Phenotypic analysis of nylon-wool-adherent suppressor cells that inhibit the effector process of tumor cell lysis by lymphokine-activated killer cells in patients with advanced gastric carcinoma

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Abstract. The causes of down-regulation of cytotoxic immune responses in cancer patients have not been fully evaluated. We previously demonstrated that T-cell-growth-factor-activated peripheral blood lymphocytes (PBL) with the surface phenotype CD8+ CD11b−, from patients with widespread metastasis of gastric carcinoma, inhibited the effector process of lymphokine-activated-killer (LAK)-cell-mediated cytolysis. In this study, we examined suppressor cell activity in freshly prepared PBL from 18 patients with advanced gastric carcinoma, and 10 normal healthy individuals. The suppressor cell activity was assayed by recording whether or not PBL inhibited directly the effector process of LAK cell cytotoxicity. Most of the PBL suspensions from cancer patients showed that they contained a population of cells that can directly inhibit the effector phase of tumor cell lysis of the cytotoxic cells. To analyze further the PBL responsible for the suppression, the cells were passed over a nylon-wool column. Nylon-wool-adherent cells significantly augmented the suppression, while the cells passing through abrogated the suppressive effect. Most nylon-wool-adherent cells from 10 normal healthy controls did not inhibit the cytotoxic reaction. To determine further the suppressor-effector population in nylon-wool-adherent cells, negative-selection studies using CD8-, CD4- or CD11b-coated magnetic beads, and positive-selection studies using CD8- or CD4-coated magnetic beads were performed. Finally, the results suggest that the suppressor-effector cells comprise at least two different surface phenotypes: CD8+ T and CD8− CD11b+ cells. The possible role of CD4+ T cells and HLA-DR+ LeuM3+ macrophages as suppressor cells was ruled out in nylon-wool-adherent cells. CD8+ T and possibly CD8− CD11b+ cells apparently suppressed the efferent limb of the antitumor immunity. The selective immune suppression mediated by these cells may partly be concerned with escape mechanisms of gastric carcinoma from the host immune surveillance system.

Key words: Suppressor cells – Nylon-wool-adherent cells – Lymphokine-activated killer cells – Gastric carcinoma

Introduction

A decrease in cytotoxic immune response has been demonstrated in cancer patients. The impaired immune response becomes more apparent as the tumor load increases (Koyama et al. 1989; Ebihara et al. 1989, 1990, 1991). It has been suggested that the decreased cytotoxic immune response may be due to the concomitant presence of suppressor and cytotoxic lymphocytes rather than to the absence of cytotoxic cells (Koyama et al. 1989; Ebihara et al. 1989, 1990, 1991; Mukherji et al. 1986; Bykovskaya et al. 1990). Although the population of suppressor cells or the precise mechanisms involved in suppression have so far remained obscure, multiple models for suppressor cell activities have been employed by many laboratories with different perspectives. In most cases, however, the suppressive activity has appeared to be more pronounced against the induction phase of cytotoxic immune responses (Mukherji et al. 1986; Bykovskaya et al. 1990), lymphoproliferative responses (Jerrells et al. 1978; Han 1980; Landy et al. 1983; Koyama et al. 1985b) and immunoglobulin synthesis (Clement et al. 1984; Kanof et al. 1987; Kansas and Engelman 1987). Suppressor T cells, which are effective in the efferent or afferent limbs of tumor immunity in animal models, have been extensively studied (Takei et al. 1976; Fujimoto et al. 1978;...

We have attempted to study the condition in which suppressor cells are made active, and the nature of such cells involved in the inhibition of antitumor immunity. The present communication described circulating suppressor cells with the surface phenotype CD8+ or possibly CD8+CD11b+ from freshly prepared PBL in patients with advanced gastric carcinoma. The suppression of immunity by these cells is clearly directed at the effector process performed by LAK cells.

Materials and methods

Patients and their PBL. Eighteen patients with advanced gastric carcinoma (stage IV) with distant metastasis (such as liver or lung metastasis, or peritonitis carcinomatosa) were studied. The patients ranged in age from 37 to 77 years (mean 61.3 years). The control group consisted of 10 healthy volunteers, ranging in age from 47 to 64 years (mean 55.4 years). PBL from patients' heparinized blood samples were separated by standard Ficoll/Hypaque (Pharmacia, Uppsala) density-gradient centrifugation described previously (Koyama et al. 1983b, 1989; Ebihara et al. 1989, 1991).

Separation of PBL on a nylon-wool column. This was performed according to the method of Julis et al. (1973). PBL suspensions in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, Md.), 20 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES; Wako Junyaku Co., Ltd., Tokyo) and 100 μg/ml kanamycin (complete medium) were filtered at room temperature through 0.2-μm nylon-wool (BioTest AG, Dreieich, Germany) packed into a 5-ml glass syringe. Approximately (4-5)×107 cells in 0.5 ml were then loaded onto a sterile nylon-wool column. The columns were incubated for 60 min at 37°C, and then washed slowly with complete medium until the first 10 ml effluent had been collected. Adherent PBL were then recovered by gentle washing of the nylon-wool in cold Hanks' solution in a petri dish. The cells were pelleted and resuspended in complete medium and their viability was determined.

In vitro culture for the generation of LAK cells. LAK cells were generated in the PBL culture for 10 days in complete medium with 100 U/ml recombinant interleukin-2 (IL-2; Shionogi Co. Ltd., Osaka, Japan) in 5% CO2 in air at 37°C, as described previously (Ebihara et al. 1989, 1990, 1991; Koyama et al. 1989).

Assay for LAK cell activity. LAK cells activities were tested against natural-killer (NK)-resistant Daudi cells. The Daudi cells were kindly donated from the Institute of Hygienic Science, Tokyo. The tumor cells were labeled with 51Cr by the usual method as described previously (Koyama et al. 1989, 1990, 1991; Ebihara and Koyama 1990). The cytolytic activity of LAK cells was examined by using 4- to 51Cr-release assays. The labeled target cells (5×103) were mixed with effector cells at three effector target cell ratios (E/T ratios) of 5:1, 10:1 and 20:1, and were distributed in a final volume of 200 μl to each well of a V-bottomed 96-well Nunc microplate (no. 2.49662, Roskilde, Denmark) in quadruplicate. After incubation, the plates were centrifuged and cytolysis was evaluated by counting 0.1-ml samples in a gamma counter. Specific lysis was expressed according to the following formula:

$$\text{specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

The maximum releasable radioactivity was determined by repeated freezing and thawing of the labeled cells, and amounted to 80%-90% of the total radioactivity incorporated into the cells. Spontaneous release, determined from Daudi cells incubated in the complete medium, was always 5% or less of the maximum release in 4 h.

Assay for suppressor cell activity of patients' PBL on the cytolytic reaction of LAK cells. This was performed to the modified method described previously (Ebihara et al. 1989, 1990, 1991; Koyama et al. 1981, 1982, 1985a). Briefly, as the source of suppressor cells, the desired numbers of PBL from patients (for experiments) or normal individuals (for control) were added to a mixture of 2.5×104 LAK effector cells and 5×103 51Cr-labeled targets in a final volume of 0.2 ml in each well of a 96-well microtiter plate. The mixture was incubated at 37°C for 4 h for cytolysis of Daudi cells. All assays were autologous cultivation, that is, the PBL as the suppressor cell source were obtained from the blood of the same patients who donated the LAK cells. To standardize results and permit evaluation of the degree of suppression, the following formula was used:

$$\text{suppression} = \left(1 - \frac{\Delta^-}{\Delta^+}\right) \times 100$$

where Δ− is change in the percentage cytotoxicity after the addition of a suppressor cell source from patients to the culture of target cells mixed with effector cells, and Δ+ is the change in percentage cytotoxicity of target cells mixed with effector cells without cells of the suppressor cell source.

Monoclonal antibodies and two-color flow-cytometric analysis. Fluorescein-isothiocyanate (FITC)-conjugated anti-Leu2a (CD8), anti-Leu3a (CD4) and anti-HLA-DR, and phycoerythrin (PE)-conjugated anti-Leu15 (CD11b), anti-Leu4 and anti-Leu-M3 were provided by Becton Dickinson. Kolt 2 (CD28) was obtained from Nichirei Co. Ltd. (Tokyo). Secondary antibody staining was with the PE-conjugated goat anti-(mouse IgG) antibody (Biomedex, Foster City, Calif.). PBL were stained with fluorochrome-conjugated monoclonal antibody for 30 min at 4°C and were washed twice, as described previously (Ebihara et al. 1989, 1991; Koyama et al. 1989, 1992; Ebihara and Koyama 1990). The cells stained with FITC- and PE-conjugated antibodies were analyzed for double labeling by flow cytometry (FACScan, Becton Dickinson). To analyze the PBL, the cells were gated on light scattering to include both lymphocytes and monocytes.

Negative or positive immunomagnetic selection of suppressor cells from nylon-wool-adherent cells. For negative selection, an indirect technique was used. Briefly, adherent PBL were incubated with CD8 or CD4 or CD11b for 30 min at 4°C, was washed three times. The primary-antibody-labeled cells were then incubated with sheep anti-(mouse IgG)-conjugated immunomagnetic beads (Dynabeads M-450, product no. 110.01, Dynal, Oslo, Norway) at 4°C for 60 min with end-over-end rotation at a beads: cell ratio of 10:1. Immunomagnetic-bead-rossetted cells were removed using a magnetic particle concentrator (Dynal MPC-1, Oslo, Norway), and unrosetted cells remaining in suspension were harvested by pasteur pipette. For positive selection, a direct technique was used. Briefly, CD8- or CD4-conjugated beads (Dynabeads M-450 CD8 or CD4, product no. 111.07 or 111.15, Dynal, Oslo, Norway) were added to the nylon-wool-adherent cells. The mixture of cells and beads was incubated for 60 min at 4°C. Hanks' solution supplemented with 2% FCS was added to make the volume up to 10 ml, and beads with any attached cells were removed magnetically. Unrosetted cells remaining in suspension were harvested by pasteur pipette and were further used for functional assays. Rossetted cells were resuspended in 100 μl Hanks' solution supplemented with 2% FCS, and were incubated with 1 unit (10 μl) Detachbeads (product no. 125.01, Dynal, Oslo, Norway) for 60 min at room temperature. Detached beads were removed magnetically as described above.