Application of an Interleukin 2 Slow Delivery System to the Immunotherapy of Established Murine Colon 26 Adenocarcinoma Liver Metastases

Toshiyoshi Fujiwara, Kenichi Sakagami, Junji Matsuoka, Shigehiro Shiozaki, Susumu Uchida, Keiji Fujioka, Yoshihiro Takada, Tadashi Onoda, and Kunzo Orita

ABSTRACT

We evaluated the antitumor effect of an interleukin 2 (IL-2) slow delivery system, the IL-2 minipellet, using a murine hepatic metastasis model. The IL-2 minipellet consists of atelocollagen derived from natural bovine skin together with lymphokine-activated killer cells and 1 x 10^7 units of recombinant IL-2. Administration of the IL-2 minipellet was performed via the s.c. position. Administration of the IL-2 minipellet was evaluated for its efficacy against hepatic metastases from colon 26 adenocarcinoma in the BALB/c mice. Both the administration of the IL-2 minipellet alone and its combination with the injection of 5 x 10^7 lymphokine-activated killer cells resulted in significant reductions of the number of metastatic nodules. Moreover, increased survival of mice bearing colon 26 adenocarcinoma was noted in these two treatment groups. To investigate the mechanism of the IL-2 minipellet activity, we tested the lytic potential of splenocytes obtained after administration of the IL-2 minipellet in a ^51^Cr release assay. Cytotoxicity against YAC-1 cells and colon 26 cells was significantly augmented on Day 2 after minipellet administration. These results demonstrated that local administration of the IL-2 minipellet into the hepatic circulation was extremely effective against metastatic liver cancer.

INTRODUCTION

IL-2, a glycoprotein released by activated T-lymphocytes (1), is a member of a class of essential immunoregulatory molecules and also has important antitumor functions, for example, the augmentation of NK activity (2), the expansion of CTL (3), and the production of γ-interferon (4). In 1982, Grimm et al. (5) first reported that peripheral blood lymphocytes could be activated in the presence of IL-2, without any additional antigen or mitogen, into killer cells capable of lysing a wide range of fresh, NK-resistant tumor cell targets. IL-2 based treatment using these killer cells, termed LAK cells, has been shown to have significant effects on some kinds of cancers in both experimental animal models and humans (5).

Recently, large quantities of recombinant IL-2 have become available through the cloning and expression of the complementary DNA for this protein in Escherichia coli bacteria (6). Since it is technically difficult to obtain a large number of lymphocytes from cancer patients for the generation of LAK cells, treatment with high-dose IL-2 alone has been used in several clinical trials (7). IL-2 in very high doses could mediate tumor regression in mice by the in vivo generation of LAK cells and CTL (8). However, the severe toxicity of systemic administration of IL-2, mainly due to the vascular leak syndrome, has been a common problem with the high-dose regimen (9). In order to circumvent some of these problems, we have developed a new biodegradable IL-2 slow delivery system, the IL-2 minipellet. A previous study showed that the local administration of the IL-2 minipellet to target sites could produce significant antitumor activity in murine solid tumors (10).

In the present study, we found that the local administration of the IL-2 minipellet into the spleen, which then gave IL-2 access to the hepatic circulation, was effective in reducing the number of established metastatic nodules of colon 26 murine adenocarcinoma and in prolonging the mean survival time of tumor-bearing mice. In addition, to help to determine the precise mechanism of the antitumor effect detected, we investigated the lytic activity of splenocytes against several different target cells after administration of the IL-2 minipellet.

MATERIALS AND METHODS

Mice. The 6-wk-old male inbred BALB/c mice used in all experiments were purchased from the Shizuoka Experimental Animal Center (Hamamatsu, Shizuoka, Japan). Mice were maintained under specific-pathogen-free conditions in our laboratory.

Recombinant IL-2. Recombinant human IL-2 was kindly provided by Ajinomoto Co., Ltd. (Tokyo, Japan). It had a specific activity of 4.82 x 10^8 units/mg of protein.

IL-2 Minipellet. The IL-2 minipellet used in all experiments was manufactured and supplied by Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). Atelocollagen, which was supplied by Koken Co., Ltd. (Tokyo, Japan), has little immuno-genicity following protease treatment and was used as the carrier material for IL-2 (11). An aqueous solution (10 ml) containing 4.8 x 10^6 units of recombinant IL-2 and 3.8 g of atelocollagen was homogenously mixed to obtain a uniform gel mixture. The gel mixture was subjected to molding and then drying to produce a cylindrical pellet with a diameter of 1 mm. The pellet was cut so as to obtain minipellets with a length of 10 mm (Fig. 1). Each IL-2 minipellet contained 1 x 10^7 units of IL-2.

Collection of Serum Samples and Assay of IL-2 Activity. A left subcostal incision was made in each mouse under ether anesthesia, and the spleen was delivered into the s.c. position. After 1 wk, the IL-2 minipellet was administered into the spleen, and serial blood samples were collected by direct intracardiac puncture. The samples were centrifuged at 3000 rpm for 10 min, and the serum was collected and stored at −25°C until the IL-2 assays were performed. Serum IL-2 activity was then measured by assaying [H]thymidine incorporation by an IL-2-dependent cell line (CTLL-2), as reported previously (12).

Splenocytes. Splenocytes were removed aseptically and gently crushed in complete medium with the flat end of a sterile syringe. Complete medium consists of RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 0.03% glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 5 x 10^-5 M 2-mercaptoethanol. The cells were passed through nylon mesh and then placed in buffered ammonium chloride solution to produce osmotic lysis of erythrocytes. The splenocytes were then centrifuged and washed 3 times (8).

Preparation of LAK Cells. Fresh splenocytes were incubated in recombinant IL-2 (1 x 10^7 units/ml) in complete medium for 72 h at 37°C in a moist atmosphere with 5% CO₂. The cells were then washed at a concentration of 2.5 x 10^6 cells/ml into culture flasks (Flacon No. 3024), incubated, and then washed 3 times (8).
and (e) the IL-2 minipellet and 5 x 10^7 LAK cells. The pellet was externalized to the s.c. position. On Days 7 and 10 after tumor inoculation, the mice were divided into 5 groups which were treated with the following test agents: (a) minipellet without IL-2 (placebo); (b) 1 x 10^6 units of aqueous IL-2; (c) 5 x 10^7 LAK cells; (d) the IL-2 minipellet; and (e) the IL-2 minipellet and 5 x 10^7 LAK cells. The pellet was inserted into the s.c.-positioned spleen with a conventional injection technique. Aqueous IL-2 and LAK cells were also administered into the spleen. At the conclusion of the experiments (Day 21), mice received trypsinization before use.

Experimental Therapy Model. Six-wk-old BALB/c mice were anesthetized, and an upper median incision was made. A 30-gauge needle was used to inject 1 x 10^6 colon 26 cells in 0.2 ml of medium into the portal vein via the superior mesenteric vein. Gentle pressure was applied for a period of 1 min to prevent hemorrhage and tumor cell extravasation. A left subcostal incision was also made, and the spleen was externalized to the s.c. position. On Days 7 and 10 after tumor inoculation, the number of metastatic nodules on the liver surface was counted against the black background of normal liver parenchyma. Following an injection of India ink solution via the lateral tail vein and were then killed. The livers were extracted and placed into Fekete's solution for 2 min to prevent hemorrhage and tumor cell extravasation. Following this, the number of metastatic nodules on the liver surface was counted (14). In survival experiments, mice were followed until death and then autopsied.

Chromium Release Assay. The cytotoxic activity of splenocytes was tested in vitro in a standard 4-h 51Cr release assay. After the inoculation of mice with colon 26 adenocarcinoma cells, splenocytes to use as effector cells were obtained daily beginning on Day 8 from mice treated with the following agents: (a) placebo; (b) the IL-2 minipellet on Day 7; and (c) the IL-2 minipellet on Days 7 and 10. NK-sensitive YAC-1 cells, NK-resistant EL-4 cells, and colon 26 cells were used as the target cells. The YAC-1 and EL-4 target cells were labeled with 200 Ci of Na_2^{51}CrO_4 for 12 h after the cells had become attached. The colon 26 target cells (1 x 10^4 cells/well to various numbers of effector cells in triplicate at 37°C in 5% CO_2 for 4 h. The colon 26 target cells (1 x 10^4 cells/well) were cultured as monolayers in a 96-well flat-bottomed plate and labeled with Na_2^{51}CrO_4 for 12 h after the cells had become attached. Then the cells were washed 3 times, and the effector cells were added at various effector/target ratios and incubated for 4 h. Supernatants from the cultures were harvested and counted in a gamma counter.

Target cells incubated in medium alone or with 1 M NaOH were used to determine the spontaneous and maximal release of chromium, respectively. The percentage of lysis was calculated by the following equation:

% of lysis = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}}

Statistical Analysis. The significance of difference in the number of liver metastases between groups and in the survival experiments were determined by the Wilcoxon rank sum test. Two-tailed P values are presented for all experiments.

RESULTS

Serum Concentration of IL-2 after Administration of the IL-2 Minipellet. Serial determination of serum IL-2 activity revealed that the serum IL-2 concentration began to increase gradually after administration of the IL-2 minipellet, and a peak serum level of about 100 units/ml was seen after 2 h. Serum IL-2 activity subsequently declined very gradually and remained detectable for 72 h (Fig. 2).

Antitumor Effect of the IL-2 Minipellet on Colon 26 Adenocarcinoma Liver Metastases. Multiple colon 26 liver metastases were induced in BALB/c mice as described in “Materials and Methods.” The IL-2 minipellet was administered into the s.c. spleen on Days 7 and 10, on the basis of the observation that serum IL-2 activity was maintained for 72 h after a single IL-2 minipellet was administered in the pharmacokinetic study described above. A characteristic experiment is shown in Fig. 3. Neither 1 x 10^6 units of aqueous IL-2 alone nor 5 x 10^7 LAK cells alone reduced the number of metastases when compared with placebo-treated control mice (the mean number of metastatic nodules was 85.3 ± 8.9 and 85.5 ± 8.4 versus 86.9 ± 17.4, respectively; P = not significant). In the group treated with combinations of the IL-2 minipellet and LAK cells, however, a significant decrease in the mean number of metastases was observed which exceeded 80% (the IL-2 minipellet plus LAK cells, 15.2 ± 9.2; P < 0.01). In addition, treatment with the IL-2 minipellet alone was also capable of reducing the number of metastases significantly as well as when combined with LAK cells (the IL-2 minipellet alone, 16.5 ± 16.7; P < 0.01), and there was no significant difference between the two groups.

![Fig. 1. The IL-2 minipellet is about 1 mm in diameter and 10 mm in length. It contains 1 x 10^6 units of recombinant IL-2.](image_url)

![Fig. 2. Serum IL-2 activity in mice after the intrasplenic administration of the IL-2 minipellet.](image_url)
groups receiving minipellets. Representative livers from each group are shown in Fig. 4.

Survival Benefit of the IL-2 Minipellet in Mice Bearing Colon 26 Adenocarcinoma. Mice bearing colon 26 adenocarcinoma were treated according to the same protocol as described in the experiment evaluating hepatic metastases. As shown in Fig. 5, in mice treated with the IL-2 minipellet alone, a significant extension of survival was achieved compared with placebo-treated controls (the median survival time was 38.8 ± 3.3 days versus 30.8 ± 2.4 days; \( P < 0.05 \)), corresponding to the effect of the treatment on hepatic metastases. In contrast, \( 1 \times 10^6 \) units of aqueous IL-2 had no effect on the duration of survival (data not shown).

In Vitro Cytotoxic Activity of Splenocytes Obtained from Tumor-bearing Mice Treated with the IL-2 Minipellet. The IL-2 minipellet was administered into the s.c. spleen on Days 7 and 10 after the intraportal inoculation of colon 26 adenocarcinoma cells, and splenocytes were obtained and tested daily for lytic activity. The results of representative assays are shown in Figs. 6 to 8. NK activity began to rise on Day 1 after the administration of the IL-2 minipellet. The highest activity was achieved on Day 2, and this was followed by a gradual decrease.

However, in response to the administration of an additional IL-2 minipellet on Day 3, a rapid increase of NK activity was seen on Day 5 again. Although LAK activity was slightly augmented on Day 3, the increase was far less than for NK activity. The lytic potential against colon 26 cells also began to increase on Day 1 and reached a peak on Day 2. However, in contrast with NK activity, the administration of the IL-2 minipellet on Day 3 did not induce further cytotoxicity against colon 26 cells.
activity of splenocytes. "Cr-labeled EL-4 cells served as the LAK target.

**DISCUSSION**

The treatment of liver metastases is extremely important for the prognosis of patients with advanced colorectal cancer, who have undergone the surgical resection of the primary tumor. For a localized metastatic nodule restricted to a single lobe, secondary surgical resection can be the most effective treatment and offers the possibility of a cure. However, in the case of multiple metastases, it is impossible to remove all of the lesions surgically. Moreover, although systemic administration and hepatic arterial infusion of anticancer agents have been used to treat patients with unresectable liver metastases, the results are still less than ideal (IS).

Recent developments in bioengineering have expanded the opportunities for using many new substances as drug carriers and have established a new concept termed the DDS. This means the use of drug carriers to induce new effects of conventional drugs by changing their pharmacokinetics. For instance, the therapeutic potential of liposomes or microspheres containing anticancer drugs or biological effector molecules has been investigated in experimental models (18, 19). We utilized this technology for IL-2 therapy by applying a minipellet with biodegradable and biocompatible atelocollagen derived from highly purified bovine dermal collagen as the carrier material. The development of this new IL-2 slow delivery system, the IL-2 minipellet, has been previously reported (20).

Since the IL-2 minipellet is prepared without heating or organic solvent, IL-2 is not inactivated during the formulation procedure. IL-2 (1 x 10⁶ units) is incorporated uniformly throughout the atelocollagen matrix. The release of embedded IL-2 is made by the combination of matrix degradation and drug diffusion. The IL-2 serum concentration profile demonstrated that IL-2 could be released slowly without burst effect.

We have previously shown that the local administration of the IL-2 minipellet alone in a solid murine tumor model of methylchlanthrene-induced fibrosarcoma was effective in both inhibiting tumor growth and prolonging survival, even though there was no transfer of LAK cells (10). In another experiment, however, we also revealed that the s.c. injection of the IL-2 minipellet alone in the back of C57BL/6 mice was insufficient to reduce the number of spontaneous pulmonary metastases of Lewis lung carcinoma from the hind footpad, and the concomitant i.v. transfer of LAK cells was necessary (21). Based on these initial studies, we considered that the IL-2 minipellet should be administered locally into the target site and, this time, investigated the effects of modified local administration of the IL-2 minipellet to the hepatic circulation via implantation into the spleen in mice with hepatic metastases of colon 26 adenocarcinoma.

In our experiments, first, the pharmacokinetics of IL-2 after the intrasplenic administration of the IL-2 minipellet was determined. The half-life of IL-2 following i.v. injection is approximately 3 to 4 min in mice (16). Our current study showed that the IL-2 minipellet decreased the peak serum IL-2 level and prolonged the duration of detectable serum IL-2 activity. These observations suggest the potential value of this DDS, since the toxicity of IL-2 is thought to be related to the maximum serum concentration, and since the duration of exposure...
of T-cells to IL-2 appears to be very important in inducing a useful antitumor effect (22). It appears that the IL-2 minipellet should enhance the efficacy and minimize the toxicity of IL-2 by changing its pharmacokinetic profile.

Administration of the IL-2 minipellet and 5 \times 10^7 LAK cells into the spleen resulted in a marked reduction in the mean number of hepatic metastases of colon 26 adenocarcinoma by more than 80% relative to control placebo-treated mice. The number of metastases was also reduced significantly by the IL-2 minipellet alone. These reductions were observed despite the relative ineffectiveness of therapy with aqueous IL-2 or LAK cells alone. Thus, our results demonstrated that, on the therapy of hepatic metastases of colon 26 adenocarcinoma, the IL-2 minipellet alone was sufficiently effective when administered into the hepatic circulation directly, and that the adoptive transfer of exogenous activated cells was unnecessary. The therapeutic benefit was also associated with a survival benefit. The increase in the overall survival of tumor-bearing mice was significant but not so long, despite the drastic reduction of metastatic nodules. One of the possible reasons for this discrepancy could be the number of doses of the IL-2 minipellet that were given. Administration of more than 2 doses has prolonged the survival further.

In order to further investigate the mechanism of these effects, we examined lytic activity of splenocytes against several types of tumor target cells after the intrasplenic administration of the IL-2 minipellet. Cytotoxicity against YAC-1 cells (i.e., NK activity) and colon 26 cells was enhanced on Day 2 after administration of the IL-2 minipellet. Further administration on Day 3 could augment NK activity again; however, lytic activity against colon 26 cells did not respond to the additional IL-2 minipellet. Although exogenous IL-2 given in vivo has been reported to induce LAK cells from endogenous lymphoid cells and to lead to their proliferation (8), we found that the LAK activity of splenocytes could not be augmented sufficiently. Weber et al. (23) have reported that in some models IL-2-mediated antitumor activity could be attributed to Lyt-2+ cytotoxic T-cells. In immunohistochemical studies, we identified an accumulation of Thy-1.2+ cells and Lyt-2+ cells on the borders of metastatic nodules (data not shown). Thus, in our experimental model, the effector cells might be cytotoxic T-cells, which were primed by the tumor-bearing condition and activated by the IL-2 minipellet.

Our findings described in the present paper strongly suggest that IL-2 should be administered locally into the target site and that the IL-2 minipellet is an appropriate DDS for the local administration of IL-2. The results of clinical trials of the systemic administration of IL-2 alone or in combination with LAK cells in patients with advanced cancer have been rather disappointing because of various serious adverse effects. We propose that the injection of the IL-2 minipellet locally into the hepatic artery as an embolic material in patients with metastatic liver cancer would be a useful therapeutic method. A clinical trial of this new approach is now in progress.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. T. Yasuda for his helpful advice and N. Ohmura and Y. Tarumi of Sumitomo Pharmaceutical Co. for producing the IL-2 minipellet. We also thank J. Hamuro and T. Shimamura of Ajinomoto Co. for providing recombinant IL-2, T. Miyata of Koken Co. for his generous technical advice, and K. Nasu for her technical assistance.

REFERENCES

Application of an Interleukin 2 Slow Delivery System to the Immunotherapy of Established Murine Colon 26 Adenocarcinoma Liver Metastases

Toshiyoshi Fujiwara, Kenichi Sakagami, Junji Matsuoka, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/21/7003

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.