Local injections of OK432 can help the infiltration of adoptively transferred CD8+ T cells into the tumor sites and synergistically induce the local production of Th1-type cytokines and CXC3 chemokines

Abstract The effect of local injections with streptococcal preparation OK432 on the antitumor effect induced by adoptive immunotherapy (AIT) was investigated. Draining lymph node cells on day 14 after B7-P815 inoculation were used for AIT after in vitro stimulation. AIT on days 7 and 10 showed no effect on the growth of s.c. established P815 mastocytoma, but local injections with OK432 into the tumor sites on days 3, 6 and 9 resulted in a moderate antitumor effect. On the other hand, the combination therapy significantly suppressed tumor growth, and the tumor-bearing mice survived longer than those receiving only one of the treatment modalities. The significant infiltration of CD4+ or CD8+ T cells and multiple necrosis in the tumor sites were observed only when the tumor-bearing mice were treated with the combination therapy. In addition, a transfer experiment using labeled effector cells revealed these infiltrated CD8+ T cells and CD4+ T cells to be derived from the donor and the host respectively. More importantly, the combination therapy clearly led to higher expression of the mRNA for Th1-type cytokines and CXC3 chemokines in the tumor sites than resulted from each of the treatment modalities alone. Collectively, these results indicate that local injections with OK432 can help the infiltration of adoptively transferred CD8+ T cells into the tumor sites and synergistically induce the local production of the Th1-type cytokines and CXC3 chemokines.

Key words Adoptive immunotherapy · OK432 · Tumor-infiltrating lymphocytes · Th1-type cytokines · CXC3 chemokines

Introduction
A large number of animal models have been developed to evaluate the requirements for eradicating established tumors by adoptive immunotherapy (AIT) with tumorspecific T cells [8]. In many cases, the infusion of tumor specific T cells with or without interleukin(IL)-2 can not inhibit the development of tumors, especially in s.c. established tumors. One plausible explanation is that very small amounts of the transferred cells could migrate to the tumor sites, and the migration of tumor-specific T cells to the tumor sites thus appears to be a crucial step to elicit a sufficient antitumor effect in vivo. Local inflammation could help the infiltration of antitumor T cells into the tumor sites, as suggested by several reports [10, 20, 23, 30].

A streptococcal preparation, OK432, has been shown to be a biological response modifier, and has been applied clinically against various types of cancers [12, 28, 36, 41]. It has been proposed that its immunomodulating mechanism relies on the activation of natural killer cells, macrophages, polynucleonuclear cells and CD4+ T cells [13, 25, 34, 40]. In addition, OK432 has been reported to induce multiple cytokines including IL-1, IL-2, IL-6, tumor necrosis factor (TNF) α, interferon (IFN) γ and IL-12 [6]. We have also demonstrated that peritumoral injections of OK432 can significantly enhance the immunotherapeutic potential of the tumor-draining lymph node (dLN) cells by augmenting their Th1-type responses [22], and that the effect of dendritic cells, used as an antitumor vaccination, can be significantly augmented by locally utilizing Th1-type cytokines from OK432-reactive CD4+ T cells [35]. Moreover, OK432 has been reported to help the infiltration of T cells into the injection sites and improve the survival rate of cancer patients when concomitantly used with chemotherapy [11, 39].
A recent study indicated that chemokines take part in the infiltration of lymphocytes and the suppression of tumor growth. Chemokine gene transfer can elicit the accumulation of activated T cells and tumor rejection by the induction of Th1-type responses [4]. In particular, CXC3 chemokines such as interferon-inducible protein 10 (IP-10) and the “monokine induced by IFNγ” (Mig), all of which are induced by IFNγ, can lead to an antitumor effect by enhancing the recruitment of effector lymphocytes into the tumor sites and causing tumor necrosis associated with vascular damage [26, 27].

In this study, we investigated the effect of local injections with OK432 on the antitumor effect induced by AIT against a syngeneic tumor established s.c. and such local injections were found to elicit the antitumor effect efficiently. Analysis of the infiltrating cells suggested that local injections of OK432 can help the infiltration of adoptively transferred CD8+ T cells. More importantly, the OK432 treatment was found to help the local production of the Th1-type cytokines and CXC3 chemokines in the tumor sites. The implications of these findings are also discussed.

Materials and methods

Mice

Female DBA/2 mice, 6–7 weeks old, were purchased from Japan SLC (Shizuoka, Japan). All mice were kept in specific-pathogen-free conditions and were used for the experiments at 8 weeks of age.

Cell preparation

To prepare lymph node cells, the lymph nodes were removed aseptically and teased into suspension in a complete medium consisting of RPMI-1640 (Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, Utah 50 μM), 2-mercaptoethanol, 20 mM 4-HEPES, 30 μg/ml gentamicin (Gentamicin; Schering Corporation, Kenilworth, NJ), and 0.2% sodium bicarbonate was used as the complete medium.

Tumors

P815 is a mastocytoma of DBA/2 origin and B7-P815 is a subline transfected with human CD80 (kindly provided from Dr. Lewis L. Lanier, Department of Immunology, DNAX Research Institute, Palo Alto, Calif.). P388D1 is a macrophage-like cell line that was isolated from a methylcholanthrene-induced lymphoid neoplasm of a DBA/2 mouse. All tumor cell lines were maintained in vitro in complete medium.

OK432

OK432, a penicillin- and H2O2-killed lyophilized preparation of the Su strain of Streptococcus pyogenes, was kindly provided from the Chugai Pharmaceutical Co., Tokyo, Japan. OK432 was suspended in phosphate-buffered saline (PBS) at a dose of 0.1 mg/ml.

Preparation of antitumor effector cells for AIT

DBA/2 mice were inoculated s.c. with B7-P815 tumor cells (1 × 10⁶) bilaterally into the abdomen. Two weeks later, the dLN cells were harvested and cultured in the presence of inactivated P815 tumor cells for 3 days, as previously reported [9]. To inactivate the tumor cells, they were cultured with 100 μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo) for 90 min.

Selective depletion of CD8+ T cells

To deplete CD8+ T cells, the cultured cells were incubated with the supernatant of a hybridoma producing anti-CD8 monoclonal antibody (mAb) (116-13.1: mouse IgG2a) for 40 min at 4 °C. After three washes with the complete medium, the cells were placed in contact with Dynabeads coated with goat anti-(mouse IgG) (Dynal, Oslo, Norway) by centrifuging at 200 g for 10 min at 4 °C. After magnetic separation, non-attached cells were collected and used as CD8+ T-cell-depleted effector cells. Selective depletion was confirmed by a flow-cytometric analysis.

Assay of cytolytic activity

The effector cells were incubated with 1 × 10⁶ 51Cr-labeled target cells in 200 μl complete medium in a round-bottomed 96-well microtiter plate. After 4 h of incubation, 100-μl samples of the supernatants were harvested. The radioactivity of the supernatants was measured by a gamma counter and the percentage specific 51Cr release was calculated according to the following formula:

\[
\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100
\]

The spontaneous release was determined from a sample of the target cells incubated without effector cells, while the maximal release was determined from the sample of the target cells incubated with 10% Triton-X (Wako, Osaka, Japan). All samples were assayed in triplicate and the values were shown as the means ± SD.

AIT model

DBA/2 mice, which had been inoculated s.c. with P815 cells (2 × 10⁵) laterally into the abdomen, were injected in the tumor sites with 0.01 mg OK432 in a volume of 100 μl on days 3, 6 and 9 after tumor inoculation. As a control, some mice were locally injected with PBS. On days 7 and 10 after tumor inoculation, some mice were injected intravenously (i.v.) with the antitumor effector cells (1 × 10⁶). Thereafter, the tumor growth and survival of the tumor-bearing mice were recorded every 3 days. The tumor size (mm²) was calculated as the product of two perpendicular diameters.

Preparation of TIL (tumor-infiltrating lymphocytes)

DBA/2 mice, which had been s.c. inoculated with P815 cells (5 × 10⁵) laterally into the abdomen, were injected in the tumor sites with 0.01 mg OK432 on days 2, 4, and 6. On day 7, the mice were injected i.v. with the effector cells (1 × 10⁶). On day 10, the tumors were harvested. To prepare tumor-infiltrating lymphocytes (TIL), the tumor cell samples were resuspended in 5 ml 45% Percoll (Sigma Chemical Co., St. Louis, Mo.) and then layered on 5 ml 67.5% Percoll. The gradient was centrifuged at 2500 rpm for 25 min at 20 °C. The lymphocytes at the interface were harvested and washed with complete medium.

Flow-cytometric analysis

For flow cytometry, a cell suspension was incubated with the following mAb and then analyzed by FACScan (Becton Dickinson, Mountain View, Calif.). Phycoerythrin-conjugated anti-(mouse CD4) mAb was purchased from Gibco BRL (Gaithersburg, Md.) and allophycocyanin-conjugated anti-(mouse CD8) mAb was purchased from PharMingen (San Diego, Calif.).