induced by HIV is mainly involved in the functional impariment of $CD4^+$, macrophages and polymorphonuclear neutrophils(PMNs). It was noticed that phagocytosis of bacterial by PMNs of HIV-in-

收稿日期:2000-10-30,修回日期:2001-04-13
* 基金项目:国家自然科学基金(30060091);海南省自然科学基金(39910)
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fected patients was significantly lower, but this was contributed to the increased susceptibility to bacterial infection in HIV-infecred patients with low $CD4^+$ subset-cell counts[1,2]. In the presence of AIDS, the marker expression on PMNs was modulated in the cytokines tests[3]. It was found that PMN from HIV^+ subjects showed an increase in expression of TNF-alpha and IL-6 genes[4], an altered ability to produce specific cytokines in response to lipopolysaccharide[5] and an induced increase of TNF-alpha by monocytes and up-regulation of monocytes phagocytosis[6]. As mentioned above, the function of PMNs was imparied or altered by virus. Since it was once considered that HIV did not directly infect PMNs, the phenomena were therefore considered to be due to reduced release of cytokines, such as granulocyte colony-stimulation factor[7]. However an important fact has been noticed that HIV DNA might be detected in PMNs of HIV-infected patients[8].

Apoptosis is an active death process genetically encoded to eliminate abnormal or unwanted cells and accompanied in the pathogenesis process of AIDS that is always associated with the alteration of gene expression. In present studies, simian immunodeficiency virus (SIV_{mac239}) was incubated with PMNs from macaque monkeys. The infection of PMNs and expression of *p*53 and *bcl*-2 genes were analyzed. Apoptosis in PMNs was therefore to be evaluated.

1 Materials and Methods

1.1 Separation of PMNs

Peripheral blood was taken out from macaque monkeys and spun down at 1000 r/min for 5 min. Plasma were taken off and supplemented with equal amount of saline. Blood was gently overlaid on the surface of 10 mL of lymphocyte separation medium (LSM) and centrifuged at 2 000 r/min for 45 min. PMNs on the surface of red blood cells were collected, transferred to 20 mL of 3% dextran in saline and drawn up into 35 mL syringe. The syringe was put in an inverted position for 35 min and granulocyte rich supernatant was saved in 50 mL tube. Equal volume of saline was added and mixed gently. Following spun 10 min at 1 000 r/min, the pellet was lysed with 2 mL of water for 30 s and diluted immediately by pouring saline into tube. The cells were washed twice and standardized to 1×10^6 cells/mL with RPMI 1 640 medium.

1.2 Incubation of PMNs and treatment with SIV

PMNs with a density of 1.8×10^6 cells per mL were aliquot into 25 cm^2 flasks and maintained in RPMI-1640 medium(containing 10% fetal calf serum and 5% autoplasma, 100 U penicillin/mL, 100μg streptomycin/mL and 1 nmol/L GM-CSF purchased from SIGMA). In SIV infected group, PMNs were treated with 1/10 volume of SIV_{mac239} supernatant of free-cells(1×10^3 $TCID_{50}$/mL). PMNs were incubated at 37℃ in a humidified atmosphere with 5% CO_2.

1.3 Determination of viability of PMNs

At the incubation time 0, 24, 48, 72, 96 and 120 h, 0.5 mL of PMNs suspension from each group was collected into an Eppendorf tube. The supernatants were taken off after centrifugation at 1 000 r/min for 10 min and the pellets were put into freezer until use. After all samples were collected, the viability of cells was detected with CyQUANT assay which has been used as a convenient, rapid and sensitive procedure for determining the density of cells in culture. Based on the principle of the assay, the CyQUANT dye exhibits strong fluorescence enhancement when bound to cellular nucleic acids and the relative fluorescent intensity may therefore be used to represent the survival cells. The CyQUANT Cell Proliferation Assay kit was purchased from Molecular Probes. The procedure of this assay was performed by following the instruction of the kit.

1.4 Amplification of *gag* gene

4×10^6 PMNs were removed from each culture flask at 0 and 24 h respectively after SIV load, washed 4 times with PBS pH 7.4 and treated with lysis buffer(0.2 mol/L Tris HCL pH 8, 0.1 mol/L Na_2 EDTA, 1% SDS, 100μg/mL poteinase K) for 16 h at 37℃. The DNA were extracted with phenol: chloroform 2x and precipitated with 3 mol/L sodium acetate and ethanol overnight at −20℃. After washed with 75% ethanol, the quantified DNA was used for PCR reaction. 0.2μg of PMN DNA was added into a reaction mixture consisting of 6 μL of 25 mmol/L $MgCl_2$, 10μL of 10x PCR buffer. 2μL of 10 mmol/L dNTP's, 2.5 U Taq Polymerase, 1 μL of 50 μmol/L each of primer (Sense: 5' AAT GAG GAG GCT GCA GAT TGG GAC T

3'; Antisense: 5' GCA CTA GCT TGC AAT CTG GGT TAG C 3'; Band size: 381 bp) and H_2O to 100μL. Amplification was performed at 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min. The reaction was followed by 72℃ for 7 min after 30 cycles. The amplified DNA fragment was analyzed by 1.5% agarose gel electrophoresis and were visualized by ethidium bromide staining.

1.5 Western blot immunodetection

At 0 and 24 h postinfection, whole-cell extracts were prepared for Western immunoblot analysis: 10^6 cells were incubated in 10μL of lysis buffer containing 0.2% Trition X-100, 500mmol/L NaCl, 500mmol/L sucrose, 1 mmol/L EDTA, 0.15 mmol/L specimen, 0.5mmol/L spermidine, 10 mmol/L HEPES (pH 8.0), 200 μmol/L phenylmethysulfonyl fluoride, 2μg leupeptin/mL, 2 μg pepstatin/mL, 24 IU aprotinin/mL and 7 mmol/L β-mercaptoethano 1.40 mg of total proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane for immunodetection. Mouse monoclonal antibodies were raised against human *p53* and *bcl*-2 (Santa Cruz Biotechnology). HRP-conjugated goat anti-mouse lgG was used as the secondary reagent. The antigen-antibody complexes were revealed by enhanced chemiluminescence (ECL; Amersham). SDS-PAGE molecular weight markers (Bio-Rad) verified the correct location of the visualized bands after being exposured to an X-ray film. Quantitative analyses of bands on X-ray film wre performed by an automated digitizing system (UN-SCAN-IT, Silk Scientific, inc.)

2 Results

2.1 Influences of SIV on the survival of PMNs

After treatment with SIV, the cells viability was determined by the method of CyQUANT assay at different incubation time (Fig. 1). From Figure 1, a pronounced decrease of the cell viability in SIV infected group could be noticed as the incubation time prolonged. The viabilities of cells in control groups were from 62.1% (24h) through 23.8% (122h) compared with incubation time 0 hour. However, those in SIV infected groups were markedly decreased from 31.1% (24h) through 14.2% (122h).

2.2 Detection of *gag* gene

At 0 and 24 h after PMNs treated with SIV, *gag* gene were amplified by PCR. The results whowed that an amplified band could be visualized at 24 h postinfection group but others (Fig. 2). It was also noticed that *gag* gene could be amplified form samples of 50% (5 out of 10 cases).

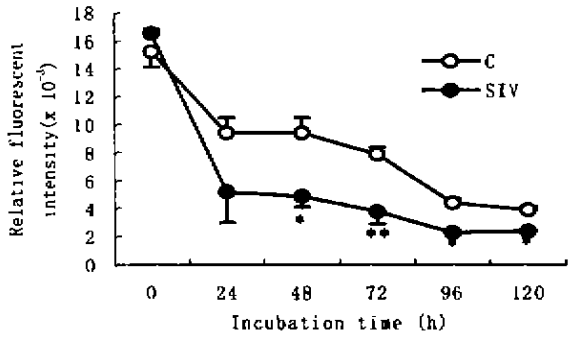

Fig. 1 Viability of PMNs after infected with SIV at different incubation time points

C, control; SIV, SIV infected group; * $P<0.05$ and * * $P<0.01$ compared with control at same time point. n = 4.

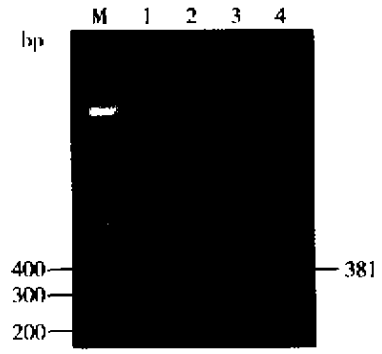

Fig. 2 Detection of *gag* gene in PMNs infected with SIV for 24 h

PMNs were separated and incubated with SIV; DNA were extracted and amplification of gag gene were performed at 0 and 24 h; M, Maker; lane 1, Control at incubation time 0 h; 2, Control at incubation time 24 h; 3, SIV infected group at incubation time 0 h; 4, SIV infected group at incubation time 24 h.

2.3 Expression of *p53* gene

The expression of *p53* gene was determined by Western blot at 0 and 24 h postinfection of PMNs. The result in Figure 3 demonstrated an obvious increase of p53 protein at 24 h in SIV infected group. The graph in Figure 3 was selected from 3 similar results.

2.4 Expression of *bcl*-2 gene

The expression of *bcl*-2 gene in SIV infected group was also detected by Western blto. Figure 4 showed that the synthesis of bcl-2 protein in both SIV infected group

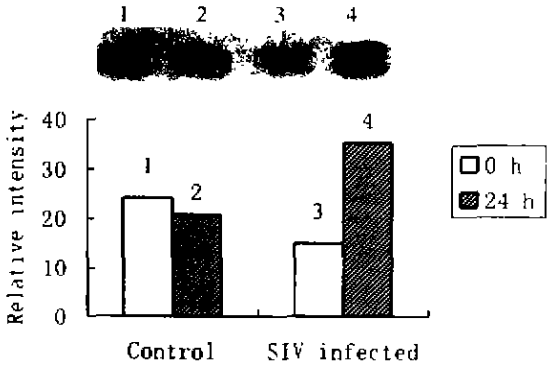

Fig.3 The expression of *p53* gene was detected by Western blot assay Relative intensity for each group was determined by an automated digitizing system. PMNs were separated and incubated with SIV. Western blot were performed at 0 and 24 h after infection. Lane 1, Control at incubation time 0 h; lane 2, Control at incubation time 24 h; lane 3, SIV infected group at incubation time 0 lane 4, SIV infected group at incubation time 24 h.

and control were upregulated at 24 h under the current condition of incubation, but the level of bcl-2 in control was much higher than that in SIV infected control (the pixel ratio of control to SIV-infected group after digitizing was 2.01). The graph in Figure 4 was selected from 6 similar results.

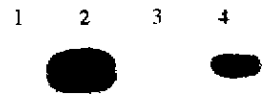

Fig.4 The expression of *bcl-2* gene was detected by Western blot assay PMNs were separated and incubated with SIV. Western blot were performed at 0 and 24 h after infection. Lane 1, Control at incubation time 0 h; 2, Control at incubation time 24 h; 3, SIV infected group at incubation time 0 h; 4, SIV infected group at incubation time 24 h.

3 Discussion

HIV affects mainly the function of $CD4^+$ cells, but other immune cells are also impaired, for instance macrophages[9] and PMNs. PMNs and monocytes/macrophages represent the first defence line against invading microorganisms. The impairment of PMNs by SIV will eventually lead to the death of cells. In the present studies, the viability of PMNs infected with SIV was remarkably decreased as incubation time prolonged that indicating the survival of PMNs was influenced by the SIV load.

Although it was once considered that PMNs were not directly infected by HIV, the functional impairment of PMNs was not fully explained so far[1~6]. *gag* gene was existed at 5′ terminal of genome of reverse transcriptive virus and frequently used for the detection of HIV or SIV infection. The fact that *gag* gene could be amplified from PMNs treated with SIV suggested that PMNs might be infected within 24 h. It was noticed that *gag* gene could not be amplified from every sample. The ratio of infection in PMNs was about 50% (5 out of 10 cases). One of the reasons might be due to the individual differences that could explain why few cases of direct infection of PMNs were reported before. There still were evidences indicating that the detectable HIV DNA of PMNs infected with HIV was found to be 44.4% in patients with neutroenia whereas 28.0% in those with a normal level of neutrophils[8]. These data suggest that the impairment of PMNs may be associated with infection of PMNs by HIV.

The cell number in tissues in maintained by a critical balance between cell proliferation and programmed cell death or apoptosis. Growing evidences have confirmed that apoptosis in HIV infection is related to the alteration of genes expression. p53 protein is important for apoptosis induced by some pathogens even it is not necessary to the death of normal cells. One group's studies established that HIV-1 gp 120 was potentially cytotoxic to human cells through the induction of p53, which might eventually lead to induction of apoptosis[10]. Our previous studies have also demonstrated an elevated level of p53 protein in SIV infected lymphocytes[11]. In the present studies, the expression of p53 was significantly enhanced at 24 h infection. It was thereby estimated that the elevated level of p53 protein might promote the process of apoptosis of PMNs. The mechanism involved has been documented that an interaction between p53 and virus Tat protein favored the formation of p53 dimers and thus led to the cells tosard apoptosis[12]. Our results on the expression of *p53* gene were consistent with what we mentioned above, in which more PMNs died in the case of SIV infection.

It was well known that bcl-2 is a kind of suppressive protein in the induction of apoptosis. It was noticed from this experiment that the expression of *bcl-2* was also altered in SIV infected group at 24 h incubation. The relative low level of bcl-2 in SIV infected group indicated that

the production of an inhibitory factor of apoptosis might be suppressed during the process of apoptosis produced by SIV. The data from other laboratories have indicated a change in the expression of apoptosis-regulating gene(including bcl-2) in tumor cells infected with SIV[13] or HIV[14]. Bcl-2 protein can inhibit the activity of cysteine-containing aspartate-specific protease(caspase) and further apoptosis. The decreased bcl-2 protein might be involved in the mechanisms of apoptosis in SIV infection.

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