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Human T Cell Leukemia Cell Death by Apoptosis-inducing Nucleosides from CD57$^+$ HLA-DR^{bright} Natural Suppressor Cell Line

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Apoptosis-inducing nucleosides (AINs) released from CD57$^+$HLA-DR^{bright} natural suppressor (57.DR-NS) cell line, derived from human decidual tissue, were isolated from 57.DR-NS cell culture supernatant by the combination of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Apoptotic cell death was strongly induced in human T cell leukemia Molt4 cells treated with AINs, absolutely depending on DNA strand breaks, with activation of the caspase cascade, especially caspase-3. The administration of AINs to Molt4 tumor-bearing severe combined immunodeficiency (SCID) mice resulted in drastic suppression of tumor growth, with a decrease of tumor size and the appearance of apoptotic signals in tumor tissue. Thus, AINs are candidates for development as anticancer agents.

Key words: Decidual cell — Natural suppressor cell — Apoptosis-inducing nucleosides — DNA strand breaks — Caspase-3

In our previous studies on humoral tumor immunity,[1–3] we demonstrated that human natural antibodies against Forssman antigen lysed several human malignant cells and that the specific active immunization of tumor-bearing rats with Forssman antigen resulted in tumor regression. On the other hand, cellular antitumor activity is mainly dependent on natural killer (NK) cell-mediated functions.[4] Natural suppressor (NS) cells carrying CD57 surface markers have been shown to regulate various lymphoid responses, sharing some features with NK cells.[5, 6] Furthermore, NS cells not only act as strong inhibitors of lymphoproliferative responses,[7, 8] but also inhibit the proliferation of tumor cells.[9, 10] Decidua cells positive for NK cell markers, including the majority of CD56 and a minority of CD57, defined morphologically as large granular lymphocytes (LGL), have been found in abundance in human and murine decidua, where they play an immunoregulatory role in implantation and invasion into the endometrium of the blastocyst.[11–16]

In our previous reports,[17–21] we described the CD57$^+$HLA-DR^{bright} natural suppressor (57.DR-NS) cell line, cloned from human decidual tissue.[22] The 57.DR-NS cell line could induce apoptosis not only in human leukemia cells,[23] but also in human gastric carcinoma cells,[24] mediated by factors of low molecular weight released into the culture fluid. Six components were isolated by means of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The six active components were determined to be nucleosides and modified nucleosides in nature by the combination of nuclear magnetic resonance and mass spectrometric analysis.[23] We collectively termed them "apoptosis-inducing nucleosides (AINs)." However, the molecular mechanisms of AINs-induced apoptosis in target cells remained unknown. In the present study, we examined the initial molecular mechanisms, such as DNA strand breaks and activation of caspase-3, during AINs-induced apoptosis of human leukemia Molt4 cells. Furthermore, the antitumor effects of AINs on human leukemia-bearing severe combined immunodeficiency (SCID) mice were examined by monitoring tumor cell growth and cell death.

MATERIALS AND METHODS

Cells and cultures 57.DR-NS cells have been maintained in RPMI 1640 (Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS), 0.25 m*M* Na pyruvate, 2 m*M* L-glutamine, 0.75% Na bicarbonate and 100 μg/ml of kanamycin at 37°C in a humidified incubator containing 5% CO_2. Human T cell leukemia cell line Molt4 cells were also maintained in the same medium. To collect the culture supernatant, 10^7 57.DR-NS/Molt4 cells were inoculated into a semi-bulk culture petri dish (diameter 15 cm, Nalge Nunc International, IL) in the medium described above. The culture supernatant (500 ml/10 dishes/experiment) was removed 72 h later, centrifuged and filtered through a 0.45 μm Millipore disposable filter unit (Millipore, Tokyo).

Isolation of AINs The 57.DR-NS cell culture supernatant collected (500 ml) was applied to an octadecyl column (Bond Elut C18, size 60 ml, Varian, CA). The active components retained in the column were eluted with acetonitrile and the eluate was evaporated under a stream of N_2. Then, the sample was spotted on a TLC plate (F254, Merck, Darmstadt, Germany) and developed with chlo-

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roform/methanol/water (C/M/W, 60:40:8, v/v/v). The components that migrated between the solvent front and the band of phenol red, previously added to the culture medium, were extracted with C/M 1:1 (v/v) followed by evaporation under a stream of N_2. Finally, the residue was subjected to C18-reverse phase HPLC (Beckman, CA) with a TSK gel ODS-80TM column (Tosoh, Tokyo), eluted with a linear gradient from 0 to 5% acetonitrile in 0.1% trifluoroacetic acid during 360 min at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected. The elution profile was monitored in terms of the absorbance at 214 nm. Fractions in each major peak (P) were pooled, freeze-dried and examined for activity. Control samples were prepared similarly, using Molt4 cell culture supernatant, and designated P_m.

Measurement of DNA strand breaks The strand unwinding rate correlated well with the number of breaks, or alkali-sensitive sites induced in DNA of target cells treated with DNA-damaging stress.[25–27] Total fluorescence was derived from cultures in which DNA unwinding was prevented and background fluorescence, from cell suspensions after sonication to permit complete DNA unwinding. The percent residual double-stranded DNA after alkali treatment was calculated as follows; Percent of double-stranded DNA=(sample fluorescence−background fluorescence)/(total fluorescence−background fluorescence)×100. Therefore, triplicate assay tubes were prepared for measurements of DNA fluorescence (total, background and test conditions).

Molt4 cells (10^6) were incubated with the AINs components, P_1, P_2 and P_4 at various doses (8, 40, 200 and 1000 μM), then resuspended in 0.2 ml of 10 m*M* sodium phosphate buffer (pH 7.2) containing 0.25 *M* inositol and 1 m*M* $MgCl_2$ and lysed by adding 0.2 ml of a solution containing 9 *M* urea, 10 m*M* NaOH, 2.5 m*M* diaminocyclohexane tetraacetate, and 0.1% sodium dodecyl sulfate to each tube for 10 min at 0°C. At this time, 0.4 ml of 1 *M* glucose and 14 m*M* 2-mercaptoethanol were added to the total fluorescence tubes to prevent DNA unwinding. All tubes then received 0.2 ml of 0.2 *N* NaOH, which raised the pH of the test and background samples to pH 12.8 and that of the total fluorescence tubes to pH 11.2. Background aliquots were then briefly sonicated. Individual samples were incubated at 0°C for 30 min and then at 18°C for intervals from 0 to 90 min. DNA unwinding was stopped by adding 0.4 ml of 1 *M* glucose and 14 m*M* 2-mercaptoethanol to test and background tubes. Ethidium bromide, 6.7 μg/ml in 13.3 m*M* NaOH, was added to each tube. Finally, the relative fluorescence intensity was measured in a filter fluorometer (excitation 520 nm, analyzer 590 nm).

Flow cytometric assessment of sub-G1 DNA content After the treatment of 5×10^5 Molt4 cells with P_1, P_2 or P_4 at the dose of 200 μM for 24, 48 and 72 h, the cells were harvested by trypsinization and fixed in 70% ethanol. The cells were stained in propidium iodide solution containing 50 μg/ml of propidium iodide, 40 m*M* sodium citrate, 0.1 μg/ml RNaseA and 1% Triton X-100 in phosphate-buffered saline (PBS). The cells were incubated for 60 min in the dark, and then analyzed by flow cytometry (Becton Dickinson FACScan, CA).

Assessment of DNA fragmentation After the treatment of Molt4 cells in the same manner as above, the cells were homogenized in homogenization buffer (0.3 *M* Tris pH 8.0, 10 m*M* EDTA, 0.1 *M* NaCl and 0.2 *M* sucrose) at 65°C for 30 min and 1/10 vol. of 3 *M* Na acetate (pH 5.0) was added. The mixture was kept at 0°C for 1 h, then total DNA was extracted with phenol/chloroform, precipitated with ethanol and incubated for 1 h at −70°C. The DNA pellets were resuspended in 20 μl of distilled water with DNase-free RNase and incubated at 37°C for 1 h. An aliquot of DNA sample was electrophoresed on 2% agarose gel containing ethidium bromide.

Morphological observation of apoptosis The Molt4 cells or tumor tissue sections treated with various doses of P_1, P_2 and P_4 were fixed in 4% paraformaldehyde-PBS at 4°C overnight. The fixed target cells or sections on slides were subjected to terminal deoxynucleotide transferase-mediated dUTP-nick end labeling (TUNEL) using an ApopTag kit according to the manufacturer's instructions (Oncor, Gaithersburg, MD). The samples on slides were incubated with TdT enzyme to catalyze digoxigenin-dUTP and dATP binding to 3′ ends of fragmented DNA to form a heteropolymer of digoxigenin-11-dUTP and dATP at 37°C for 2 h, followed by anti-digoxigenin-peroxidase antibody for 1 h at room temperature. The samples on slides were visualized by addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB) and photographed under a light microscope.

Assay of caspase-3 activity The examination of caspase-3 activity was performed by using a Caspase-3 assay kit according to the manufacturer's instructions (PharMingen, CA). In brief, 5×10^5 Molt4 cells were treated with 200 μM of P_1, P_2 or P_4 and harvested 24, 48 or 72 h later. The cell suspension (2×10^5 cells/100 μl) was mixed with 400 μl of PBS, and 10 μg of Ac-DEVD-AMC (N-acetyl-DEVD-7-amino-4-methylcoumarin) was added. The fluorogenic AMC liberated from Ac-DEVD-AMC was measured by flow cytometry using UV excitation at 380 nm.

Inhibition of caspase activity Molt4 cells (5×10^5) were incubated with 200 μM P_1, P_2 or P_4 alone or in the presence of 100 μg/ml of the caspase inhibitor Z-Asp-CH_2-DCB (carbobenzoxy- L-aspart-1-yl-[(2,6-dichlorobenzoyl)-oxy]methane) or the caspase-3 inhibitor, Ac-DEVD-CHO (N-acetyl-DEVD-aldehyde), purchased from Peptide Inst. Inc. (Osaka), for 72 h at 37°C in a 5% CO_2 incubator. Then, the cells were harvested and subjected to flow-cytometric analysis and DNA fragmentation assay.

Examination of anti-tumor activity of AINs on Molt4 tumor-bearing SCID mice Three-week-old SCID mice were purchased from CLEA Japan (Tokyo). Molt4 cells (10^8) were subcutaneously inoculated into the neck in ten SCID mice per experiment. Two weeks later, when the tumor was sufficiently grown, similar-sized tumor-bearing mice were randomly assigned into two groups of five each. In one group, P_1, P_2 and P_4 were dissolved at 400 μg each in 0.2 ml of PBS (total 1.2 mg/mouse/injection) and administered eighteen times to the Molt4 tumor-bearing mice alternately into the tumor and the tail vein. In the other group (control), P_m at the same dose was administered similarly. The anti-tumor activity with AINs was monitored by measuring the tumor size (longitude×latitude, cm^2) once a week for 3 weeks. Finally, the tumor tissues were removed and examined by TUNEL and DNA fragmentation methods.

Statistical analysis Differences from control values were analyzed by using Student's *t* test. Probability values <0.05 were considered to be significant.

RESULTS

Preparation of AIN samples by TLC and HPLC Culture supernatant of 57.DR-NS cells was partially purified on a preparative octadecyl column followed by TLC. The bioactive fraction that migrated to the area above phenol red on TLC was further subjected to reverse-phase HPLC. Six major peaks (P_1–P_6) were obtained by HPLC (Fig. 1A). The structures of the six active components were determined in our previous study[23] as follows: P_1, 2′-deoxyuridine; P_2, ribothymidine; P_3, 2′-O-methyluridine; P_4, thymidine; P_5, 2′-O-methylinosine; P_6, 2′-O-methylguanosine.

On the other hand, the elution pattern of Molt4 cell culture supernatant in HPLC gave a quite different profile from that of 57.DR-NS cell culture supernatant (Fig. 1B), affording only an inactive peak (P_m). No active peaks corresponding to P_1–P_6 from 57.DR-NS cell culture supernatant were obtained.

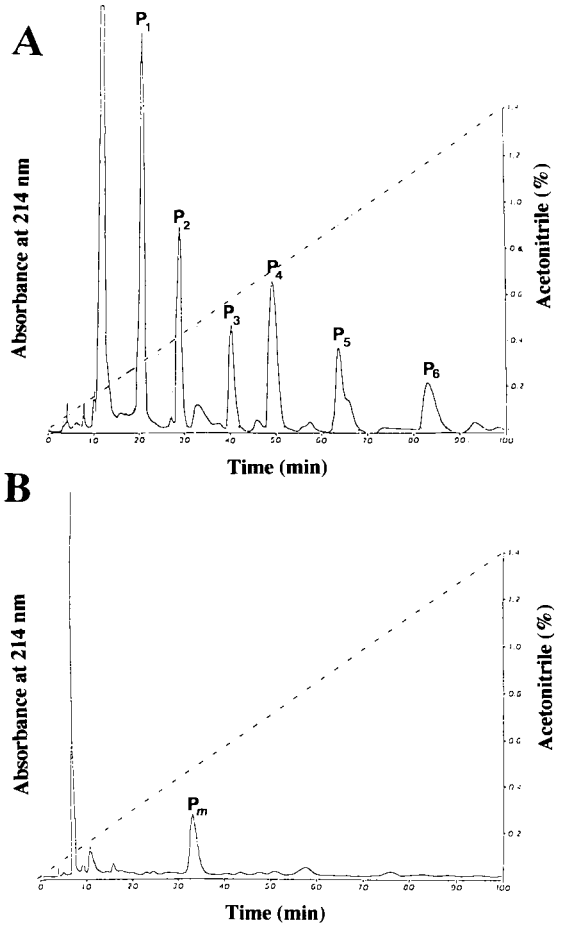

Fig. 1. Elution patterns in HPLC. In chart A, six active peaks (P_1–P_6) were obtained from 57.DR-NS cell culture supernatant. In chart B, one nonactive peak (P_m) was obtained from Molt4 cell culture supernatant.

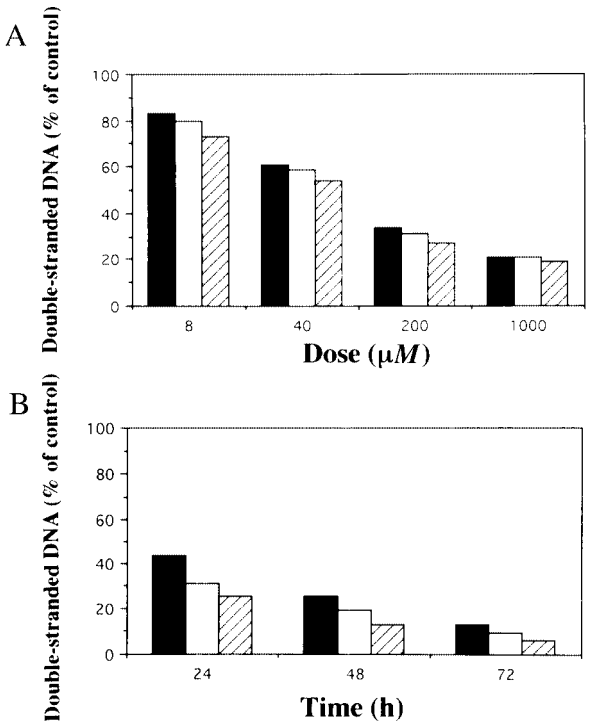

Fig. 2. Dose-response relationship and time course of DNA strand breaks. DNA unwinding in Molt4 cells began after incubation with P_1, P_2 or P_4 and progressed in a dose- and time-dependent manner. ■ P_1, □ P_2, ▨ P_4.

DNA strand breaks in target cells by AINs The assay was based on the observation that the rate of DNA unwinding in alkaline solution is inversely related to the length of intact double-stranded DNA segments. Incubation with 8, 40, 200 and 1000 μM P_1, P_2 and P_4 for 24 h caused DNA strand breaks in the target Molt4 cells in a dose-dependent manner (Fig. 2A). Furthermore, alkali-resistant double-stranded DNA decreased to less than 50% of control values at 24 h on incubation with 200 μM P_1, P_2 and P_4 (Fig. 2B). The accumulation of DNA strand breaks in the target Molt4 cells progressed in a time-dependent manner up to 72 h (Fig. 2B).

Induction of apoptosis in target cells treated with AINs Flow cytometric analysis showed that the ratio of sub-G_1 DNA content (M_1) of Molt4 cells treated with P_1, P_2 and P_4 at the dose of 200 μM for 24, 48 or 72 h increased to 5.1, 30.3 or 90.1% (P_1), 5.2, 40.2 or 70.2% (P_2) and 4.6, 70.2 or 81.1% (P_4) respectively in a time-dependent manner (Fig. 3A). The control values were 5.3, 6.0 and 10.2% (C) at the dose of 0 μM, respectively. The DNA fragmentation assay of Molt4 cells was performed over 24 to 72 h, following the treatment of target cells with 200 μM P_1, P_2 or P_4 (Fig. 3B-a). DNA ladders were observed at 72 h (Fig. 3B-a, lanes 9, 10 and 11). No ladders were found at

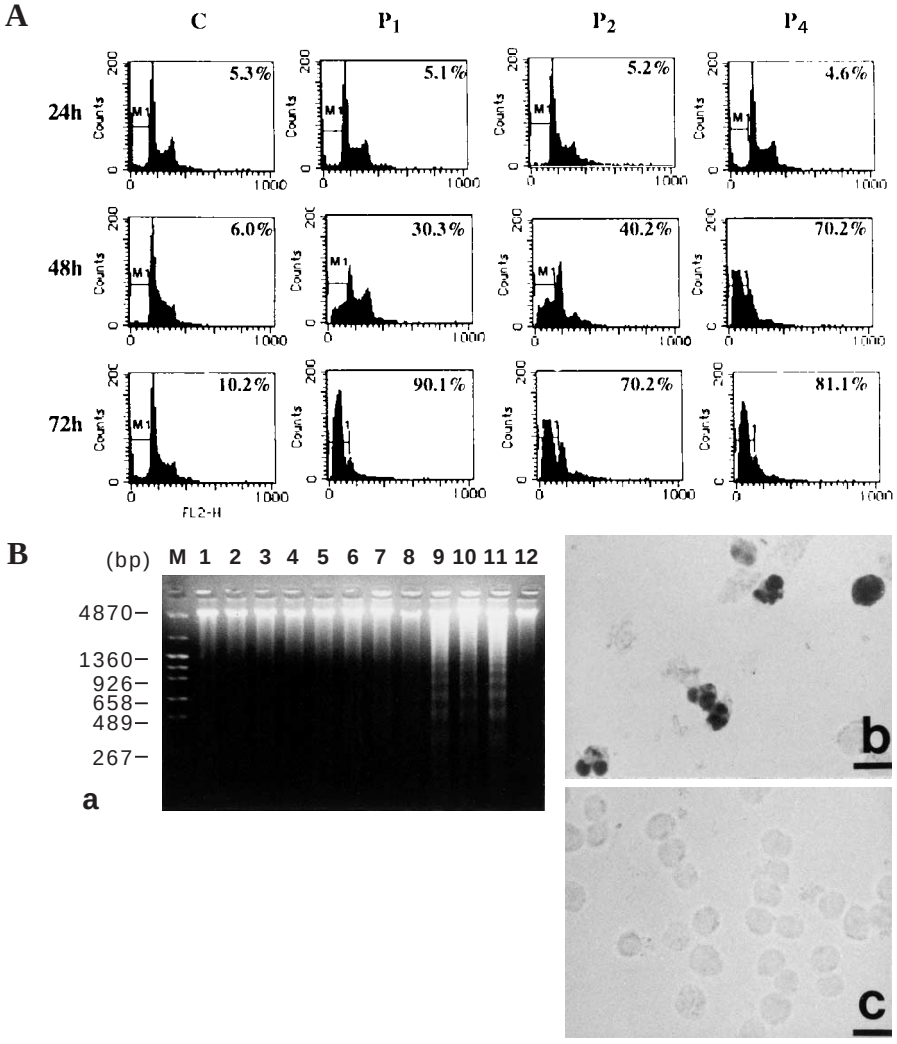

Fig. 3. Analysis of time course of apoptosis with flow cytometry, DNA fragmentation and TUNEL methods. The accumulation of sub-G_1 DNA content in Molt4 cells treated with 200 μM P_1, P_2 and P_4 increased in a time-dependent manner (A). Vertical and horizontal axes show the number of cells (counts) and the intensity of fluorescence (FL2-H), respectively. DNA fragmentation appeared at 72 h after incubation with 200 μM P_1, P_2 and P_4 (B-a, lanes 9, 10 and 11), but not at 24 h (lanes 1, 2 and 3) or 48 h (lanes 5, 6 and 7) or in the control (lanes 4, 8 and 12). Markers were run in lane M; the numbers represent base pairs (bp). The profiles of apoptosis in Molt4 cells treated with 200 μM P_1 (B-b) or without any AIN (B-c) were examined by the TUNEL method. Bars indicate 25 μm in B-b and B-c.

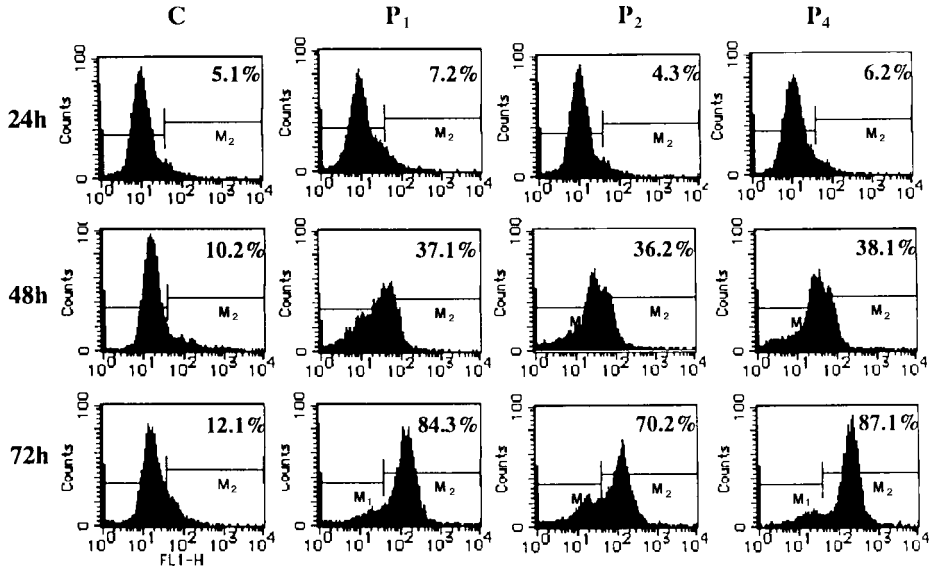

Fig. 4. Flow cytometric analysis of time course of caspase-3 activity. The number of cells positive for caspase-3 activity in Molt4 cells incubated with 200 μM P_1, P_2 or P_4 increased in a time-dependent manner. Vertical and horizontal axes show the number of cells (counts) and the intensity of fluorescence (FL1-H), respectively.

24 and 48 h (lanes 1, 2, 3 and 5, 6, 7), or in the control (lanes 4, 8 and 12).

The morphological changes in Molt4 cells treated with 200 μM P_1, P_2 or P_4 for 72 h were also examined. Molt4 cells treated with P_1 were stained by the TUNEL method, showing DNA fragments in the nucleus and a part of the cytoplasm, in addition to blebbing of membranes and shrinkage of cells (Fig. 3B-b). No such changes or staining were observed in the control cells (Fig. 3B-c). The results demonstrated that the P_1, P_2 or P_4 component of the AINs isolated by HPLC could induce apoptosis in Molt4 cells in a time-dependent manner, corresponding to the cytometric analysis of the ratio of sub-G_1 DNA content of Molt4 cells treated with these AINs (Fig. 3A).

Activation of caspase-3 in target cells treated with AINs To identify the main protease species in the apoptotic pathway, cleavage of a fluorogenic substrate (Ac-DEVD-AMC) of caspase-3 was examined by flow cytometry (Fig. 4). The number of cells positive for caspase-3 cleavage activity (M_2) at 24 h was 7.2% (P_1), 4.3% (P_2) and 6.2% (P_4) in Molt4 cells treated with 200 μM P_1, P_2 or P_4, but increased to 37.1 or 84.3% (P_1), 36.2 or 70.2% (P_2) and 38.1 or 87.1% (P_4) at 48 and 72 h, respectively. The control values were 5.1, 10.2 or 12.1% (C), respectively. Thus, it was concluded that the apoptotic pathway in Molt4 cells treated with P_1, P_2 and P_4 is mediated, at least in part, by the activation of caspase-3.

Blocking of apoptosis in target cells by inhibitors of caspases and caspase-3 To characterize further the caspase-associated apoptosis, we tested the effect of inhibitors of caspases (Z-Asp-CH_2-DCB) and of caspase-3 (Ac-DEVD-CHO) on the development of apoptosis. DNA fragmentation was induced in Molt4 cells treated with P_1, P_2 or P_4 at the dose of 200 μM for 72 h (Fig. 5A, lanes 1, 2 and 3). But the addition of 100 μg/ml of inhibitor (Z-Asp-CH_2-DCB or Ac-DEVD-CHO) prevented DNA fragmentation in Molt4 cells (lanes 4, 5 and 6 or lanes 7, 8 and 9), and no DNA fragmentation was seen in the control (lane 10). In addition, flow cytometric analysis showed that the accumulation of sub-G_1 DNA content (M_1) in Molt4 cells treated simultaneously with 200 μM P_1, P_2 or P_4 and 100 μg/ml of Ac-DEVD-CHO in 72 h was inhibited to marginal levels (Fig. 5B-b) compared with that in Molt4 cells treated with 200 μM P_1, P_2 or P_4 (Fig. 5B-a). Thus, it was confirmed that caspase-3 plays a critical role in P_1, P_2 and P_4-induced apoptosis of Molt4 cells.

Curative effects of AINs in Molt4 tumor-bearing SCID mice The average tumor growth curves in a group treated with a mixture of P_1, P_2 and P_4 and a P_m-treated control group are shown in Fig. 6A. The average tumor sizes were much smaller in the former group ($P<0.001$), indicating that the growth of Molt4 tumor cells was strongly inhibited by the administration of AINs. All five tumor-bearing mice survived and three out of five were completely cured, in contrast with the rapid growth of tumor tissues in all five control mice. Even in the two mice that were not cured, the apoptotic signals detected by DNA fragmentation and TUNEL methods were spread in all areas of tumor tissues. On completion of the experiment after 3 weeks, the tumor tissue was dissected from an AINs-

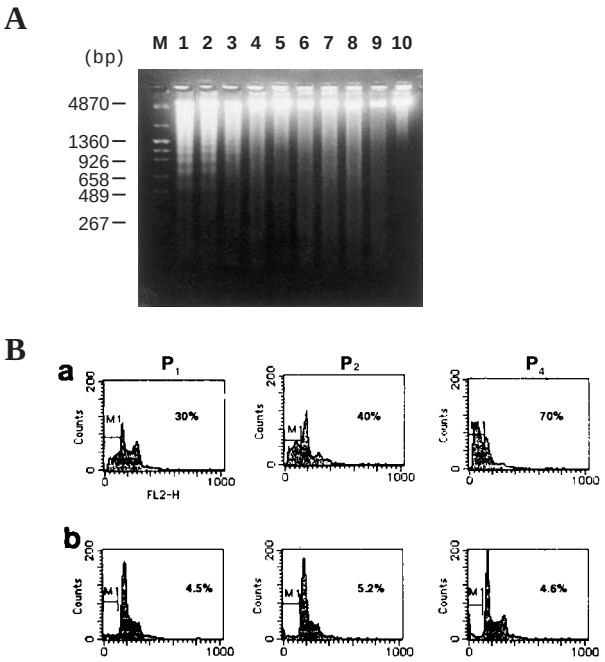

Fig. 5. Analysis of induction and inhibition of apoptosis in terms of DNA fragmentation and flow cytometry. Panel A shows DNA fragmentation in Molt4 cells at 72 h on incubation with 200 μM P_1, P_2 and P_4 (lanes 1, 2 and 3), but not after further addition of inhibitors of caspases (lanes 4, 5 and 6) and of caspase-3 (lanes 7, 8 and 9) or in the untreated control (lane 10). Markers were run in lane M. The sizes of markers are shown as numbers of base pairs (bp). Panel B shows the result of flow cytometric analysis, demonstrating the accumulation of sub-G_1 DNA content in Molt4 cells treated with 200 μM P_1, P_2 and P_4 for 72 h (a), but not in the presence of an inhibitor of caspase-3 (b).

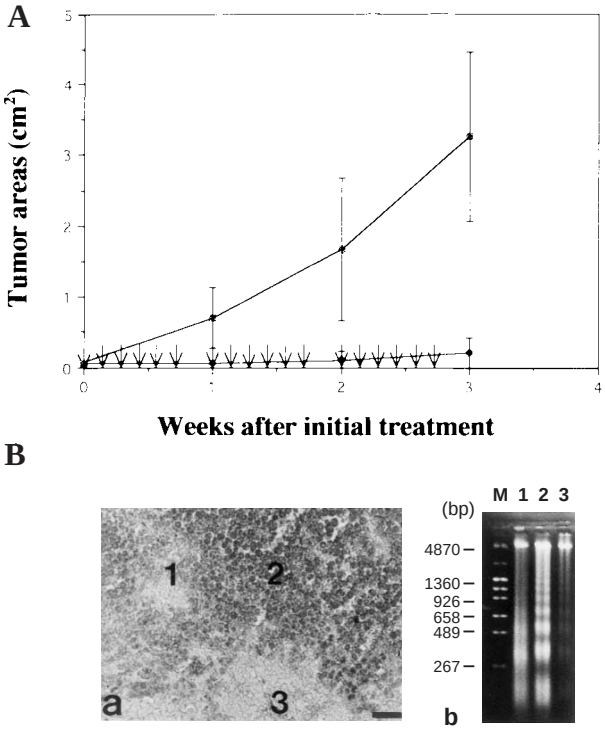

Fig. 6. Therapeutic effects of AINs on Molt4 tumor-bearing SCID mice. Panel A. A mixture of P_1, P_2 and P_4 from 57.DR-NS cell culture supernatant or P_m component from Molt4 cell culture supernatant as a control was administered 18 times to Molt4 tumor-bearing SCID mice alternately into the tumor and the tail vein, starting at 2 weeks after subcutaneous inoculation of Molt4 cells. Each curve shows the average of the tumor sizes in five Molt4 tumor-bearing SCID mice. The tumor growth in the AINs-treated group (+) is much less than that of the control group (−). Vertical and horizontal axes show the average tumor size and the time (weeks) after the initial treatment. Bars show standard deviation. The arrowheads show the points of administration. ◆ AINs(+), ■ AINs(−). Panel B. The tumor tissue was dissected from a mouse treated with AINs and separated into three parts, a-1, 2, 3. Panel a shows the result of TUNEL staining. The bar indicates 50 μm. Panel b shows DNA fragmentation in the three parts (lanes 1, 2 and 3, respectively). Markers were run in lane M. The sizes of markers are shown as numbers of base pairs (bp).

treated mouse and separated into three parts, according to the TUNEL staining grade: 1, positive staining region; 2, strongly positive staining region; 3, weakly positive staining region (Fig. 6B-a). DNA fragmentation was found in all parts (Fig. 6B-b).

DISCUSSION

Some investigators have postulated that macrophages produce and release thymidine and other nucleosides in order to regulate the proliferation of adjacent cells.[28, 29] Further studies[30] showed that deoxyribonucleosides and thymidine are toxic to human malignant T lymphoid cells, since malignant T cell lines have high levels of deoxyribonucleoside phosphorylating activity and low levels of deoxyribonucleotide dephosphorylating activity, resulting in the accumulation of triphosphate forms of deoxyribonucleotides. 8-Chloroadenosine can arrest Molt4 cell growth *in vitro*[31] and inhibit human lung tumor growth in nude mice *in vivo*,[32] probably via apoptotic mechanisms.[33] Furthermore, 1-β-arabinofuranosylcytosine has been used for the treatment of acute myelogenous leukemia, inducing DNA fragmentation in leukemia cells.[34, 35] Deoxyadenosine derivatives, such as 2-chloro-2′-deoxyadenosine, also induce apoptosis in quiescent lymphocytes, and are available for the treatment of indolent lymphoproliferative diseases.[36, 37] Thus, the modulation of apoptotic cell death may be an effective antitumor strategy.

It has been clarified that murine metrial LGLs can lyse Yac target cells[12] and that NK cell populations in human

early pregnancy decidua respond to IL-2 and acquire killer activity against K562 leukemia cells and trophoblast cells including choriocarcinoma cells.[38, 39] We have demonstrated that 57.DR-NS cells originated in human decidual tissue induce apoptosis not only in human leukemia K562 cells, but also in human gastric carcinoma GCIY cells through AINs, though they have no effect on human lung normal WI-38 cells.[23, 24] In the present study, we examined the apoptotic mechanisms in Molt4 cells exposed to AINs released from 57.DR-NS cells and further investigated the therapeutic effects of AINs in Molt4 tumor-bearing SCID mice. By means of flow cytometry, DNA fragmentation and TUNEL methods, we demonstrated that P_1 (2′-deoxyuridine), P_2 (ribothymidine) and P_4 (thymidine) isolated by HPLC from 57.DR-NS cell culture supernatant could induce apoptosis in Molt4 cells (Fig. 3). Then, we demonstrated that the antitumor efficacy of AINs in Molt4 tumor-bearing SCID mice was dependent on tumor cell death through the induction of apoptosis throughout the tumor tissue (Fig. 6).

We speculate that the AINs might be incorporated into Molt4 cells and converted progressively to their triphosphate forms, causing deoxynucleotide imbalance and DNA strand breakdown (Fig. 2), and activating the poly-ADP ribose polymerase[40] and p53-dependent pathways.[41] The activation of the caspase cascade is ubiquitously involved in various apoptotic mechanisms,[42–44] so we examined the involvement of caspase-3 in the induction of apoptosis in Molt4 cells by AINs as shown in Fig. 4, using Ac-DEVD-CHO as a specific caspase-3 inhibitor. The generation of DNA fragments and accumulation of sub-G_1 DNA content was completely inhibited in Molt4 cells treated with P_1, P_2 or P_4 in combination with the inhibitor (Fig. 5). This result confirmed the involvement of caspase-3, which is activated by sequential proteolytic events that cleave the 32-kDa precursor at aspartic acid residues to generate an active heterodimer of 20- and 12-kDa subunits,[45] corresponding to our recent findings.[46] The active caspase-3, in turn, cleaves the inhibitor of caspase-activated DNase (ICAD) and then activates the caspase-activated DNase (CAD), which migrates to the nucleus and degrades DNA.[47] We consider that AINs are good candidates for anticancer agents, and we are planning further studies on AINs-induced apoptosis in various malignant target cells.

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