Original articles

Autologous tumor killing and natural cytotoxic activity of tumor-associated macrophages in cancer patients

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Summary. Tumor-associated macrophages (TAM) isolated from pleural effusions and ascites fluids of cancer patients were tested for cytotoxicity against freshly isolated autologous tumor cells and K562 in a 4-h 51Cr-release assay, and in vitro effects of OK432 (a streptococcal preparation) and partially purified human leukocyte interferon (IFN) on their cytotoxicities were examined. Positive cytotoxicities against K562 were recorded for TAM samples from 2 of 23 pleural effusions and 3 of 10 ascites specimens. Tumor-associated macrophages were not cytotoxic to autologous tumor cells, while low but significant lysis was observed with tumor-associated lymphocytes (TAL) samples from 2 of 13 pleural effusions and 1 of 6 ascites specimens. An induction or augmentation of autologous tumor killing activity by OK432 was observed in 2 of 10 TAM and 8 of 11 TAL samples. In contrast, IFN failed to induce autologous tumor killing activity, although IFN-enhanced lysis of K562 was detected in 1 of 7 TAM and 2 of 9 TAL samples. These results indicated that autologous tumor killing and natural cytotoxic activities were defective in macrophages and lymphocytes at the site of the tumor growth, and both activities were strongly enhanced by OK432 rather than IFN.

Introduction

Tumor-associated lymphocytes (TAL) isolated from solid tumors [12], ascites fluids [19], and pleural effusions [15, 16] have been shown to express low or no natural killing activity. Similarly, low natural cytotoxic activity of tumor-associated macrophages (TAM) has been documented in ovarian cancer patients [10, 13]. It is, however, difficult to interpret the data on cytotoxic activity against tumor cell lines, since tumor cells have been shown to acquire susceptibility to natural killer (NK) cells through growth in vitro [2, 3]. For better evaluation of antitumor activity of cancer patients, studies on autologous combinations of effector and target cells have been performed. Fresh tumor cells are relatively resistant to lysis by autologous peripheral blood lymphocytes (PBL) and TAL [1, 14, 17, 24]. However, little is known about autologous tumor killing activity of TAM [6, 10, 23].

It has been reported that both PBL and TAL treated with interferon (IFN) are frequently cytotoxic to autologous fresh tumor cells in patients with ascitic tumors in vitro [1]. In contrast, IFN treatment of PBL has enhanced lysis of allogeneic, but not autologous, fresh tumor cells from solid neoplasms [7, 21]. We have reported that OK432, a heat- and penicillin-treated lyophilized powder of the Su strain of Streptococcus pyogenes A3, augments NK and autologous tumor killing activity of TAL from pleural effusions [16, 18, 19]. The present study was designed to investigate autologous tumor killing and natural cytotoxic activities of macrophages isolated from carcinomatous fluids and in vitro effects of OK432 and IFN on their cytotoxicities.

Materials and methods

Patients. Fluid specimens were obtained from 23 patients with carcinomatous pleural effusions and 10 patients with malignant ascites. Of these patients 16 had lung cancer, 8 breast cancer, 5 malignant melanoma, 2 rectal cancer, and 2 had uterine cancer. The patients had not received prior treatment with any anticancer agents at the time of the study. Peripheral blood specimens from 22 healthy donors were used as controls.

Preparation of effector cells. Effector cells were prepared as described in detail elsewhere [15, 16]. Mononuclear cells were isolated from heparinized peripheral blood by centrifugation on Ficoll-Hypaque gradients. The cells at the interface were washed and suspended in RPMI-1640 supplemented with 25 mM HEPES, 2 mM l-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS; Gibco, Glasgow, Scotland); this is referred to subsequently as complete medium. Specimens of pleural effusions and ascites fluids were centrifuged, then the cells were washed, suspended in complete medium, and layered on discontinuous gradients of 75% and 100% of Ficoll-Hypaque. After centrifugation at 400 g for 30 min, mononuclear cells were collected from the 100% interface, tumor cells and mesothelial cells from the 75% interface, and erythrocytes, polymorphonuclear cells, and aggregated tumor cells from the bottom. Mononuclear cells having less than 5% tumor cells as judged by morpho-
logical examination of Wright-Giemsa-stained smears were accepted for use.

After incubation of mononuclear cells for 1 h at 37 °C in plastic dishes precoated with FCS, nonadherent cells were collected. The preparation contained more than 96% lymphocytes as judged by Wright-Giemsa staining and morphology. After the dishes had been thoroughly washed, adherent cells were collected by incubating the dishes with Versene (1:5000, Gibco) for 15 min at room temperature and by gentle scraping with a rubber policeman. The recovered cells contained more than 96% monocytes/macrophages as assessed by nonspecific esterase staining and morphology. Every fraction was more than 97% viable according to the trypan blue dye exclusion test.

**Tumor cells from carcinomatous fluids.** Cell suspension enriched tumor cells obtained above were contaminated by mesothelial cells, monocytes/macrophages, and lymphocytes. The cell suspension was layered on discontinuous gradients of 25%, 15%, and 10% Percoll (Pharmacia Fine Chemicals, Uppsal, Sweden) in complete medium, then centrifuged at 25 g for 7 min at room temperature [17, 18]. Tumor cells and depleted lymphoid cells were collected from the bottom, suspended in complete medium, and incubated in plastic dishes for 30–60 min at 37 °C. After incubation nonadherent cells were collected, washed, and resuspended in complete medium. Usually, the nonadherent cells contained mainly tumor cells with less than 5% contaminating nonmalignant cells as judged by morphological examination of Wright-Giemsa smears, and were more than 95% viable according to the trypan blue dye exclusion test. The cells having less than 5% contamination with nonmalignant cells were accepted for use as tumor cells.

**Treatment with OK432 and IFN.** OK432 was supplied by Chugai Pharmaceutical Co., Tokyo, Japan. The unit of "KE" is used to express the strength of the preparation, 1 KE corresponding to 0.1 mg dried streptococci. Human leukocyte IFN, partially purified and frozen, was obtained from Immunoloski Zabod, Zagreb, Yugoslavia. The specific activity was $2 \times 10^{6}$ U/mg protein. Effector cells at a concentration of $1 \times 10^6$/ml in complete medium were incubated alone or with OK432 (0.5 KE/ml) or IFN (10$^6$ U/ml) for 20 h at 37 °C, as described previously [18, 25, 26]. After incubation the cells were washed and resuspended in complete medium. The viabilities of incubated cells were more than 95%, and no significant difference was observed in the recovery of viable cells incubated alone and with OK432 or IFN.

**Target cells.** Fresh tumor cells and K562 human myeloid leukemia cell line [8] were used as targets. Target cells were incubated overnight with 100 μCi Na$_2^{51}$CrO$_4$ (specific activity 100–350 μCi/μg Cr; Radiochemical Centre, Amersham, Buckinghamshire, England) at 37 °C, as previously described [17, 18]. After incubation the cells were washed four times, suspended in complete medium and further incubated for 3 h at 37 °C. The cells were then washed twice and resuspended at a concentration of $5 \times 10^6$/ml in complete medium. The specimens more than 90% viable as assessed by trypan blue dye exclusion test were accepted for use as target cells.

**Cytotoxicity assay.** A 4-h $^{51}$Cr-release assay was performed as described in detail elsewhere [17, 18], unless otherwise stated. Briefly, 100 μl labeled target cells ($5 \times 10^3$) and 100 μl effector cells in different numbers were added to wells of round-bottomed microtiter plates (Nunc, Roskilde, Denmark). After a 4-h incubation the supernatant was collected and the radioactivity was counted in an autometric gamma counter. The specific percentage lysis for each assay was calculated from the following formula for triplicate samples: Specific percentage lysis = [(test cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm)] × 100. The ranges of spontaneous release from K562 and fresh tumor cells were 2%–16% and 6%–36% of the total isotope count, respectively.

**Statistical analysis.** The results were evaluated for statistical significance by Student's t-test and Fisher's exact probability test. A specific percentage lysis greater than 8.0% was always statistically significant at $P<0.05$ and considered to be positive.

**Results**

**Lysis of K562 cells**

Macrophages isolated from carcinomatous fluids expressed low or no cytotoxicity against K562. Positive reactions were recorded for TAM in 2 of 23 pleural effusion samples and 3 of 10 ascites fluid specimens, with the mean specific percentage lysis of 4.3 ± 0.9% (mean ± SEM) and 6.6 ± 2.6% at an effector-to-target (E:T) ratio of 20:1, respectively (Fig. 1A). The levels of cytotoxicities of TAM were comparable to that (4.5 ± 0.6%) of normal peripheral blood monocytes (PBM), and the frequency of positive reactions was similar to that (9%) of normal PBM. On the other hand, TAL showed significant lysis of K562 in 8 of 23 pleural effusion and 3 of 10 ascites samples, while the mean values of cytotoxicities by TAL in both pleural effusion (8.6 ± 1.9%) and ascites (10.5 ± 4.3%) samples were lower than that (37.1 ± 3.1%) of normal PBL ($P<0.005$).

**Fig. 1A, B.** Cytotoxic activity against K562 (A) and fresh tumor cells (B) of unstimulated effector cells. Cytotoxic activity was measured at an effector-to-target ratio of 20:1 in a 4-h assay. Specific percentage lysis over 8.0% was estimated to be positive ($P<0.05$).