Effect of Intraperitoneal Neutrophils Induced by OK432 on Malignant Ascites

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Abstract

Purpose. To evaluate the efficacy of a streptococcal preparation, OK432, on malignant ascites in mice.

Methods. PC-C203U (PC203) is a preparation of another strain of the streptococcal family, with the lowest antineoplastic action. To examine the survival curves of mice after the inoculation of BAMC-1 tumor cells, we gave intraperitoneal OK432, PC203, or saline as a control. Intraperitoneal neutrophils were counted by cytospin, and interleukin-1β (IL-1β), interleukin-6 (IL-6), and macrophage inflammatory protein (MIP)-1α were measured by enzyme-linked immunosorbent assay, and 8 h after the administration of OK432, PC203, and saline. Using electron microscopy, we examined the greater omental milky spots, where the in situ proliferation of neutrophils or macrophages takes place.

Results. The OK432 group had the best survival and the control group the worst. The ratio of intraperitoneal neutrophils to BAMC-1 was highest in the OK432 group and lowest in the control group. Quantitative IL-1β, IL-6 and MIP-1α levels were correlated closely with survival. Electron microscopic examination of the milky spots revealed massive proliferation of neutrophils in the OK432 group, but not in the PC203 or control groups.

Conclusion. OK432 effectively activated intraperitoneal neutrophils and a series of immunological chain reactions through an increase in IL-1β, IL-6, and MIP-1α levels. Milky spots could have important antitumor effects in terms of the spread of neutrophils.

Key words OK432 · Neutrophil · Immunopotentiator · Cytokine · Milky spots

Introduction

OK432 is considered to be one of the strongest immunopotentiators activating the immune system of the host, resulting in neoplasm degeneration. This strain has been used widely in clinical medicine, and its benefits proven by prospective randomized clinical studies in various medico-biological fields such as gastric cancer, lung cancer, and malignant effusion.1–7 The mechanism of the antitumor effect induced by OK432 has been described,8–12 but it is still not fully understood. OK432 is a heat- and penicillin-treated lyophilized powder of the SU strain of Streptococcus, which has lost the production activity of streptolysin S (SLS). PC-C203U (PC203) is a preparation of another strain of the streptococcal family, reported by Okamoto et al. to have the least antineoplastic action.13

BAMC-1 is an ascetic neoplasm grown in female BALB/c mice, which is particularly sensitive to OK432.14,15 The purpose of this study was to clarify the mechanism of OK432, in comparison with that of PC203, using this mouse model, focusing on cytokine or chemokine production and the accumulation of intraperitoneal neutrophils. We paid special attention to the milky spots of the greater omentum, which are important for intraperitoneal immunity,16–20 and where the migration of neutrophils occurs.

Materials and Methods

Mice and Tumor Cell Line

Female, pathogen-free BALB/c mice were purchased from Charles River Japan and Clea Japan (Tokyo), and maintained under specific pathogen-free conditions in the experimental medical center of Jichi Medical School. BAMC-1, methylcholanthrene-induced fibrosarcoma in BALB/c mice, were established in the Re-
search Laboratories of Chugai Pharmaceutical. The tumor cell lines were kindly provided by Chugai Pharmaceutical (Tokyo, Japan) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

**Therapeutic Effect of Immunopotentiators Against BAMC-1 Tumors**

BALB/c mice, 8 weeks of age, were inoculated intraperitoneally with BAMC-1, $1 \times 10^5$ cells/mouse. OK432 was suspended in saline at a concentration of 0.2 Klinische Einheit (KE)/0.5 ml; 1 Klinische Einheit of OK432 being equivalent to 0.1 mg of dried cells. An intraperitoneal injection of OK432, 0.2 KE/0.5 ml saline, was given on days 0 (4 h after tumor inoculation), 2, 4, 6, 8, and 10 after the inoculation of tumor cells. A corresponding amount of PC203 was given in an identical manner, and 0.5 ml saline was used as the negative control ($n = 10$ each). Animals were observed until 60 days after the inoculation.

**Analysis of the Grade of Neutrophil Accumulation**

Nine mice were inoculated with BAMC-1 in the same way as described above. Animals were divided into three groups of three mice each and given a bolus intraperitoneal injection of OK432, PC203, or saline intraperitoneally, at the doses described above, 48 h after the inoculation. The animals were killed 4 h later, and 3 ml of saline was injected into the peritoneal cavity to wash out cells in the peritoneal fluid, 50 µg of which were smeared onto a slide glass using cytopsin (700 rpm for 5 min). Slides were stained with the Wright-Giemsa method. The neutrophils and tumor cells were obtained by observing slides under 1000× magnification, and counting was repeated ten times for each slide.

**Assessment of Cytokine Levels by Specific ELISA**

Eighteen mice were inoculated with BAMC-1 in the same way. These animals were divided into three groups of six mice each, given a bolus injection of OK432, PC203, or saline intraperitoneally, at the same doses described above, 48 h after the inoculation. Animals were killed 4 h later and 3 ml of saline was injected into the peritoneal cavity. Centrifugation of 1 ml of this saline was done at 15000 rpm for 1 min to assay for interleukin (IL)-$1\alpha$, IL-6, and macrophage inflammatory protein (MIP)-$1\alpha$. The concentrations of IL-1$\beta$, IL-6, and MIP-1$\alpha$ in the suspension were assayed using an enzyme-linked immunosorbent assay (ELISA) kit. Mouse IL-1$\beta$ and IL-6 ELISA systems were purchased from Amersham Pharmacia Biotech (Tokyo, Japan) and the mouse MIP-1$\alpha$ immunoassay system was purchased from R&D Systems (distributed by Funakoshi, Tokyo, Japan). Standard curves of IL-1$\beta$, IL-6, and MIP-1$\alpha$ were obtained at concentrations of 15.6–1000, 50–1250, and 4.7–300 pg/ml, and the minimum detectable doses were 2.0, 15, and 1.5 pg/ml, respectively. Standards and samples were assayed in duplicate. Assay plates were read at 450 nm with a microplate reader (SPECTRAMax 340; Molecular Devices, Sunnyvale, CA, USA).

**Morphological Analysis of Activated Cells on Milky Spots**

Six mice were inoculated with BAMC-1 in the same way. These animals were divided into three groups of two mice each, and given a bolus intraperitoneal injection of OK432, PC203, or saline at the same doses described above, 48 h after the inoculation. The animals were killed 4 h later and the greater omentums with their milkey spots were removed, and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 5% sucrose, for 60 min at 4°C. After fixation, the samples were washed several times in the same buffer. For morphological investigation of neutrophils in the milkey spots, enzyme cytochemical peroxidase staining was done because neutrophils contain abundant myeloperoxidase in their intracellular azurophilic granules. The specimens were incubated in a reaction medium to detect peroxidase activity for 30 min at 22°C. This reaction medium consisted of 0.5 mg/ml diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6, with 0.01% H$_2$O$_2$ (Graham and Karnovsky, 1966). After the cytochemical reaction, the samples were postfixed with 1% osmium tetroxide, dehydrated, then embedded in Epon. Ultrathin sections were made, stained with uranyl acetate and lead citrate, and examined under a Hitachi H-7000 electron microscope (Hitachi, Hitachinaka, Japan) operated at 75 kV.

**Statistical Analysis**

Data are presented as mean ± standard deviation (SD). The differences in survival rates were analyzed with the Kaplan-Meier method and evaluated by the log rank test. The results for tumor cells, neutrophils, and quantitative IL-1$\beta$, IL-6, and MIP-1$\alpha$ were evaluated for significance by the Kruskal-Wallis test and Fisher’s protected least significant difference. Calculations were done with software for Macintosh (Statview, Abacus Concepts, Berkeley, CA, USA). $P$ values of less than 0.05 were considered significant.