

Controlled Local Delivery of Interleukin-2 by Biodegradable Polymers Protects Animals from Experimental Brain Tumors and Liver Tumors

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Purpose. The purpose of our study was to develop an injectable polymeric system for the long-term localized delivery of bioactive interleukin-2 for antitumor immunotherapy.

Methods. IL-2 was encapsulated into gelatin and chondroitin-6-sulfate using an aqueous-based complex coacervation. CTLL-2 cells were used to measure the bioactivity of released IL-2 and radiolabeled IL-2 was used for release studies in the rat brain and mouse liver. Antitumor efficacy studies were carried out in primary (9L gliosarcoma) and metastatic (B16-F10 melanoma) brain tumor models in rats and mice, respectively, as well as a murine liver tumor model (CT26 carcinoma). Survivors of the metastatic brain tumor challenge were rechallenged with tumor in the opposite lobe of the brain to confirm that antitumor immunologic memory had developed.

Results. Bioactive IL-2 was released for over 2 weeks *in vitro* and *in vivo* IL-2 release showed significant IL-2 levels for up to 21 days. Polymeric IL-2 microspheres injected intratumorally were statistically more effective in protecting animals challenged with fatal tumor doses in the brain and the liver than placebo or autologous tumor cells genetically engineered to secrete IL-2. Immunologic memory was induced following IL-2 microsphere therapy in the B16-F10 brain tumor model that was capable of protecting 42% of animals from a subsequent intracranial tumor challenge, suggesting that tumor destruction was mediated by the immune system.

Conclusions. Local IL-2 therapy using novel polymeric carriers, aimed at stimulating long-lasting antitumor immunity, may provide an improved method of treating a variety of cancers.

KEY WORDS: polymer microspheres; interleukin-2; controlled release; immunotherapy; brain cancer; liver cancer.

INTRODUCTION

Advances in molecular biology have spurred renewed interest in using the body's own defenses to fight cancer, as several potent stimulators of the immune system (cytokines) have been cloned and are now available for use. Interleukin-2 (IL-2) was one of the first cytokines to be cloned and has a number of activities associated with it, including the enhancement of T cell growth, T cell activation, monocyte activation and natural killer cell activation (1). IL-2 is currently indicated for systemic administration to treat adult patients with melanoma and metastatic renal cell carcinoma. A major limitation of this treatment, however, is the severe toxicity caused by high doses of systemic IL-2 (2).

The major conceptual problem with systemic cytokine administration is that lymphokines are known to exert their immunologic effects locally, in a paracrine fashion (2). That is, under normal physiologic conditions, appropriate lymphokines are produced in high concentrations local to the site of antigen where they serve as the necessary costimulatory signal for induction of an appropriate immune response. This paracrine function cannot be achieved when cytokines are administered systemically. Toxicity from systemic administration of IL-2 can also be severe and would be particularly limiting in the treatment of brain tumors, as the availability of macromolecules to the brain is limited by the blood-brain barrier (3).

Most recent efforts to deliver molecules, such as IL-2, locally to tumors in both the brain (4,5) and the periphery (for review see (6)) have focused on the use of autologous tumor cells genetically engineered to secrete cytokines, often with promising results. Local cytokine delivery by genetically engineered cells is not only physiologically more relevant, but also allows high concentrations of the stimulatory molecule at the tumor site while greatly reducing systemic exposure. However, while conceptually elegant, this approach is difficult to implement in the clinic due to the labor intensity and high cost involved with the genetic transduction of each patient's tumor cells and due to the extensive characterization of the transductants required before they may be used (7). In addition, there may be inherent variability with cytokine-producing cells that are rapidly destroyed by the immune system following administration.

In this article, we discuss the development of an injectable polymeric system capable of delivering active recombinant IL-2 in a controlled fashion following intratumoral injection for up to 21 days *in vivo*. Moreover, we show that local IL-2 delivery from microspheres, made by the complex coacervation of gelatin and chondroitin sulfate, can effectively protect rats and mice from lethal tumor challenges in three different tumors models (two model brain tumors and one model liver tumor). Gelatin (denatured collagen) and chondroitin sulfate (CS) were used to encapsulate IL-2 for three reasons. First, collagen and chondroitin sulfate are primary components of extracellular matrix (ECM) and, thus, systems

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ABBREVIATIONS: CNS, central nervous system; IC, intracranial; B16/IL-2, replication-incompetent B16-F10 tumor cells genetically engineered to secrete IL-2; CT26/IL-2, replication-incompetent CT26 tumor cells genetically engineered to secrete IL-2.

made using gelatin and CS should be biocompatible. Second, gelatin and CS can be made to coacervate and entrap proteins under mild, all-aqueous conditions that should improve activity retention of sensitive proteins, such as IL-2. Finally, tumors typically secrete degradative enzymes, including collagenase, to degrade ECM in order to grow and metastasize (8). In doing so, actively growing tumors may cause an increased rate of polymer degradation, thereby exposing themselves to higher IL-2 concentrations.

Polymeric microspheres may provide a more controlled product with respect to cytokine release profiles and total cytokine dose than can be achieved with genetically engineered cells or by *in vivo* gene therapy, thereby providing an improved method of administering therapeutic gene products (i.e. proteins). Furthermore, the use of polymeric microspheres to deliver IL-2 may simplify therapy by eliminating the need for transfection of each patient's cells with cytokine genes. The development of such a system would allow clinicians to administer cytokine immunotherapy at the time of surgical debulking of an existing tumor, obviating the need to reoperate many days to weeks later to administer genetically engineered autologous cells. Because gelatin (denatured collagen) and chondroitin sulfate both occur naturally in the body, the polymeric delivery system described in this study may be suitable for the immunotherapy of many solid tumors.

MATERIALS AND METHODS

Synthesis of Polymeric IL-2 Delivery Vehicle

IL-2 was encapsulated into polymeric matrices (IL-2 microspheres) by the complex coacervation of gelatin and chondroitin-6-sulfate in the presence of IL-2. Edible porcine gelatin (100 Bloom) was supplied by General Foods (Atlantic Gelatin, Woburn, MA). Chondroitin-6-sulfate and glutaraldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Briefly, 3 ml of a 4% gelatin solution in distilled water at 37°C was mixed with 1 mg of lyophilized IL-2 dissolved in 3 ml of 0.2% chondroitin sulfate in phosphate buffer solution at room temperature. Coacervation was achieved by the addition of the gelatin solution to a rapidly mixed IL-2/chondroitin sulfate solution. Nascent microspheres were cross-linked with 0.125% aqueous glutaraldehyde solution for 20 min and then poured into 10 ml of a 0.1M aqueous glycine solution to stop the cross-linking reaction and quench the excess aldehyde groups. Crosslinked microspheres were collected by centrifugation and washed with phosphate-buffered saline. Placebo microspheres were prepared identically, but in the absence of IL-2.

The mass-average diameter of the microspheres was determined using a Coulter Multisizer II (Coulter Electronics, Luton, Beds, England). Greater than 200,000 microspheres were sized per measurement ($n = 3$ batches).

Preparation and Encapsulation of ^{125}I -Labeled IL-2

Na^{125}I in 0.1N NaOH was obtained from NEN Life Science Products (Boston, MA). Recombinant IL-2 (Aldesleukin, 22 million IU per 1.3 mg protein) was kindly supplied by Chiron Corporation (Emeryville, CA). ^{125}I was covalently attached to IL-2 by reaction in a pre-coated iodination tube (Iodogen®, Pierce, Rockford, IL) for 10 min. Unreacted ^{125}I was separated from ^{125}I -labeled IL-2 ($[^{125}\text{I}]\text{IL-2}$) by column

chromatography (Econo-Pak 10DG column, BioRad, Hercules, CA). Radiolabeled $[^{125}\text{I}]\text{IL-2}$ was subsequently encapsulated into gelatin/chondroitin sulfate microspheres following the standard encapsulation procedure described above and used to determine the IL-2 encapsulation efficiency and the *in vivo* release kinetics of IL-2 following intracranial or intrahepatic injection in rats and BALB/c mice, respectively.

Tumor Cells

9L gliosarcoma cells and CT26 colon carcinoma cells were obtained from ATCC and B16-F10 melanoma cells were obtained from the National Cancer Institute Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD). Tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and penicillin/streptomycin in humidified incubators at 37°C and gassed with 5% carbon dioxide. The B16-F10 and CT26 cell lines were transduced with the murine IL-2 gene by using the replication-defective MFG retroviral vector, as previously described (9). The amount of IL-2 produced by transformed tumor cells was quantified routinely using a standard ELISA kit (Endogen, Cambridge, MA). Cultured tumor monolayers were harvested with 0.025% trypsin, counted, and resuspended in DMEM prior to inoculation. Transduced tumor cells were exposed to 5000 rads (1 rad = 0.01 Gy) from a ^{137}Cs source (Gammacell model 62 irradiator, Nordin International, Inc., Kanata, Ontario, Canada) discharging approximately 1300 rads/min to render them replication-incompetent immediately prior to injection.

Animals

The use of animals in this study was carried out in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Fisher 344 rats weighing 200–250 g, 6–8 week old BALB/c mice, and 6–8 week old C57/B6 mice were obtained from Harlan (Indianapolis, IN) and housed in an approved animal facility. Animals were allowed free access to Purina Rodent Chow and water.

Intracranial and Intrahepatic Injections

Rats and mice were anesthetized prior to all surgical procedures. Rats were anesthetized by an intraperitoneal injection of 1 ml of a stock solution containing ketamine hydrochloride (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol. Mice were anesthetized by an intraperitoneal injection of 0.1 ml of this stock solution diluted 1:3 in 0.9% aqueous NaCl solution.

For intracranial injection of tumor cells or polymer microspheres, the head of mice or rats was shaved and prepared with 70% ethanol and iodine-containing solution. A midline incision was made using a scalpel and a 1 mm burr hole was made at the site for injection. All intracranial injections were made in the left parietal lobe of the brain using a stereotactic frame (the coordinates in mice were 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture; coordinates in rats were 5 mm posterior and 3mm lateral to the bregma). Tumor cells or polymer microspheres were delivered over a period of 3 min by a 26-gauge needle inserted to a depth of 3 mm in both mice and rats. The needle was removed following injection and the site was irrigated with 0.9% NaCl solution and closed with 4.0 Vicryl sutures.

For intrahepatic injection of tumor cells or microspheres in BALB/c mice, the abdominal region was shaved and prepared with 70% ethanol and iodine-containing solution. A transverse incision was used to open the peritoneal cavity and the left lobe of the liver was exposed. Using an operative microscope, 10 μ l of HBSS containing 5×10^4 wild type CT26 cells (CT26 WT) were injected beneath the liver capsule using a 30-gauge needle. Compression of the liver capsule at the injection site was maintained for one minute to prevent extravasation. 4.0 Vicryl suture was used to close the abdominal incision. Using this method and cell dose, a solitary intraparenchymal tumor can be readily identified by the naked eye by day 2 following tumor injection, and 90% of untreated tumor-bearing animals die by day 28 due to massive hepatic tumor growth (all animals die by day 55). Animals treated by concomitant injection of CT26/IL-2 or IL-2 microspheres received a total injection volume of 13 μ l.

In Vivo IL-2 Release from Polymer Microspheres

Polymer microspheres containing [125 I]IL-2 were injected intracranially into Fisher 344 rats and into the left lobe of the liver of BALB/c mice to determine the *in vivo* release kinetics of [125 I]IL-2 from the polymeric carrier. Control animals received a bolus injection of free [125 I]IL-2.

Groups of rats were given either an intracranial injection of free [125 I]IL-2 (i.e. not encapsulated into polymeric carrier) or an identical dose of [125 I]IL-2 encapsulated within polymer microspheres in a total volume of 10 μ l of PBS. Animals were sacrificed at predetermined time points and their brains removed and frozen prior to sectioning with a cryostat microtome. 20 μ m sections were mounted on glass slides, air-dried in a sterile hood and wrapped in a single layer of plastic wrap along with an 125 I standard plastic strip (Amersham Pharmacia Biotech, Piscataway, NJ). The slides were subsequently exposed to a phosphorus imager screen (FUJIFILM Medical Systems USA, Stamford, CT) in a sealed cassette and the phosphorus screen processed in a phosphorus imager (MacBas 1000, FUJIFILM Medical Systems USA, Stamford, CT) to yield the IL-2 concentration profiles in each tissue section.

To confirm long-term IL-2 delivery by polymer microspheres in tissues other than the brain, groups of BALB/c mice were injected intrahepatically with either free [125 I]IL-2 or [125 I]IL-2 microspheres and the total reactivity in the injection lobe followed over time. While still under general anesthesia, three mice from each group were exsanguinated immediately following intrahepatic injection and their blood and organs harvested in the following order: blood (via cardiac puncture), injection lobe of liver, remainder of liver, heart, lungs, spleen, kidneys, and brain. The organs and blood were weighed and the amount of radioactive IL-2 in each was determined using a gamma counter. The remaining animals were allowed to recover from anesthesia and were returned to their cages. Subsequently, animals from each group were sacrificed at various time points (3 animals per time point per group) and the [125 I]IL-2 activity was measured in the injection lobe of the liver, various other organs, and the blood following the above procedure.

Efficacy of Local IL-2 Delivery in Intracranial and Hepatic Tumor Models

For *in vivo* testing against brain tumors, animals were challenged intracranially with wild-type 9L gliosarcoma cells

(Fischer 344 rats) or B16-F10 melanoma cells (C57/B16 mice). Animals were treated with a simultaneous local injection of placebo microspheres (negative control) or IL-2 microspheres. The total dose of bioactive IL-2 delivered within polymer microspheres was approximately 0.4 μ g for mice and 4 μ g for rats. There were two additional groups in the mouse brain tumor model study: one group of mice received replication-incompetent B16-F10 cells engineered to secrete IL-2 (B16/IL-2) as a positive control and another group received an equal number of replication-incompetent B16-F10 cells (negative control group). Outcome was measured by survival, and all animals were autopsied at the time of death.

For *in vivo* testing against liver tumors, the anti-tumor effect of locally delivered IL-2 (by either IL-2 microspheres or CT26/IL-2 cells) was measured by its ability to protect animals challenged intrahepatically with CT26 WT from tumor growth. BALB/C mice were challenged by intrahepatic injection with 5×10^4 CT26 carcinoma cells and simultaneously treated by a local injection of placebo microspheres (negative controls, $n = 9$), 10^6 replication-incompetent CT26 cells engineered to secrete IL-2 (CT26/IL-2, positive controls, $n = 10$), or IL-2 microspheres (treatment group, $n = 10$). 10^6 replication-incompetent CT26/IL-2 cells had been determined to yield the optimal anti-tumor effect in a preliminary dose escalation study (data not shown). The total dose of bioactive IL-2 delivered within polymer microspheres was approximately 0.4 μ g. Three weeks after intrahepatic tumor challenge, animals were euthanized and examined for tumor presence and tumor volumes were measured.

Intracranial Memory Immunity Studies in IL-2 Microsphere-Treated Survivors

C57/B6 mice that survived a B16-melanoma intracranial challenge as a result of local IL-2 microsphere therapy were rechallenged ($n = 12$), along with naive control animals ($n = 10$), by stereotactic injection with a lethal dose of B16 in the contralateral hemisphere of the brain 75 days after the original treatment with IL-2 microspheres to determine if anti-tumor immunologic memory had developed. Animals were returned to their cages following surgery and survival assessed. Autopsy was performed to determine cause of death.

Histology

C57/B6 mice challenged intracranially with B16-F10 WT and treated with placebo microspheres, IL-2 microspheres, or B16/IL-2 cells were sacrificed at various time points following challenge for histologic analysis of the challenge site. Brains were harvested, fixed in 10% formalin, serial sectioned in the coronal plane and embedded in paraffin. Microscopic sections were cut at 6 microns and stained with hematoxylin and eosin. Independent pathologic review of all specimens was performed.

RESULTS

IL-2 Encapsulation into Polymer Microspheres

Injectable polymeric matrices containing entrapped IL-2 were made by the complex coacervation of positively-charged gelatin with negatively-charged chondroitin-6-sulfate. Polymer matrices containing IL-2 (IL-2 microspheres) were

