Interleukin-2 Prevents Programmed Cell Death in Adult T-Cell Leukemia Cells

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Adult T-cell leukemia (ATL) is a prototype of the lymphoma/leukemia syndromes involving immunologically mature T-lymphocytes. The first retrovirus described in humans, HTLV-I, is causally related to the disease. In this study, we examined whether ATL cells die *in vitro* through programmed cell death (PCD), which has been shown to occur in cells affected by several other acute and chronic leukemias. When ATL cells from peripheral blood were cultured in serum-free complete medium, a substantial proportion of them spontaneously died by PCD. After 48 h of culture, approximately 30% of the total DNA was fragmented. Electrophoresis indicated that the DNA of the ATL cells had been cleaved into regular oligonucleosome fragments each comprising approximately 180-200 base pairs. This process was significantly promoted by methylprednisolone and the protein kinase A (PKA) activator Sp-cAMPS in at least some cases. Since all ATL cells possess interleukin-2 receptors on the cell membrane, the effect of IL-2 on spontaneous PCD was assessed. PCD after 48 h of culture was inhibited by 30-50% by 100 U/ml interleukin-2 (IL-2). This effect of IL-2 to prevent spontaneous PCD was dose- and time-dependent. These findings suggest that the viability of ATL cells *in vivo* is regulated positively and negatively by intrinsic IL-2, glucocorticoid and regulators of PKA activity. Furthermore, the process of cell death may be involved in the development of the disease.

Key words: Apoptosis — Programmed cell death — Adult T-cell leukemia — Interleukin-2

Adult T-cell leukemia/lymphoma (ATL) is a prototype of the lymphoma/leukemia syndromes involving peripheral, mature CD4⁺ T-lymphocytes.^{1,2)} The hallmark of the disease is the clonal integration of proviral DNA into the cellular DNA of ATL cells and the presence of antibodies against the virus. A causal relationship between HTLV-I infection and ATL has also been suggested by epidemiological data.^{1,2)} However, the very long incubation period of the disease and the determinants of the relatively rare occurrence of the disease among carriers of the virus have yet to be explained.

Eukaryotic cells generally die either by necrosis or by programmed cell death (PCD) or apoptosis.³⁻⁸⁾ The latter process of death is characterized by (a) chromatin condensation and (b) degradation of cellular DNA into oligonucleosome-sized fragments. PCD occurs in thymocytes stimulated with either calcium ionophore, antibodies against the TcR-CD3 complex, glucocorticoids or gamma irradiation. In addition, T-cell-mediated cytotoxicity is associated with this cell death process. Recently, spontaneous and inducible PCD has been reported in certain neoplastic cells, indicating a potential use for it in cancer therapy.⁴⁾ Recent studies have suggested that neoplastic lymphoid cells of T- and B-lymphocyte origin are inherently sensitive to PCD.⁴⁾

The growth of ATL cells may be regulated *in vitro* and possibly also *in vivo* by certain growth factors.^{1,2)} For example, it has been reported that some ATL cells proliferate in response to interleukin-2 (IL-2), and IL-4 *in vitro*.⁹⁻¹¹⁾ However, these cases, especially IL-2-responsive ones, are rather exceptional and in the majority of cases, tumor cells do not proliferate when stimulated with IL-2 despite their possession of IL-2 receptor α and β chains.^{1,9,10)} In IL-2 unresponsive cases, it is nevertheless possible that IL-2 signals the tumor cells through the receptor. Therefore, we hypothesized that growth factors act in the prevention of PCD, rather than in promoting the proliferation of ATL cells. In this report, we first show that both spontaneous and reagent-induced PCD takes place in ATL cells *in vitro* and then demonstrate that IL-2 may prevent this process.

MATERIALS AND METHODS

Cell isolation and incubation Heparinized peripheral blood was collected from seven patients diagnosed as ATL on the basis of standard clinical and hematological criteria and from five healthy volunteers. To remove phagocytes, the blood was incubated with 1/10 volume of silica solution, KAC-2 for 2 h at 37°C. Mononuclear cells (MNC) were then isolated by differential centrifugation using Lymphoprep. In the case of normal controls, T-cells were further separated from MNC by passing the

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samples through a nylon-fiber column. The cell preparations were adjusted to a concentration of 2×10^6 /ml in serum-free complete medium: Dulbecco's modified Eagle's medium/RPMI-1640 (1:1) supplemented with 10% Nutridoma-HU. MNC preparations from ATL cases comprised more than 90% CD4⁺ cells as assessed by flow cytometry using a Cytoron. Contamination of the preparations by esterase⁺ cells was less than 2%. MNC were incubated either with or without PCD-inducing reagents such as $1 \mu\text{M}$ 6 α -methylprednisolone (mPSL), or 10^{-4} M Sp-cAMPS¹²⁾ in either the presence or the absence of 100 U/ml IL-2 for 48 h. The dose response and time course of the effects of IL-2 were assessed in cultures containing 0–1,000 U/ml IL-2 harvested after 48 h of culture or in cultures containing 100 U/ml IL-2 harvested after 0, 24, 48, and 72 h, respectively.

Quantitation of DNA fragmentation DNA fragmentation was estimated as described previously. Briefly, 3×10^6 cells in complete medium were centrifuged (200g, 5 min, 4°C) and the pellet was lysed in 400 μl of hypotonic lysing buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton X-100). The lysate was centrifuged at 13,000g for 10 min, and the pellet and supernatant were both treated with 12.5% TCA at 4°C overnight. After centrifugation at 13,000g for 5 min, DNA in the precipitates was extracted into 80 μl of 5% TCA by heating at 90°C for 10 min. Perchloric acid (20 μl) was added to the samples to a final concentration of 0.5 N. The amount of DNA in the precipitated samples was determined by means of the diphenylamine reaction.^{13, 14)} Freshly prepared diphenylamine reagent (250 μl) containing acetaldehyde was added to each sample and the tubes were incubated at 30°C for 18 h. Aliquots were transferred to flat-bottomed 96-well polystyrene microtiter plates and A₅₇₀ was measured on an automated plate reader (ELISA Processor II, Behring, Germany). The amount of DNA in the samples was calculated from a standard curve prepared from salmon sperm DNA diluted in 0.5 N HClO₄. Fragmentation was calculated as the percentage of total DNA (supernatant and pellet) which was recovered as low-molecular-weight DNA in the supernatant.

Agarose gel electrophoresis of DNA fragments DNA was extracted from each cell lysate with an equal volume of phenol/chloroform (1:1). DNA in the aqueous phase was precipitated at –70°C in 2.5 vol of ethanol and 0.3 M sodium acetate for more than 1 h. Precipitates were collected by centrifugation (13,000g, 15 min, 4°C), washed twice with ice-cold ethanol, dried and resuspended in 200 μl of 10 mM Tris-HCl:25 mM EDTA buffer, pH 8 (Tris-EDTA). The samples were treated with 20 $\mu\text{g}/\text{ml}$ RNase for 1 h at 30°C and then with 50 $\mu\text{g}/\text{ml}$ proteinase K for 3 h at 50°C. DNA was extracted again with phenol/chloroform, re-precipitated with

sodium acetate and ethanol, and resuspended in 10 μl of Tris-EDTA. Loading buffer (2 μl : 30% glycerol, 0.25% bromphenol blue and 0.25% xylene cyanol FF) was added and the samples were electrophoresed in 1.2% agarose containing ethidium bromide (1 $\mu\text{g}/\text{ml}$) for 80 min at 100 V in a TBE:1 mM EDTA buffer, pH 8. DNA was visualized under UV light and photographed with a Polaroid camera. The sizes of DNA fragments in the samples were compared with standard size fragments in a 1 kb DNA ladder.

Cell viability and morphology To estimate cell viability, a trypan-blue dye exclusion test was employed as described.¹⁵⁾ Morphology of the cells was examined on May-Grünwald Giemsa stained cytospin preparations (Shandon-Elliott cytocentrifuge, Astmoor, UK).

Reagents Diphenylamine, salmon sperm DNA, and mPSL were purchased from Sigma Chemical Co. (St. Louis, MO). Sp-cAMPS¹²⁾ was obtained from BIOLOG Life Science Institute (Bremen, Germany). Nutridoma-HU, KAC-2, and Lymphoprep were obtained from Boehringer Mannheim (Mannheim, Germany), Japan Antibody Institute (Takasaki) and Nycomed Pharma AS (Oslo, Norway), respectively. Nylon-fiber columns were purchased from Wako Co. Ltd. (Osaka). The 1 kb DNA ladder was from BRL (Gaithersburg, MD). Recombinant human IL-2 was a generous gift from Shionogi Pharmaceutical Co. Ltd. (Osaka).

RESULTS

DNA fragmentation in ATL cells No apoptotic cell death was observed in freshly collected ATL cells as assessed by analysis by agarose gel electrophoresis (Fig. 1), quantitation of DNA (less than 3.6% of total DNA) and light microscopy (Fig. 2A). The percentage of DNA fragmentation of fresh T cells from normal individuals was $6.1 \pm 1.0\%$ ($n=5$). After 48 h of incubation in serum-free complete medium, substantial PCD of ATL cells was evident (22–52% of total DNA, Table I). The mean percentage of DNA fragmentation in PCD-affected cells after culture was $30.3 \pm 10.5\%$ (mean $\pm$ SD, $n=7$). On the other hand, the percentage of fragmented DNA of normal T cells remained at a low level after 48 h incubation ($9.7 \pm 1.3\%$, $n=5$). Electrophoretic analysis indicated that the DNA of the ATL cells had been cleaved into regular oligonucleosome fragments each comprising approximately 180–200 base pairs (Fig. 1). Spontaneous PCD of ATL cells was also confirmed by morphological examination, which revealed a decrease in cell size, chromatin condensation and cytoplasmic blebbing (Fig. 2B). Glucocorticoid reportedly induces PCD in thymocytes and in several types of malignant B-cells.^{3–8)} Our results showed that mPSL apparently enhanced PCD in all ATL cells (34 – 59% , $46.5 \pm 10\%$, $n=6$,

$P=0.02$) (Table I). In five out of the six cases, the DNA was fragmented in over 50% of control cultures without mPSL (this was not observed in case 4). It is known

Fig. 1. Electrophoresis pattern of DNA fragments isolated from ATL cells (case 1). DNA from lysed cells was isolated from 3×10^6 fresh ATL cells (lane 2), or an equal number of ATL cells cultured for 48 h in medium only (lane 3), or medium containing 100 U/ml IL-2 (lane 4), $1 \mu\text{M}$ mPSL (lane 4), or 10^{-4} M Sp-cAMPS (lane 5). A 1 kb DNA ladder is shown in lane 1.

that PCD can be induced by activating cyclic adenosine monophosphate-kinase I (cAKI) in some cells including thymocytes, peripheral T cells, and a myeloid leukemic cell line.¹⁶⁻¹⁸ Sp-cAMPS, a specific cAK stimulator,¹² apparently induced PCD in cases 1 and 4 (44 and 21% with the control described above, Table I).

Effects of IL-2 on DNA fragmentation Several cytokines have been reported to affect PCD positively or negatively.¹⁹⁻²⁸ Since ATL cells frequently possess specific receptors for IL-2,¹⁰ the effect of IL-2 on PCD induced in those cells was investigated. As shown in Table II, ATL cells were protected from spontaneous PCD by 30-50% ($39.6 \pm 6.2\%$, $n=7$, $P<0.05$) by the addition of

Table I. Effects of mPSL and Sp-cAMPS on DNA Fragmentation in ATL Cells

Case	DNA fragmentation (%)		
	Spontaneous	mPSL	Sp-cAMPS
1	25	45	36
2	22	34	24
3	27	42	21
4	52	59	63
5	23	41	20
6	28	58	ND
7	35	ND	ND

Cells were cultured for 48 h in the absence (spontaneous) or presence of mPSL or Sp-cAMPS. Then, DNA fragmentation was quantified as described in "Materials and Methods." ND, not done.

Fig. 2. Morphology of ATL cells before and after incubation. ATL cells were cultured in serum-free medium with or without 100 U/ml IL-2 for 48 h. Fresh cells (A) and the cells cultured in the absence (B) and presence (C) of IL-2 are shown (case 3).

Table II. The Inhibition of Spontaneous DNA Fragmentation in ATL Cells by Interleukin-2

Case	DNA fragmentation (%)		% inhibition
	Spontaneous	IL-2	
1	24.9	14.9	40.2
2	22.0	13.9	36.8
3	27.0	16.5	40.7
4	52.0	29.7	42.9
5	23.5	14.8	37.0
6	28.0	14.0	50.0
7	49.0	34.5	29.7

Cells were cultured for 48 h in the absence (spontaneous) or presence of IL-2. Then, DNA fragmentation was quantified as described in "Materials and Methods."

Table III. Effects of Interleukin-2, mPSL and Sp-cAMPS on Viability of ATL Cells

Case	Effect on % viability			
	None	IL-2	mPSL	Sp-cAMPS
1	91	88	77	74
2	88	92	70	76
3	83	85	79	71
4	65	68	46	55
5	71	ND	71	59
6	84	87	76	ND
7	55	70	ND	ND

Cells were cultured for 48 h in the absence (None) or presence of IL-2, mPSL or Sp-cAMPS. Then viability of cells was assessed by means of the trypan-blue dye-exclusion test. ND, not done.

100 U/ml IL-2 to cultures. This was confirmed by electrophoretic analysis of DNA from ATL cells (Fig. 1) and morphological examination of the cells (Fig. 2C).

Effects of PCD-inducing agents and IL-2 on the viability of ATL cells Before incubation, the viability of ATL cells, as tested by trypan-blue dye exclusion, always exceeded 98%. It declined to 55–91% ($76.7 \pm 13.3\%$, $n=7$) after incubating the cells for 48 h (Table III). This decline of viability was inhibited by IL-2 in case 7. mPSL and Sp-cAMPS reduced the viability of the cells in most ATL cases ($69.8 \pm 12.2\%$, $n=6$ and $67.0 \pm 9.4\%$, $n=5$, respectively). It should be noted that the extent of decrease in viability which occurred spontaneously or which was induced by mPSL and Sp-cAMPS was generally less than the extent of DNA fragmentation induced by those agents. This implies that DNA fragmentation precedes membrane damage.

Fig. 3. Dose response of the effects of interleukin-2 on spontaneous DNA fragmentation of ATL cells. ATL cells (cases 1, 3, and 4) were cultured for 48 h in the presence of 0, 10, 100, and 1,000 U/ml of IL-2. Then, the extent of DNA fragmentation of the ATL cells was determined as described in "Materials and Methods." Data are given as the mean $\pm$ SD of three determinations.

Dose- and time-dependent effects of IL-2 on spontaneous PCD The dose-dependent effect of IL-2 on spontaneous PCD in ATL cells was studied by incubating the cells with 0, 10, 100 and 1,000 U/ml of IL-2. As shown in Fig. 3, IL-2 dose-dependent protection from spontaneous PCD was observed in all 3 cases of ATL tested. The time-dependency of the effects of IL-2 on PCD was next examined. As shown in Fig. 4, spontaneous DNA fragmentation was already apparent after 24 h of incubation in all 3 cases tested. However, it was not until after 48 h of incubation that the protective effect of IL-2 against spontaneous PCD became significant. Spontaneous PCD increased day by day throughout 72 h of culture and the protective effect of IL-2 was apparent until 72 h of incubation. In case 4, spontaneous PCD did not occur from 24 h of culture to 72 h after the start of culture in the presence of IL-2. This suggests that, in this case, PCD after 24 h of culture was almost completely blocked by IL-2.

Fig. 4. Time course of the effects of interleukin-2 on spontaneous DNA fragmentation of ATL cells. ATL cells (cases 1, 2, and 4) were cultured in the presence or absence of 100 U/ml IL-2 and harvested at 0, 24, 48, and 72 h of incubation. Then, the extent of DNA fragmentation of the ATL cells was determined as described in "Materials and Methods." Data from the cultures with or without IL-2 are indicated by shaded and solid columns, respectively. Data are given as the mean $\pm$ SD of three determinations.

DISCUSSION

Spontaneous ATL cell death by PCD was demonstrated for the first time *in vitro*, although PCD induced by anti-Apo monoclonal antibody has already been reported in ATL cells.²⁹ This process was promoted by mPSL and Sp-cAMPS in some cases. Furthermore, spontaneous PCD in ATL cells was apparently inhibited by exogenously added IL-2.

Various cytokines regulate PCD positively or negatively in particular cell types *in vitro*.^{15, 19-28} Tumor necrosis factor- α (TNF) and IL-1 β prevent PCD in monocytes, whereas TNF induces PCD in the other cells. IL-2 prevents anti-CD3-induced cell death in human thymocytes, whereas IL-2 participates in the induction of PCD in mature $\alpha\beta$ T lymphocytes. IL-5 prevents PCD in mature eosinophils. PCD in myeloid leukemia cell line M1, induced by the introduction of wild-type p53, is inhibited by IL-6. Colony-stimulating factors promote the survival of hematopoietic precursor cells by suppressing PCD. In the past decade, many cytokines have been tested for their ability to initiate or promote the proliferation of ATL cells.⁹⁻¹¹ Above all, IL-2 and IL-4 have been reported to stimulate the proliferation of ATL cells, but only in selected cases.⁹⁻¹¹ Therefore, we hypothesized that IL-2 and perhaps IL-4 play a role in preventing PCD

rather than in promoting tumor cell growth. As expected, IL-2 prevented spontaneous PCD in ATL cells in this study. We assume that this also occurs *in vivo*. Spontaneous fluctuations of the tumor cell count, the extent of lymphadenopathy and organ involvement are frequently observed in the clinical course of ATL, especially in chronic ATL.^{1,2} These phenomena could result from changes in the levels of intrinsic glucocorticoids, regulators of protein kinase A (PKA) activity or cytokines such as IL-2, which regulate PCD either positively or negatively.

According to a recent review by Raff, PCD seems to occur by default in some mammalian cells, unless suppressed by signals from other cells.⁶ That is, cells are programmed to destroy themselves unless they continuously receive signals from other cells not to do so, although how general this is remains to be studied. The significance of PCD in the development of ATL is not apparent. However, it is intriguing to speculate that competition for a survival signal such as IL-2 ensures that the fittest tumor cells are selected and that less fit cells are eliminated. During the course of ATL, there may be a balance between proliferation and PCD, but selection may favor cells with more receptors for survival signals such as IL-2, or those with more efficient signal transduction pathways.

Recently, it has been reported that PKA and PKC affect PCD positively and negatively, respectively, in several experimental systems.^{4, 16-18} Therefore, PKC activation induced by IL-2 could involve the prevention of PCD in ATL cells, although whether PKC is activated by IL-2 is still being debated.^{29, 30} The molecular mechanism of PCD is largely unknown. However, recent studies of nematode have revealed that two genes, *ced-3*

and *ced-4*, function in PCD and one gene, *ced-9*, acts as a brake for the suicide program.^{31, 32} In mammals, *bcl-2* in the inner mitochondrial membrane resembles *ced-9* and can protect cells from PCD.³³ IL-2 might be involved in the expression of genes that initiate or regulate PCD in ATL cells.

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