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Attenuated *Salmonella typhimurium* with interleukin 2 gene prevents the establishment of pulmonary metastases in a model of osteosarcoma

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Abstract

Purpose: The current management of osteosarcoma (OS) entails an aggressive preoperative and postoperative chemotherapeutic regimen with limb salvage surgery. Despite these efforts, relapse-free survival is less than 60% in patients with classic OS, whereas most patients relapse with pulmonary metastases. In these studies, we sought to prevent the establishment of pulmonary metastases from OS with a single oral dose of *SalpIL2*.

Methods: Mice were administered attenuated *Salmonella typhimurium* with (*SalpIL2*) and without a gene for human interleukin 2 (*Sal-NG*) 7 days before challenge with 2×10^5 OS cells via tail vein. Three weeks after injection, mice were harvested for splenic lymphocytes and tumor enumeration.

Results: Prophylaxis with attenuated *SalpIL2* significantly reduces pulmonary metastases in number and volume ($P < .0001$ and $P < .0001$) with respect to saline controls. Furthermore, splenic natural killer cell populations were increased 396% with *SalpIL2* ($P < .0007$) and 426% with *Sal-NG* ($P < .0003$) compared to nontreated groups.

Conclusions: Host natural killer response is greatly amplified and maybe partially responsible for the effective immune response against the formation of pulmonary metastases. A single oral dose of *SalpIL2* may be a novel form of adjuvant therapy for patients after early detection of primary OS.

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Pediatric osteosarcoma (OS) is a rare but highly aggressive bone cancer. Three-year survival from primary OS has improved from 20% in 1970 to nearly 70% with current treatment regimen of high-dose methotrexate, cisplatin, ifosfamide, and doxorubicin [1-3]. Positive long-term prognosis for patients is associated with greater than 90% necrosis of primary tumor after chemotherapy and low intracellular content of the metastatic protein ezrin. In patients who present with metastatic disease detectable by computerized tomography (CT), less than 30% disease-free survival has been achieved [4,5]. A cytokine produced by $CD4^+$ T cells, interleukin 2 (IL-2), promotes lymphocyte proliferation and enhances cytolytic function of cytotoxic T lymphocytes and natural killer (NK) cells [6]. In some cases,

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intravenous (IV) IL-2 treatment has resulted in complete regression of the primary OS tumor, though severe side effects have been noted, including fever, nausea, capillary leak syndrome, and death [7-9]. Investigators have attempted to limit the toxicity of IL-2 by altering the dose, schedule, and routes of administration; however, little reductions in adverse effects have been noted. In the attempt to limit toxicity as seen with IV IL-2, our laboratory developed a biologic vector for local IL-2 delivery. The avirulent, but highly immunogenic, *Salmonella typhimurium* strain χ4550 was chosen as a vector for IL-2. *Salmonella typhimurium* is a facultative intracellular organism known to colonize the liver, spleen, lymph nodes, and lungs after oral ingestion. Its innate biodistribution properties make it an ideal delivery system for local recombinant IL-2 delivery. The gene for human IL-2 was ligated into the plasmid pYA292, renamed pIL2, and electroporated into χ4550, and the resulting bacteria were renamed *SalpIL2*. In previous experiments, *SalpIL2* has been shown to reduce hepatic metastases of colon adenocarcinoma, the volume and mass of primary neuroblastoma, and OS pulmonary metastases [10-15]. Experiments described in these studies attempt to determine the antitumor capabilities of *SalpIL2* in a prophylactic model of pulmonary metastasis in OS.

1. Materials and methods

1.1. Bacteria

Attenuated *S. typhimurium* χ4550 and plasmid pYA292 were given by Dr Roy Curtiss III, Washington University, St Louis, Mo. χ4550 is an adenylate cyclase (cya), cyclin adenosine monophosphate receptor protein (crp), and aspartate semialdehyde dehydrogenase (asd) mutant that renders it avirulent but highly immunogenic. Plasmid constructs with and without a truncated gene for human IL-2 were electroporated into χ4550 using well-described techniques and renamed *SalpIL2* and *Sal-NG* [11]. Standardized glycerol stocks of approximately 10^8 colony forming units (CFU)/mL were prepared by creating growth curves for overnight cultures in Luria broth (Difco Laboratories, Detroit, Mich) to an optical density at 600nm of 0.560 and placed in liquid nitrogen. For experiments, cryovials were thawed to room temperature, serial diluted, and plated on MacConkey's agar plates to verify CFU concentration. Use of *S. typhimurium* with a truncated gene for human IL-2 was approved by the University of Minnesota (Minneapolis, Minn) Institutional Biosafety Committee (no. 541 and 542).

1.2. Animals

Six-week to eight-week-old female balb/c mice were acquired from Harlan-Sprague-Dawley (Indianapolis, Ind), fed standard mouse chow and water ad libitum, and housed in microisolator cages with 12-hour light/dark cycles under the strict care of the University of Minnesota Research Animal Resources. All experiments were approved by the University of Minnesota Institutional Animal Care and Uses Committee (no. 0409A63728).

1.3. Tumor preparation and tumor cell line

The murine OS cell line K7M2 was acquired from the American Type Culture Collection and maintained in 25 mL Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum, 1% penicillin, streptomycin, and L-glutamine (Sigma Chemical, St. Louis, Mo) at 37°C at 5% CO_2. Media was changed twice weekly, and cells were not allowed to become confluent. Tumor cells were incubated with 0.3% trypsin EDTA (Invitrogen, Carlsbad, Calif) at 37°C at 5% CO_2 for 3 minutes or until nonadherent. Tumor cells were serially washed in Hanks' balanced salt solution (Invitrogen) before enumeration via trypan blue exclusion (Sigma Chemical) on a phase contrast hemacytometer (Hausser Scientific, Horsham, Pa). Injection solutions were diluted to a concentration of 2×10^6/mL and placed on ice before injection. All tumor preparations were more than 90% viable and used within 1 hour of preparation.

1.4. K7M2 pulmonary metastases model

Seven days before tumor injection, mice were orally gavaged with their respective treatments (n = 5), 200 μL Hanks' balanced salt solution for controls or 3×10^7 CFU of either *Sal-NG* or *SalpIL2*. Previously, we established a treatment model for pulmonary metastases to examine the antitumor mechanisms of *SalpIL2*. In triplicate experiments, animals were anesthetized by intraperitoneal injection of 2:1 xylazine, 20 mg/mL (Phoenix Pharmaceuticals, St Joseph, Mo), and ketamine, 100 mg/mL (Abbot Laboratories, North Chicago, Ill). On day 0, animals were prepared and administered 2×10^5 K7M2 mouse OS cells using well-described techniques [15]. Mice were selected at random from their respective groups for IV tail vein injection. In all experiments, the mice were evaluated for presence of metastases 3 weeks postinjection by euthanasia followed by an intratracheal injection of 1.5 mL of 15% India ink solution via a blunt-ended needle. The stained lungs were carefully resected and rinsed in Feket's solution overnight. Tumors were enumerated and classified by the diameter of the nodules; volume was calculated by $4/3\pi r^3$, assuming the metastases were a sphere. Spleens were aseptically removed and placed in 60 × 15-mm culture dishes for fluorescent-activated cell sorting (FACS) analysis of splenic lymphocytes.

1.5. Splenic lymphocyte preparation

Splenic lymphocytes were isolated by mechanically mincing spleens in DMEM containing 10% fetal goat serum. Splenic homogenates were filtered through a