Intrathoracic administration of OK432 in cancer patients: Augmentation of autologous tumor killing activity of tumor-associated large granular lymphocytes

Atsushi Uchida¹, Michael Micksche¹, and Takashi Hoshino²

1 Institute for Applied and Experimental Oncology, University of Vienna, A-1090 Vienna, Austria
2 Department of Immunology, Fukui Medical School, Fukui, 910-11, Japan

Summary. Ten patients with carcinomatous pleural effusions were treated with single intrapleural (i.pl.) injections of the streptococcal preparation OK432 on day 0 and the effects of i.pl. OK432 on the lysis of fresh or cryopreserved autologous tumor cells isolated from the pleural effusions were observed on day 7. In eight patients tumor cells in the effusions had decreased or disappeared by day 7. The other two patients, however, had no clinical evidence of therapeutic benefit from i.pl. OK432. Effusion tumor cells were frequently resistant to lysis by autologous lymphocytes when tested in a 4-h^-Cr-release assay. Positive reactions were recorded for blood and effusion lymphocytes in two of ten untreated patients. Injection of OK432 i.pl. resulted in an induction or augmentation of cytotoxicity against autologous tumor cells and K562 in the effusions of seven of ten subjects by day 7. In contrast, autologous tumor killing activity of blood lymphocytes was not always modified by i.pl. OK432. Purification of large granular lymphocytes (LGL) by discontinuous Percoll gradient centrifugation enriched autologous tumor killing activity, with no reactivity in LGL-depleted, small T lymphocytes. Significant lysis of autologous tumor cells was observed with effusion LGL from seven of ten untreated patients. Seven days after i.pl. OK432 injection, effusion LGL expressed enhanced cytotoxicity against autologous effusion tumor cells, whereas T cells were still not cytotoxic to autologous tumor cells on day 7. The frequency of LGL among effusion lymphocytes was not altered by i.pl. OK432. Adherent effusion LGL were not involved in lysis of autologous effusion tumor cells in either untreated or OK432-treated patients. In vitro treatment of blood and effusion lymphocytes with OK432 induced an enhancement of autologous tumor-killing activity in patients who subsequently responded to i.pl. OK432 treatment. OK432 augmented in vitro autologous tumor killing activity of LGL, whereas T cells failed to lyse autologous tumor cells even after in vitro activation with OK432. These results indicate that i.pl. administration of OK432 to cancer patients will result in an augmentation of autologous tumor killing activity of LGL in the pleural effusions, and that this could be responsible for the antitumor activity of i.pl. OK432 therapy.

Introduction

In vitro cell-mediated cytotoxicity has been considered an expression of the host immune defense mechanisms. In the majority of studies on cytotoxicity of spontaneous and activated killer cells in cancer patients, cultured human tumor cell lines have been used as targets. It is difficult to interpret the data on cytotoxicity against cell line targets, however, since tumor cells acquire susceptibility to natural killer (NK) cells through growth in vitro [2, 4]. For the better evaluation of cytotoxicity of lymphocytes in cancer patients studies on autologous combinations of fresh effector and fresh target cells have been performed. Peripheral blood lymphocytes from approximately 25% of cancer patients have been demonstrated to be cytotoxic to autologous, freshly isolated tumor cells from solid neoplasms [9, 33-35, 37, 38] and from malignant effusions [1, 20, 28, 30]. We have recently demonstrated that cytotoxic potential for autologous tumor cells is present in the peripheral blood and pleural effusions of cancer patients, and that it is strongly associated with a minor proportion of large granular lymphocytes (LGL) and restricted to the cell population that can lyse NK-sensitive K562 cells [20, 28, 30]. Similar observations have been made with tumor biopsy cells from solid tumors, although reactivity is also present in T lymphocytes [35]. Since fresh human tumor cells are resistant to lysis by autologous unstimulated lymphocytes, several attempts have been made to induce or augment autologous tumor killing activity. The lysis of fresh human tumor cells has been observed with autologous blood lymphocytes activated in vitro by allosensitization [5, 18], by lectins [13], and by interleukin 2 (IL 2) [6, 7, 37].

OK432, a heat- and penicillin-treated lyophilized powder of the Su substrain of Streptococcus pyogenes A3, has been used as an immunomodulating agent and shown to have antitumor activity in cancer patients [22, 26]. Although the therapeutic usefulness of OK432 has been demonstrated, the mechanism responsible for its antitumor activity is not fully understood. Experimental animal studies have revealed that OK432 stimulates the cytostatic or cytotoxic activity of macrophages [14, 17], induces immune interferon (IFN) [12], and enhances NK cell activity [16]. In cancer patients administration of OK432 has been shown to result in an increase in lymphoproliferative response to mitogens and antigens [21, 22] and in NK cell activity of blood lymphocytes [26, 31]. Furthermore, in vitro treatment of blood lymphocytes and tumor-associated lymphocytes with OK432 has enhanced NK cell activity [3, 23, 31, 39] and autologous tumor killing activity [3, 29, 31] independently of IFN induction. Our previous studies have demonstrated that intrapleural (i.pl.) administration of OK432 to patients with carcinomatous pleural effusions induces an augmentation of effusion NK cell
activity and a reduction of tumor cells in the effusions [26, 27]. We have suggested on the basis of these findings that OK432-activated NK cells may interact in vivo with tumor cells in the pleural effusions of patients who have received i.pl. OK432 treatment. For a better evaluation of the biologic role of NK cells and the antitumor activity of OK432, it seems of importance to ascertain whether OK432 administration augments the autologous tumor killing activity of LGL in cancer patients. The present study was designed to investigate the effects of i.pl. administration of OK432 on cytotoxicity against autologous effusion tumor cells in patients with carcinomatous pleural effusions.

Materials and methods

OK432. OK432 was supplied by Chugai Pharmaceutical Co., Tokyo, Japan. The 'KE' unit is used to express the strength of the preparation, 1 KE corresponding to 0.1 mg dried streptococci [21, 22].

Patients and treatment. Ten patients with carcinomatous pleural effusions were entered into this study. Histologic diagnosis revealed that eight patients had adenocarcinoma and two had squamous cell carcinoma of the lung. The patients, ranging in age from 33 to 72 years, had not received any anticancer agents at the time of the study. OK432 was administered i.pl. at a dose of 10 KE in 10 ml physiological saline on day 0, and clinical and immunological parameters were evaluated on day 7. No systemic treatment was given during the entire treatment period. Pleural effusions (500–1,000 ml) and peripheral blood (20–50 ml) were obtained from the patients on day 0 and 7 just before i.pl. injection of OK432. Specimens of peripheral blood from 20 healthy normal individuals were used as roughly age- and sex-matched controls.

Blood and effusion effector cells. Effector cells were prepared as described in detail elsewhere [24, 25, 27–29]. Lymphocyte-rich mononuclear cells were isolated from heparinized peripheral blood by centrifugation on Ficoll-Hypaque gradients. The mononuclear cells in the interface were collected, washed, and suspended in RPMI-1640 medium supplemented with 2 mM glutamine, 25 mM Hepes, 100 units penicillin/ml, 100 μg streptomycin/ml, and 10% heat-inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland) (complete medium).

Specimens of pleural effusions were obtained from the patients by thoracentesis. Specimens were immediately centrifuged at 400 g for 5 min. The cells were washed, suspended at a concentration of 1 × 10^6/ml in complete medium, and layered on discontinuous gradients of 75% and 100% Ficoll-Hypaque. After centrifugation at 400 g for 30 min, lymphocyte-rich 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. The procedure was repeated if the separation was not successful as judged by morphologic examination. Mononuclear cells having less than 5% tumor cells, as judged by morphologic examination of Wright-Giemsa-stained smears, were accepted for use.

The mononuclear cells were then incubated for 1 h at 37°C in plastic dishes precoated with fetal calf serum. After incubation, nonadherent cells were removed, and the dish was washed with cold medium. Adherent cells were harvested from the dish after 15 min incubation with Versene (1/5,000; Gibco) and by vigorous washing with a pipette, then washed and suspended in complete medium. The adherent cells contained more than 95% monocyte/macrophages, as judged by morphologic examination and nonspecific esterase staining. The nonadherent cells were passed through Sephadex G10 columns at 37°C to remove further contaminating monocyte/macrophages and tumor cells. The G10-passed lymphocytes were incubated in nylon-wool columns for 1 h at 37°C and eluted with warm complete medium.

The nonadherent lymphocytes were then fractionated by centrifugation on discontinuous Percoll density gradients, as previously described [19, 23, 28, 30]. Briefly, complete medium and Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) were adjusted to 285 mosmolar H2O with distilled water and 10-fold concentrated phosphate-buffered saline, respectively. Seven different concentrations of Percoll in medium (40%–55%) were prepared by 2.5% increments. After layering of the gradients into 15-ml plastic tubes, 5 × 10^7 nonadherent lymphocytes were placed on the top of the gradients, and the tube was centrifuged at 550 g for 30 min. Cells from the seven layers were collected, washed, and suspended in complete medium. The cells from low-density fractions 2 and 3 usually contained more than 65% LGL, as judged by morphologic examination of Giemsa-stained cyt centrifuged smears. The cells from high-density fractions 6 and 7 usually contained less than 2% LGL. Every fraction was more than 96% viable according to the trypan blue dye exclusion test.

Effusion tumor cells. Cell suspension enriched for tumor cells obtained as described above were contaminated by mesothelial cells, monocyte/macrophages, and lymphoid cells. To eliminate these contaminating host nonmalignant cells, the cell suspension was layered on discontinuous gradients containing 4 ml each of 25%, 15%, and 10% Percoll in complete medium in 15-ml plastic tubes, then centrifugated at 25 g for 7 min, as previously described [28, 29]. Tumor cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. To remove residual contaminating mesothelial cells and monocyte/macrophages, the cell suspension was incubated for 30–60 min at 37°C in plastic dishes. After incubation, nonadherent cells were recovered, washed, and suspended in complete medium. The nonadherent cells usually consisted mainly of tumor cells, with less than 5% contaminating nonmalignant cells as judged by morphologic examination of Wright-Giemsa-stained smears, and were more than 93% viable according to the trypan blue dye exclusion test. Cells having less than 5% contamination with nonmalignant cells were accepted for use. Tumor cells were either used immediately or frozen according to a temperature-controlled freezing program and stored at −2–5 × 10^6/vial in 90% human AB serum plus 10% dimethyl sulfoxide in liquid nitrogen for further use. After rapid thawing, cells were gradually diluted to 10 ml with complete medium, centrifuged, and suspended in complete medium.

In vitro treatment with OK432 and IFN. Effector cells at a concentration of 1 × 10^6/ml in complete medium were preincubated alone or with 50 μg OK432/ml or 1,000 IU IFN/ml (human leukocyte IFN, specific activity 2 × 10^6 IU/mg protein, Immunološki Zavod, Zagreb, Yugoslavia) for 20 h at 37°C in a humidified 5% CO2 atmosphere, as described previously [23, 27, 29]. After incubation, the cells were harvested, washed, and suspended in complete medium.