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APOPTOSIS OF TUMOR CELLS IN LECTIN-DEPENDENT LYMPHOKINE-ACTIVATED KILLER CELL-MEDIATED CYTOTOXICITY

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By using DNA electrophoresis and propidium iodide (PI) staining flow cytometry (FACS) analysis, we studied the mechanisms of lymphokine-activated killer (LAK) cell-mediated cytotoxicity. In the presence of pokeweed mitogen (PWM), human LAK cells induced DNA fragmentation of two leukemic cell lines (U937 cells and Raji cells) and two solid tumor cell lines (SW1116 cells and Hep-2 cells), a hallmark of apoptosis. The reactions were carried out at the effector/target ratio of 1:1 in 4 h co-culture. Pretreatment with RNA and protein synthesis inhibitors (actinomycin D and cyclohexamide) did not prevent the target cells from apoptosis. As the TNF-resistant tumor cell lines such as SW1116 cells and Raji cells were also triggered to apoptosis, other factors than TNF may play the role. DNA-PI staining FACS analysis also suggested that a part of LAK cells underwent apoptosis to some extent during incubation with target cells. The results provide a new way to investigate the mechanisms of the cytotoxicity of LAK cells, and a new possibility to enhance the efficiency of adoptive tumor therapy with LAK cells.

Key words: Lectin, LAK cells, Apoptosis.

The deeper understanding of the mechanisms of lymphokine-activated killer (LAK) cell-mediated cytotoxicity, will give a new way to improve the clinic therapeutic effects of adoptive immunotherapy of cancer. It has been reported that apoptotic cell death of tumor target cells was induced by LAK cells,[1] but the condition and mechanisms of this phenomenon are still unclear. So far, we have discovered that lectin pokeweed mitogen (PWM) enhanced the proliferation and the cytotoxicity of LAK cells,[2] and lead to a remarkable tumoricidal activity to several kind of tumor cell lines.[3] In this study, we investigated the relationship of apoptosis and the lectin-dependent cell-mediated cytotoxicity (LDCC) of LAK by analysis of the DNA fragmentation of tumor target cells.

MATERIALS AND METHODS

Culture of Lymphokine-activated Killer Cells

Mononuclear cells (MNC) were isolated from heparinized peripheral blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation at 1600 rpm for 40 min. The MNC were washed with phosphate buffered saline (PBS), and incubated with 1000 U/ml of interleukin-2 (IL-2, Seleuk, Japan) in RPMI-1640 medium containing 10% of fresh human AB blood serum. Pokeweed mitogen (PWM, Sigma) was present in the primary 48 h of culture, at the final concentration of 0.3–1 μg/ml.

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Human Tumor Cell Line

The target cell used in this study were U937 cells (human monoblasma cell line), Raji cells (human B lymphoblastoid cell line), SW1116 cells (human colon carcinoma cell line) and Hep-2 cells (human nasopharyngeal carcinoma cell lines). All the cell lines were cultured in RPMI-1640 medium containing 10% of fetal bovine serum. DAPI staining showed that the culture had no mycoplasma contamination.

DNA Electrophoresis

LAK cells and tumor target cells were co-cultured in 6-well tissue culture plates at the E/T ratio of 1:1. After 4 h of incubation, the cells were collected and washed in PBS. The target cells were isolated from LAK cells by Ficoll-Hypaque density gradient centrifugation. After the centrifugation, both cells in the bottom of tube (intact cells) and in the supernatants of the tube (fragmented cells) were collected together, and washed. The DNA of these cells were isolated by using DNA extract kit (SepGene, Wako Junyaku, Japan). 10 μg of DNA were mixed with loading buffer and electrophoresed in 1.5% agarose gel. Fragmented DNA ladders were visualized under UV after ethidium bromide staining.

DNA Staining and Flow Cytometric Analysis

The percentage of apoptotic cells was determined by using the DNA-intercalating dye propidium iodide (PI, Sigma).[4] Briefly, after 4 h of incubation, the cells were gently resuspended in 1 ml hypotonic fluorochrome solution of PI (50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100) and placed at 4 ℃ in the dark overnight before analysis. The PI fluorescence of isolated nuclei was measured with a FACScan flow cytometry (Becton-Dickinson) and the percentages of apoptotic nuclei (subdiploid DNA peak in the DNA fluorescence histogram) was calculated using a Consort TM computer (Packard).

RESULTS

DNA Fragmentation of Target Cells in the LDCC of LAK Cells

As shown in Figure 1 and 2, intact DNA was observed when the target cells were incubated alone. After incubation with LAK cells in the presence of PWM, the DNA of both solid tumor cells and leukemic cells were degraded into discrete bands which by comparison to DNA markers were multiples of an approximately 200-base pair subunit. In the absence of PWM, LAK cell alone did not induce remarkable DNA fragmentation of target cells. The pretreatment of target cells with actinomycin D or cyclohexamide did not prevent the target cells from apoptosis in LDCC.

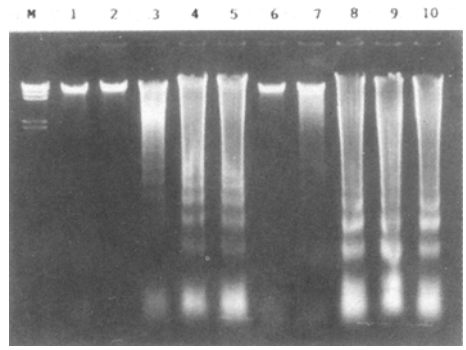

Fig. 1. DNA fragmentation of leukemic cells in LDCC.

Electrophoresis of DNA isolated from Raji cells (1–5) and U937 cells (6–10) which were treated as: (1, 6) medium control, (2, 7) incubated with LAK for 4 h, pretreated with 5 μg/ml of actinomycin D (4, 9) or 50 μg/ml of cyclohexamide (5, 10) for 2 h followed by 4 h of incubation with LAK+PWM.

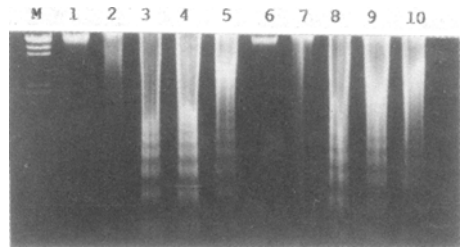

Fig. 2. DNA fragmentation of solid tumor cells in LDCC.

Electrophoresis of DNA isolated from Hep-2 cells (1–5) and SW1116 cells (6–10) which were treated as: (1, 6) medium control, (2, 7) incubated with LAK for 4 h, pretreated with 5 μg/ml of actinomycin D (4, 9) or 50 μg/ml of cyclohexamide (5, 10) for 2 h followed by 4 h of incubation wtih LAK+PWM.

Quantitative Analysis of Apoptotic Cells in LDCC

The percentage of DNA fragmentation of target cells was determined by flow cytometry. Results of a representative experiment are depicted in Figure 3, which shows a typical diploid peak in control cells and a hypodiploid peak adjacent to the left of the diploid peak in all these tumor cells incubated with LAK in the presence of PWM (LAK+PWM). The percentage of apoptotic cells was higher in culture with LAK +PWM than that with LAK alone. From the change of G_0/G_1 (diploid) peaks and G_2 (tetraploid) peaks, it seems that the decreased degree of G_0/G_1 peaks in the profiles of LAK-treated groups was much sharper than that of G_2 peaks. As the G_2 peaks mainly represented the cells of tumor, the decreased G_0/G_1 peaks may included the apoptosis of LAK cells *per se*. Meanwhile, the sharply decreased G_2 peaks in the profiles of LAK+PWM-treated groups indicated that the cell death of tumor cells mainly contributed to the lose of the fluorescence intensity.

DISCUSSION

Following the deeper study of the mechanisms of

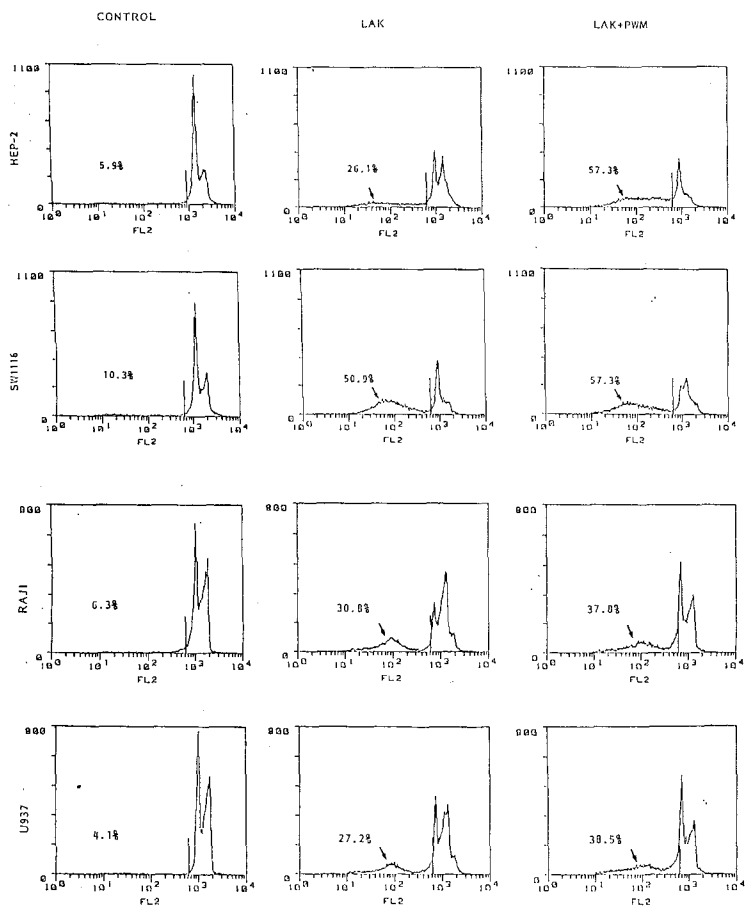

Fig. 3. DNA fluorescence histograms of apoptotic cells

PI-staining flow cytometric analysis of DNA from tumor cells which were subjected to 4 h incubation with medium control, LAK cells or LAK+PWM, respectively. The percentages of apoptotic cells were indicated in Figures.

of CTL- and NK- induced apoptosis of target cells, the apoptosis was also observed in LAK- and monocytes–mediated killing of target cells.[1, 5] In this study we found that different original tumor cells were induced to apoptosis in the LDCC of LAK cells, and this induced apoptotic cell death did not dependent on the *de novo* synthesis of intrinsic RNA and proteins.

The phenotype of PWM-pretreated LAK cells were CD3+, CD8+, and 50% of these cells were CD56+.[2] It has been detected that TNF-α was present in the culture medium supernatants of LAK cells.[6] TNF-α was found mainly mediated the slow killing of target cells by CD8+ cells.[7] As the TNF-insensitive target cells (SW1116 cells and Raji cells) were also rapidly induced to apoptosis in this study, the factors other than TNF may play a role in inducing apoptosis of target cells. It has been noticed that nitric oxide (NO) mediated the apoptosis of macrophages,[8] but we failed in detecting any significant production of NO_2 in the system of the LDCC of LAK cells. It remains to be investigated whether LAK cells produce H_2O_2, for H_2O_2 was observed to be able to induce DNA fragmentation of several kinds of cells *in vitro* (unpublished data).

PWM was found to enhance the sensitivity of target cells to LAK cell-mediated killing through binding to the surface of these cells.[9] It have been suggested that the role of PWM which bound to target cells my modify the surfacr structure of target cells and facilitate the recognition and killing of target cells by effector cells. Using LAK and PWM combination therapy, we successfully treated the human vascular endothelioma by subcutaneous injection of these effector cells.[10]

Little is known about the outcome of LAK cells in the process of killing target cells. By flow cytometry analysis, we found that during induction of apoptosis of target cells, a part of LAK cells dies as well. Similar results was also found in the killing of Daudi lymphoma by γδ T lymphocytes,[11] the interaction with the target physiologically induces the programmed cells death of the cytotoxic γδ effector. It is still difficult to see whether the death of LAK in this study was due to apoptosis, because there is no good method to separate the dead effector cells from the death target cells. The understanding of the biologic feature of LAK cells in the process of killing tumor cells will benefit the activation and culture conditions of LAK cells *in vitro*, and provide experimental basis to improve the efficiency of LAK cells *in vivo*.

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A CASE REPORT OF PRIMARY SQUAMOUS CARCINOMA OF THE ENDOMETRIUM

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A 60-year-old female patient presented to our hospital with compliant of 5-year-menopause and irregular vaginal bleeding of 3 months duration on Feb. 8, 1993. After menopause, the patient presented stink and quantities of vaginal discharge which wasn't treated. In the early Nov. of 1992, the patient noticed vaginal bleeding like menstruation which lasted more than ten days. Thereafter the vaginal discharge still continued. In Jan. 1993, the patient came to local hospital because the vaginal bleeding occurred again. She was underwent biopsy of the cervix and fractionally diagnostic curettage. The pathologic result revealed chronic cervicitis and adenocarcinoma of the endometrium, respectively. Pelvic examination showed light cervix erosion, but there were no apparent abnormalities in the uterus and the bilateral adnexa except a slightly enlarged corpus of the uterus. After the patient was admitted to our hospital, a diagnosis of squamous carcinoma of the endometrium (grade III) was made, which depended on reviewing of histologic sections obtained by diagnostic curettage at the local hospital. Because two pathologic results are entirely different in two hospital , diagnostic curettage was made again and the result was same as reviewed report. The patient underwent extend abdominal hysterectomy on Feb. 18. After operation the specimen was cut and there was a fungating mass sticking out to the endometrial cavity. The growth is about the size of 4 cm × 4 cm × 2 cm from the back wall to the fundus of the uterus. Histologic examination of the specimen revealed the results of squamous carcinoma of the uterine corpus (grade II–III) invading to superficial myometrium and chronic cervitis. It had been two years and seven months after the surgery, but there was no evidence of recurrence of the disease.

DISCUSSION

Primary squamous carcinoma of the endometrium is very rare. This growth occurs most frequently in old women. Up to now, only more than fifty cases of this disease were found in all cases reported. The dominant symptoms are irregular vaginal bleeding and discharge after menopause or menorrhagia and menostaxis which were usually followed with slight vaginal bleeding of postmenstrua. On pelvic examination the uterus may be enlarged, but the cervix is usually normal. The diagnosis is mostly dependent on histologic examination of the curettings obtained by fractional curettage, but it is necessary to differentiate from squamous carcinoma of the cervix extending to endometrium and mixed adenosquamous carcinoma. The essential treatment of this disease is surgery and radiotherapy may be given as auxiliary before or after operation. Prognosis is related with the extent of endometrium involved, the depth of myometrium invaded by the growth and the presence or absence of the extension or metastasis of the tumor.

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