Local Activation of Immune Response in Bladder Cancer Patients Treated with Intraarterial Infusion of Recombinant Interleukin-2

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Abstract

Tumor regression induced in cancer patients by i.v. infusion of interleukin-2 (IL-2) is often accompanied by severe side effects. To investigate whether local administration would affect immune response without the side effects, two 5-day cycles of continuous intraarterial intraluminal iliac artery infusion of recombinant interleukin-2 (rIL-2) were performed in 12 patients with transitional cell carcinoma (tumor stage 1, node stage 0, metastasis stage 0, and grade 1–2) of the bladder. Four groups of 3 patients were treated at each of 4 escalating doses of rIL-2 (18 × 10^4, 18 × 10^5, and 18 × 10^6 IU/m^2/day) throughout the course of the two IL-2 cycles. This treatment was effective in inducing a marked intratumoral inflammatory response, consisting mainly of T-lymphocytes and macrophages. A remarkable dose-dependent increase in the levels of soluble CD25 was observed in the urine of all patients, which was associated consistently with an enhanced number of intratumoral (sCD25+) and macrophages. A remarkable dose-dependent increase in the levels of soluble CD25 and CD25+ lymphocytes was observed in peripheral blood only at the two highest doses of rIL-2, while increased percentages of circulating HLA-DR+ and CD71+ lymphoid cells and enhancement of CD3+CD16+ T-lymphocytes were found at lower doses. Peripheral blood eosinophils were augmented in almost all patients but were rarely increased in situ. We provide evidence that continuous intraarterial infusion of rIL-2 activates host immune response, acting preferentially at the tissue level.

Introduction

rIL-2 infused i.v. at high doses in cancer patients affects host immune response and may induce tumor regression (1, 2); however, the high doses of IL-2 that are effective for therapy cause severe toxicity (1–3).

Alternative treatments with IL-2 have been evaluated that would increase host immunostimulation and decrease dose-dependent systemic toxicity. Encouraging experimental and clinical results have been obtained for some tumors with more localized administration of IL-2, capable of activating local and systemic immune response (4–9).

One anatomic region suitable for local therapy is the urinary bladder. Pizza et al. (10) reported preliminary results of intravesional injections of partially purified natural IL-2 in invasive bladder cancer. More recently, intravesical perfusion of natural IL-2 in patients with invasive inoperable bladder cancer has been reported by Huland et al. (11).

The urinary bladder derives its blood supply from the internal iliac artery, a system that can be catheterized, with a relatively low flow rate, making it appropriate for drug administration (12). We report here the results of a phase I study of continuous infusion of rIL-2 via the internal iliac artery to patients with low-stage bladder cancer to determine whether this new IL-2 treatment affects local and systemic host immune response.

Materials and Methods

Interleukin-2. rIL-2, provided by EuroCetus B.V. (Amsterdam, The Netherlands), had a specific activity of 3 × 10^6 Cetus units/mg. One Cetus unit of rIL-2 is equivalent to 6 IU.

Patients and Treatment Schedules. Twelve patients (P. L., F. C., F. S., P. E., S. E., B. R., F. T., R. A., L. A., S. G., C. T., and T. M.) entered our study between June 1988 and February 1990, after informed consent was signed. Tumor cross-section diameters were measured by ultrasonographic sonography in all patients. All had histologically proven low-stage (tumor stage 1, node stage 0, metastasis stage 0, and grade 1–2) transitional cell carcinoma of the bladder and were fully ambulatory (Karnofsky 100%) at the time IL-2 treatment was started. None of the patients had been treated previously for bladder cancer or other malignancies.

Patients were treated with continuous rIL-2 infusion into the internal iliac artery for two consecutive 5-day cycles, separated by a 48-h rest. TUR-BT was performed 4 days after the end of the second IL-2 cycle (Fig. 1).

The interpatient dose escalation scheme was the following: 18 × 10^4, 18 × 10^5, and 18 × 10^6 IU/m^2/day. Four groups of three patients each were treated at each of the four doses of IL-2 throughout the course of treatment.

Tumor fragments from all patients were fixed with formalin for conventional histologic examination. Fresh samples of tumors were obtained by biopsy at the time of diagnosis and at the time of TUR-BT; pre- and posttreatment samples from three (P. L., F. C., B. R.) and pretreatment samples from two (F. S., R. A.) of the 12 patients were inadequate and not included in the study. Normal urothelium was obtained from two of the patients (C. T., T. M.).

Peripheral Blood. Heparinized venous blood samples were collected 1 week before treatment (day −7), during IL-2 administration (days 5 and 6), 2 days after the end of the second IL-2 cycle (day 14), the day of resection (day 16), and 1 (day 47) and 2 months (day 77) after treatment (Fig. 1). Each sample was obtained at the same time of day (between 7 and 8 a.m.), and freshly isolated cells were used for all assays.

Lymphocyte and eosinophil numbers were evaluated with an automated analyzer (H6000/H6101 system, Technicon, Tarrytown, NY).

PBMCs were isolated by Ficoll density gradient (Lymphoprep; Nycomed As, Oslo, Norway), and plasma was collected and frozen at −80°C.

Urine. Urine was collected daily, before, during, and after IL-2 infusion. Urinalysis showed no significant change in the number of leukocytes and/or erythrocytes, during and after treatment. To assay sCD25, urine was centrifuged at 500 × g for 10 min and then frozen at −80°C.
Monoclonal Antibodies. PBMC phenotype was evaluated using the following MAbs: Leu4 (anti-CD3), Leu2a (anti-CD8), Leu3a (anti-CD4), Leu11c (anti-CD16), Leu12 (anti-CD19), LeuM3 (anti-CD14), anti-p55 IL-2 receptor (anti-CD25), anti-HLA-DR, and anti-transferrin receptor (anti-CD71), all purchased from Becton-Dickinson, Mountain View, CA.

Immunohistochemical characteristics of infiltrating leukocytes were analyzed using the following MABS: Pan-Leu (anti-CD45), B4 (anti-CD19), DakoMac, DakoPC (Ki67), and TAC (anti-CD25), purchased from Dakopatts, Geostrup, Denmark; OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKT6 (anti-CD1a), OKT10 (anti-CD38), and anti-HLA-DR, purchased from Ortho; Leu11c (anti-CD16) from Becton-Dickinson. Pam-1 was kindly provided by R. F. Todd (13); anti-IL-1α (Vmp18) and anti-IL-1β (Vhp20 and BR1C3), by M. Bigio and R. Rosse (14, 15); and anti-α-tumor necrosis factor (154-7.1) by Dr. B. Persuia (16).

Flow Cytometric Analysis of Peripheral Blood Mononuclear Cells. PBMC phenotype was studied by single and double flowcytometry staining by direct immunofluorescence method (17). PBMCs were incubated with different MABs for 30 min at 4°C. When cells were tested by dual antibody staining, one MAB was conjugated with fluorescein isothiocyanate and the other with phycoerythrin. After two washings, stained cells were analyzed using a FACScan (Becton-Dickinson, Mountain View, CA). The cell-associated surface antigens expressed by PBMCs of healthy donors were: CD3, 65 ± 10%; CD4, 40 ± 10%; CD8, 28 ± 9%; CD16, 17 ± 7%; CD19, 10 ± 5%; CD14, 18 ± 5%; and HLA-DR, 11 ± 4%. These normal means and SD values were calculated from results obtained from 100 volunteers.

Immunocytochemical Staining of Tumor-Infiltrating Leukocytes. Tumor-infiltrating leukocytes were characterized in situ by immunocytochemical methods. Fresh tissue fragments were embedded in OCT (Ames Division, Miles Laboratories, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −80°C until sectioning. Acetone-fixed cryostat sections were immunostained with optimal dilutions of MABs, and the slides were incubated sequentially with rabbit anti-mouse secondary antibody and a peroxidase-labeled mouse immunoglobulin, peroxidase-antiperoxidase method (Cambridge Research Laboratory, Cambridge, MA). Each incubation step lasted 30 min and was followed by a 10-min wash in phosphate-buffered saline. The sections were then incubated with 0.03% H2O2 and 0.006% 3,3′-diaminobenzidine (BDH Chemicals, Poole, United Kingdom) for 3–5 min, washed in tap water, and counterstained with hematoxylin for 1 min. Eosinophils were pressed by PBMCs of healthy donors were: CD3, 65 ± 10%; CD4, 40 ± 10%; CD8, 28 ± 9%; CD16, 17 ± 7%; CD19, 10 ± 5%; CD14, 18 ± 5%; and HLA-DR, 11 ± 4%. These normal means and SD values were calculated from results obtained from 100 volunteers.

Peripheral Blood Leukocytes. No significant change in the number of peripheral blood lymphocytes was seen during and after treatment. Increased eosinophil counts were seen in 11 of 12 patients (data not shown), sometimes 24–48 h after the beginning of IL-2 administration. This enhancement was more pronounced after the second cycle of IL-2.

The percentages of CD3+, CD4+, and CD8+ T-lymphocytes did not change significantly, while an increased percentage of CD16+ cells was observed in 9 of 11 patients (the two remaining had high baseline levels) (Fig. 2). This enhancement was observed at the end of the first or the second cycle of treatment, with individual variability, and independently of the dose of IL-2 administered. The cell population was better characterized by analyzing lymphocytes for coexpression of CD3/CD16 antigens in dual color analysis. Fig. 2 shows that the increased percentage of CD16+ cells was due mostly to enhancement of CD3+/CD16+ T-lymphocytes, except in those patients treated with the highest dose of IL-2, in whom NK cells (CD3−/CD16+) were increased. After the first 5-day cycle of therapy, the percentage of monocytes (CD14+) tended to increase in most...
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patients; a slight increase in the percentage of B-lymphocytes (CD19+) was found in 3 of 11 patients.

All modifications of PBMC distribution tended to return to baseline levels at the end of IL-2 treatment.

Analysis of cell activation markers showed no change in the percentage of CD25+ cells (baseline levels, ≤5%; data not shown) in patients treated with the first two doses of IL-2, but all patients treated with the two highest doses exhibited 2-8-fold increases in the percentages of CD3+/CD25+ lymphocytes (Fig. 3). A smaller increase of CD16+/CD25+ cells was observed in patients treated with 18 × 10⁴ IU/m²/day of IL-2 (Fig. 3). The increase in CD25+ cells paralleled a marked increase in plasma-soluble IL-2 receptor (sCD25) (Fig. 3). Peak levels were reached after the first IL-2 cycle, and a slow decline was observed after treatment.

In contrast, an increase in the percentage of lymphocytes with CD71+ (8 of 11 patients) and HLA-DR+ (6 of 11 patients) was also observed at the lower doses (Figs. 4 and 5). Interestingly, the percentage of CD71+ or HLA-DR+ cells was not increased in patients treated with the highest dose of IL-2. Similarly to CD25 antigen, enhancement of CD71 and HLA-DR was due mostly to an increase in the percentage of CD3+/CD71+ and CD3+/HLA-DR+ T-lymphocytes (Figs. 4 and 5).

NK and ADCC activities were affected variably, independently of the dose of IL-2 administered. LAK activity was seen in only 4 of 12 patients. Interestingly, modification of ADCC activity did not correlate strictly with changes in the percentage of CD16+ cells, which are the major mediators of this cytotoxic activity (19), or with variations in NK cytotoxicity. However, increased NK activity was always associated with the development of LAK activity.

**Tumor-infiltrating Leukocytes.** Prior to treatment, focal inflammatory infiltrates (CD45+ cells) were detected in tumor connective stroma and in submucosa (Table 1; Fig. 6A) in all patients. They were mainly composed of CD4+ T-lymphocytes and macrophages (DakoMac+, Paml+), with variable numbers of B-lymphocytes (CD19+), plasma cells (CD38+), and eosinophils. The neoplastic urothelium was infiltrated by scattered CD8+ T-lymphocytes and by some CD1+ Langerhans cells.

Treatment with IL-2 was followed by a detectable increase in the number of CD45+ leukocytes, both in tumor connective stroma and in neoplastic urothelium (Table 1; Fig. 6B). Macrophages and T-lymphocytes were still prevalent in submucosa and in tumor stroma, but a relative increase in the number of CD8+ lymphocytes was noted. In neoplastic urothelium, CD4+ lymphoid cells and macrophages became as numerous as CD8+ lymphocytes. Rare intraurothelial CD16+ cells were observed in 5 of 9 cases. A consistent increase in the number of eosinophils was detected in the connective stroma in only 3 of 9 cases (2 patients treated with 18 × 10⁴ and one treated with 18 × 10⁵ IU/m²/day of IL-2).

A dose-dependent increase in the number of CD25+ (Fig. 6, C and D) and/or HLA-DR+ lymphoid cells was observed after IL-2 treatment. Activated cells were demonstrated in the stroma and in the neoplastic urothelium. Some intratumor CD25+
LYMPOID CELLS WERE DETECTED, HOWEVER, EVEN IN PATIENTS WITH
THE TWO LOWEST DOSES OF IL-2, WHO HAD NO EVIDENCE OF CIRCULATING
CD25+ CELLS. AFTER TREATMENT, STROMAL AND INTRAUROTHELIAL MAC-
ROPHAGES WERE OFTEN IMMUNOREACTIVE FOR IL-1 AND/OR TUMOR
NECROSIS FACTOR (Fig. 6E), SUGGESTING AN ACTIVATED STATUS. AT THE
TIME OF TUMOR BIOPSY, LOCAL PROLIFERATION OF INFILTRATING LEUKO-
CYTES WAS MARGINAL, AS ESTIMATED BY THE LOW NUMBER OF KI67+
CELLS. IN THE TWO CASES (C. T., T. M.) IN WHICH FRAGMENTS OF
NORMAL UROTHELIUM WERE EXAMINED BEFORE AND AFTER TREATMENT,
IL-2 INFUSION INDUCED CHANGES IN NORMAL UROTHELIUM SIMILAR TO
THOSE IN THE TUMOR, SUGGESTING THAT THE INFLAMMATORY REACTION
WAS NOT DIRECTED SELECTIVELY AGAINST TUMOR CELLS. IN ALL CASES,
NORMAL AND NEOPLASTIC UROTHELIUM WAS HLA-DR− BEFORE AND
AFTER TREATMENT.

Soluble IL-2 Receptor in Urine. A marked, dose-dependent
increase in the levels of soluble IL-2 receptors (sCD25) was
observed in urine of all patients (Fig. 7). The urinary levels of
sCD25 were already increased 48–72 h after IL-2 infusion,
reaching peak levels after the first IL-2 cycle and returning to
baseline values after a few days. At the two lowest doses, a clear
dissociation was noted between high levels of sCD25 in urine
and baseline levels of sCD25 in the plasma.

DISCUSSION

Continuous i.a. infusion of rIL-2 is effective in inducing
systemic and local activation of the host immune response.
Dose-dependent increased levels of sCD25 were observed in the
urine of patients. sCD25 in urine might thus represent an
important marker for evaluating the local effectiveness of IL-2
treatment in diseases of the urogenital tract. We also observed
the appearance of numerous CD25+ cells at the tumor site and
in normal urothelium. These findings are in agreement with a
previous report by Huland et al. (11), describing the appearance
of CD25+ cells in the urine of bladder cancer patients after
intravesical perfusions of IL-2. Similarly, Cohen et al. (20)
described the augmentation of intratumoral CD25+ cells after
IL-2 therapy in melanoma patients.

Circulating CD25+ lymphocytes and sCD25 in the plasma
were increased only at the two highest doses of IL-2. Similarly,
Lotze et al. (21) and Voss et al. (22) reported marked increases
in the number of CD25+ cells and in sCD25 plasma levels in
patients treated with high doses of i.v. rIL-2.
The increased number of tissue CD25+ cells, enhanced sCD25 antigens in urine, and the simultaneous lack of detectable CD25 in peripheral blood suggest that urinary sCD25 is released by locally activated cells. Our results indicate a more pronounced activity of IL-2 after i.a. infusion at the tissue level, probably because of preferential delivery of IL-2 at this site.

The immunochemical study revealed activation of tissue macrophages, as indicated by the appearance of intracellular cytokines such as IL-1 and tumor necrosis factor. It is thus conceivable that these cytokines are directly active against the tumor and may act synergistically with IL-2 in the activation of T-lymphocytes (23, 24). We showed previously (25) that lymphoid and nonlymphoid tissue macrophages contain virtually no IL-1 or tumor necrosis factor, suggesting that the expression of these cytokines is related to a particular functional status of these effector cells.
A potential antitumor role of eosinophils was emphasized recently (26). Also, a remarkable increase in the number of eosinophils in urine was reported in bladder cancer patients after intravesical perfusion of IL-2 (11). We did not observe any increase in tumor-infiltrating eosinophils (although eosinophilia was found in peripheral blood); moreover, we were not able to establish a correlation between eosinophil infiltration and tumor regression.

The finding of only a few proliferating Ki67+ cells after IL-2 treatment may indicate that the increase in the number of infiltrating leukocytes depends on recruitment from the systemic circulation rather than local proliferation. The validity of this hypothesis can be shown only through the use of sequential biopsies.

Interestingly, the inflammatory infiltration pattern was similar in normal and neoplastic urothelium, before and after treatment. This finding further supports the idea that the local efficacy of i.a. treatment is the result of IL-2 activity at the level of the bladder.

The percentage of peripheral blood HLA-DR+, CD71+, and CD16+ T-lymphocytes was increased even with lower doses of IL-2. CD16 antigen, commonly expressed by NK cells (19), was found mostly on CD3+ cells, suggesting that continuous i.a. IL-2 treatment can modulate its expression on T-lymphocytes. Previous studies (27) showed that CD16 antigen is expressed on only a small proportion of T-cells (≤2–5%) from healthy donors and does not change significantly following short-term culture with IL-2 in vitro. Voltarelli et al. (28), however, reported a 3.5-fold increase in the percentage of CD3+/CD16+ T-cells when lymphocytes were cultured for 20 days with rIL-2. Furthermore, several CD3+/CD16+ clones have been described (27), and functional studies have shown that this T-cell subset displays ADCC, while NK and LAK activities are absent. In our study, ADCC activity increased in only a small number of patients and did not always correlate with the number of CD3+/CD16+ cells, suggesting that they are not the main effectors of ADCC activity.

Finally, our data indicate that i.a. infusion of IL-2 does not exert major effects on the level of circulating and tissue NK cells. In fact, the percentage of NK (CD3−/CD16+) cells did not increase consistently, except in those patients treated with the highest dose of IL-2. This finding, together with the evidence that the number of NK cells is enhanced by high doses of i.v. IL-2 (29), indicates that only high concentrations of IL-2 in the peripheral blood can increase the number of NK cells. Moreover, no consistent change in NK activity was observed, and no correlation between NK cell numbers and cytotoxicity against K562 was noted. This finding could be explained by the presence, among the PBMC, of either accessory cells capable of modulating NK activity negatively (30) or T-cytotoxic cells with "promiscuous" activity (31). The augmentation of NK activity always correlated with the appearance of LAK activity, corroborating the finding that most LAK cytotoxicity is mediated by NK cells (32). At the tissue level, rare CD16+ cells were sometimes present, independently of the dose of IL-2. This observation is in accord with previous results showing that serial biopsies of regressing melanoma lesions did not contain infiltrating CD16+ NK cells (20) and supports the finding that T-lymphocytes, rather than NK cells, display a preferential trafficking to the tumor site (33).

No clear correlation between the modulation of the immune response and tumor regression was observed. Whether immunological data, however, correlate with the local relapse rate of tumor will be evaluated at the end of a 5-year follow-up.

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REFERENCES


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