Eosinophils and C4 predict clinical failure of combination immunotherapy with very low dose subcutaneous interleukin-2 and interferon in renal cell carcinoma patients

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ABSTRACT

Background and Objectives. The clinical and immunologic activities of interleukin-2 (IL-2) in cancer patients have been extensively studied and described; however, in most of these studies, IL-2 was administered by intravenous bolus or continuous infusion, while the immunologic effects of IL-2 given by the subcutaneous (s.c.) route have not yet been well studied.

Design and Methods. The present study was aimed at evaluating the effects of IL-2, given at very low doses s.c. to patients with advanced renal cell carcinoma (RCC), on a number of immunologic parameters: number of total lymphocytes, number of CD4+, CD8+, CD25-positive cells, number of natural killer (NK) cells, titers of IL-2 soluble receptor (sIL-2R) and of C4, eosinophils, eosinophilic cationic protein (ECP) and eosinophilic protein X (EPX). Finally, a logistic regression model was performed to identify early immunologic parameters that correlate with a favorable or unfavorable treatment outcome.

Results. Independently from the mere report of the changes induced by immunotherapy, the analysis showed that, within the pre-treatment model, a large eosinophil number predicts the failure of IL-2 treatment; in contrast, within the post-treatment model, high C4 serum titers and, again, a large number of circulating eosinophils predict immunotherapy failure.

Interpretations and Conclusions. As far as concerns C4, its negative predictive value could be related to the fact that it is an indirect index of macrophage activation; thus, even though macrophages release substances with antitumor activity, they can also stimulate the release of sIL-2R, which may compete for exogenous IL-2. Some authors have postulated that macrophages may even stimulate tumor cell growth, or impair NK activity. Despite a great amount of uncertainty concerning the role of eosinophils, in our study, blood eosinophilia predicts a poor response to immunotherapy in patients with advanced RCC, thus supporting previous observations from our own group.

Key words: immunotherapy, immunologic parameters, eosinophils, C4; predictors

Interleukin-2 (IL-2), a cytokine secreted primarily by CD4+ T-helper and CD8+ cytotoxic T-cells in response to antigen and/or other cytokine stimulation,1 exerts a variety of biological effects on hematopoietic cells, including activation of cytotoxic T-cells, helper T-cells, macrophages, natural killer (NK) cells and B-cells.2 In vitro studies and in vivo adoptive immunotherapy studies have clearly shown that IL-2 can activate antigen-specific and non-specific T-cell responses against tumors,3,4 exert a direct cytotoxic effect on human carcinoma cells, at least in certain tumor models,5 and revert adenocarcinoma-derived mucin-related immunosuppression.6 Furthermore, IL-2 can also induce eosinophilia, probably through the production of secondary cytokines, e.g., IL-5, IL-3 and GM-CSF, by stimulated lymphocytes.7

The clinical and immunologic activities of recombinant human IL-2 (rhuIL-2) in cancer patients have been extensively studied and described; however, in most of these studies, rhuIL-2 was administered using chemotherapy guidelines such that the maximum tolerable dose was given by intravenous bolus or continuous infusion over a few days.8,9 Atzpodien et al. demonstrated that low doses of subcutaneous rhuIL-2, are both clinically and immunologically effective,10 more recently, Buzio et al. have demonstrated that very low doses of subcutaneous rhuIL-2 can induce persistent immunologic effects, with objective antitumor activity and low toxicity,11 the latter probably due to reduced induction of nitric oxide synthesis.12
The present study was aimed at evaluating the effects of rhuIL-2, given at very low doses using the protocol described by Buzio et al. to patients with advanced renal cell carcinoma, on each of the following immunologic parameters: number of total lymphocytes, number of CD4+, CD8+, CD25-positive cells, number of NK cells (identified as CD3 and CD56+ cells), titers of IL-2 soluble receptor (sIL-2R) and of C4; particular attention was paid to eosinophils and their cytotoxic products, eosinophilic cationic protein (ECP) and eosinophilic protein X (EPX), since the role of these cells and proteins, relative to IL-2-induced antitumor activity, remains highly controversial. Finally, a logistic regression model allowed us to identify early immunologic parameters that correlate with a favorable or unfavorable treatment outcome.

Design and Methods

Patients’ selection and treatment schedule

Twenty-five patients (16 males and 9 females, average age: 59.6 yrs, range: 42-71) affected by advanced RCC were treated subcutaneously with very low doses of rhuIL-2 plus interferon, according to the treatment protocol originally proposed by Buzio et al. All patients gave their informed consent to enrollment into both the clinical protocol and biological studies, according to institutional requirements. Briefly, rhuIL-2 was given subcutaneously for 5 days per week, together with recombinant interferon-α (rIFN-α) by intramuscular route twice weekly, for 4 consecutive weeks corresponding to one treatment cycle. The cycle was regularly repeated at 4 months’ intervals in all patients, irrespective of clinical response. rhuIL-2 was administered at the dose of 1 M U/m² every 12 hours, on days 1 and 2, followed by 0.5 M U/m² twice daily on days 3-5 of each week; concomitantly, rIFN-α was given as 1.8 M U/m² on days 3 and 5 of each week.

All blood samples were drawn from each patient prior to and about 60 hours after the end of the first treatment cycle.

Immunologic study

Total white blood cell count was calculated using a cell counter (Contron, San Diego, CA, USA), while differential count was performed, by a single investigator, on peripheral blood smears stained with May-Grünwald-Giemsa; the absolute number of lymphocytes and eosinophils was thus extrapolated.

Whole blood was collected in preservative-free heparin and mononuclear cells were separated by centrifugation through Ficoll-Hypaque (Pharmacia-Upjohn, Nerviano, Italy) and immediately analyzed, while serum was isolated and stored at less than <20°C for subsequent titration.

Flow cytometry

Immunophenotyping of mononuclear cells was performed using a staining panel which included monoclonal antibodies to the CD4, CD8, CD3, CD56 antigens and to the activation marker CD25 (Beckton-Dickinson, Mountain View, CA, USA). Since the CD25 antigen is expressed not only by lymphocytes, its percent and not its absolute number is considered. Both single and double staining, the latter for the NK subset, were used.

Stained cells were washed in phosphate-buffered saline (PBS) and resuspended in PBS containing paraformaldehyde. Flow cytometric analysis was performed using FACScan apparatus (Beckton-Dickinson, Mountain View, CA, USA).

Serum assay of sIL-2R and C4

The sIL-2R level was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cellfree IL-2R test kit, T-Cell Diagnostic Inc., Cambridge, UK).

C4, being an indirect index of macrophage activation, was titered using an commercial immunochemistry kit (Beckman Diagnostic System Group, Brea, CA, USA).

Study of eosinophilic function

Eosinophils were counted on a peripheral blood smear stained with May-Grünwald-Giemsa (Sigma Chemicals, St. Louis, M.O, USA) at 25x magnification, while serum ECP and EPX were assayed using a sensitive radioimmune (RIA) method (Pharmacia-Upjohn, Nerviano, Italy). The technique was based on the double antibody: sample ECP (or EPX) competed with an unvarying amount of 123iodine-labeled ECP (or EPX). When an immunoadsorbent antibody was added, free myeloperoxidase could be separated from bound myeloperoxidase by centrifugation and then decantating. Pellet radioactivity was measured in a γ-counter and was inversely proportional to sample ECP (or EPX) amount.

Treatment outcome

Clinical response to treatment, i.e., objective responses [complete responses (CR) plus partial responses (PR), stable disease (SD) or progression (P)], was assessed using commonly accepted WHO criteria, before the start of each treatment cycle. The aim of our study was to verify whether the biological modifications induced by the first treatment cycle only could be correlated with the best clinical outcome.

Furthermore, when the statistical analysis was performed according to treatment outcome, we considered objective response and stable disease together, because we reasoned that, especially in immunotherapy patients, a long-lasting control of tumor growth could be interpreted as a sign of immune response, as valuable as a tumor regression (and perhaps much more valuable than a short-lasting response).

Statistical analysis

Data management and analysis were performed with the S-Plus statistical package. Mean and standard deviation values were used as descriptive statistics. Paired and unpaired t-tests were used to compare pre-treatment versus post-treatment values and responders versus non-responders, respectively. The correlation among variables was tested using the Pearson’s product moment correlation coefficient. Finally, the independent effect of selected clinical variables with respect to clinical outcome (favorable - i.e., OR and SD, or unfavorable - i.e., P) was assessed by multiple logistic regression analysis.
Results

Clinical outcome

Of the 25 treated patients, 6 (24%) had an objective response, equally distributed between CR and PR; 12 (48%) had stable disease, while 7 (28%) had progression despite treatment.

Patients' characteristics are summarized in Table 1.

Response to treatment was evidenced after the first cycle of immunotherapy in four patients; the other two responders had a delayed response, one after two cycles and one after three cycles.

The average duration of responses and stable disease were 14.6 (range: 8-24+) and 11 (range: 4-20) months, respectively.

Immunotherapy was well tolerated, WHO grade II fever being the most common side-effect observed, easily controlled by the administration of acetaminophen; other unwanted side-effects included flu-like syndrome, pain at injection site and peripheral edema requiring treatment with diuretics. Finally, no treatment discontinuations due to toxicity have been observed so far.

Immunologic study

When all first cycles were considered together, independently of clinical response, rhul-2 + IFN treatment determined a statistically significant increase in the following immunologic parameters: number of lymphocytes, number of CD4+, CD8+ and NK cells, percent of CD25+ cells, number of eosinophils, EPX, C4 and sIL-2R titers, while the variations in ECP titers did not reach statistical significance (Table 2).

When we compared first cycle data of patients developing a favorable clinical outcome, i.e., OR plus SD, against the data from patients with progressive disease, statistically significant post-treatment differences were shown for the number of lymphocytes, eosinophils, CD4+ T cells and for the serum titers of C4 and sIL-2R, which were significantly higher in non-responding patients (Table 3).

Furthermore, when all patients were considered together, independently of the type of clinical response, C4 and sIL-2R post-treatment titers were strongly correlated (p = 0.005 by Spearman rank order correlation test). When response was taken into account, this correlation was significant in patients with progressive disease (p = 0.00005), but disappeared in the patients with OR or SD.

No significant differences were evidenced in terms of immunologic parameters between patients achieving an early or a delayed clinical response.

Multiple logistic regression analysis allowed us to identify some clinical variables as predictors of favorable or unfavorable outcome.

Indeed, eosinophils only predict progression despite treatment in the pre-treatment model (Table 4a), while, in the post-treatment model, C4 and eosinophils predict treatment failure (Table 4b).

The logistic regression model provides the probability of response as a function of the independent variables; considering the model to perform correctly when a responder is given a probability of response greater than 0.5, the performance of the pre-treatment model was 78%, while the performance of the post-treatment model was 85%.

Table 1. Summary of patients' characteristics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Men</th>
<th>Women</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>59.6</td>
<td>42-71</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous nephrectomy</td>
<td>19 (76%)</td>
<td>6 (24%)</td>
<td></td>
</tr>
<tr>
<td>Site of metastatic disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>11 (44%)</td>
<td>4 (16%)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4 (16%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>9 (36%)</td>
<td></td>
<td>Te</td>
</tr>
<tr>
<td>Nodes</td>
<td>10 (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site of nephrectomy</td>
<td>12 (48%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3 (12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECOG Performance status</td>
<td>0 (10%)</td>
<td>8 (32%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10 (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7 (28%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 (Abs. #/µL)</td>
<td>720.26</td>
<td>142.16</td>
<td></td>
</tr>
<tr>
<td>CD8 (Abs. #/µL)</td>
<td>522.44</td>
<td>184.68</td>
<td></td>
</tr>
<tr>
<td>CD25 (%)</td>
<td>20.11</td>
<td>8.05</td>
<td></td>
</tr>
<tr>
<td>NK (Abs. #/µL)</td>
<td>285.39</td>
<td>94.60</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (Abs. #/µL)</td>
<td>150.01</td>
<td>85.90</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (Abs. #/µL)</td>
<td>1,912.34</td>
<td>488.30</td>
<td>2,745.15</td>
</tr>
<tr>
<td>ECP (µg/L)</td>
<td>15.02</td>
<td>10.22</td>
<td>42.28</td>
</tr>
<tr>
<td>EPX (µg/L)</td>
<td>28.44</td>
<td>22.66</td>
<td>102.44</td>
</tr>
<tr>
<td>sIL-2R (µU/mL)</td>
<td>489.24</td>
<td>255.62</td>
<td>1,891.31</td>
</tr>
<tr>
<td>C4 (mg/dL)</td>
<td>25.39</td>
<td>4.30</td>
<td>41.99</td>
</tr>
</tbody>
</table>

Abs. #: absolute number.
Table 3. First cycle data of patients yielding a favorable clinical outcome (OR plus SD) compared to those of patients with progression showed statistically significant post-treatment differences for the number of lymphocytes, eosinophils, CD4+T cells and for the serum titers of C4 and sIL-2R, which were significantly higher in non-responding patients.

Post-treatment values

<table>
<thead>
<tr>
<th>Variable</th>
<th>Favorable outcome</th>
<th>Unfavorable outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
</tr>
<tr>
<td>CD4 (Abs. #/µL)</td>
<td>828.45</td>
<td>358.21</td>
</tr>
<tr>
<td>sIL-2R (U/mL)</td>
<td>1455.23</td>
<td>844.88</td>
</tr>
<tr>
<td>C4 (mg/dL)</td>
<td>40.45</td>
<td>3.51</td>
</tr>
<tr>
<td>Lymphocytes (Abs. #/µL)</td>
<td>2375.24</td>
<td>820.25</td>
</tr>
<tr>
<td>Eosinophils (Abs. #/µL)</td>
<td>689.70</td>
<td>355.10</td>
</tr>
</tbody>
</table>

Abs. #: absolute number.

Table 4. Multiple logistic regression analysis: eosinophil titer predicts tumor progression in the pre-treatment model (a), while C4 and eosinophils predict tumor progression in the post-treatment model (b).

(a) Pre-treatment model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>t-value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>9.78</td>
<td>2.61</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>-0.012</td>
<td>-2.03</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

(b) Post-treatment model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>t-value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>8.05</td>
<td>1.95</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>-0.13</td>
<td>-2.41</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C4</td>
<td>-0.24</td>
<td>-2.23</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Discussion

rhuIL-2 has usually been administered in the clinical setting by the intravenous route or subcutaneously at high doses.8,10 In both cases, antitumor activity has been well documented and the immunologic effects of the cytokine have been widely studied and described.21-25 In contrast, the use of very low doses of subcutaneous rhuIL-2 has only recently been proposed11 and, while its antitumor activity and low toxicity profile have been clearly documented,11,12 the effects that such a treatment can induce on the immune system of the host receiving this cytokine, have not been specifically investigated yet. The rationale of the long-term stimulation of the immune system of cancer patients by rhuIL-2, as proposed by Buzio et al., relies on the assumption that a continuously activated immune system could counteract active tumor proliferation, leading to, at least, reduced tumor growth.11 Indeed, independently of clinical outcome, rhuIL-2 administration was continued in all patients, even in those with progressive disease, since we reasoned11 that they may benefit from slower tumor growth, resulting from a sustained immunologic attack, no other active treatment option being currently available.16

Furthermore, the activation of cellular immunity has been proposed to play a major role in inducing tumor dormancy,27 thus, Wheelock et al. first stressed the importance of cellular immunity in inducing dormancy in a murine lymphoma model.28 In their study, growth restraint was dependent on both cytolytic T-lymphocytes and macrophages; the secretion of IL-2 and IFN-γ by T-cells induced the activation of cytotoxic T-cells in the early stage of induction of dormancy followed by activated macrophages at a later stage. Studies in mice with leukemia showed that allogeneic bone marrow transplantation provided an anti-leukemic effect29,30 and that such mice carried cell cycle-arrested dormant tumor cells for long periods of time.31 Khazaie et al.32 showed that injecting live B-lymphoma cells at a site refractory to tumor growth led to a long lived T-cell response that correlated with the persistence of dormant tumor cells. Indeed, besides OR, patients had fairly long lasting stable disease (on average: 11 months), with 8 patients showing SD for at least one year, a figure which is hardly explainable simply by slow neoplastic growth.

We investigated the changes induced by a single rhuIL-2 plus IFN treatment on some immunologic parameters in a population of patients with metastatic renal carcinoma: besides this conventional immunologic study, we also evaluated the effects of immunotherapy on eosinophil production and activity, a subject of great current interest and of questionable interpretation. The mere report of the changes induced by very low dose rhuIL-2 treatment, independently of response, showed a statistically significant increase in the number of NK, CD4+, CD8+ cells, total lymphocytes and eosinophils, as well as in EPX, C4 and sIL-2R serum titers and in the percent of CD25+ cells. The percent of cells expressing the CD25 antigen was preferred to the absolute number of CD25+ lymphocytes, because of the heterogeneity of cells capable of expressing this activation marker, which is also present on the surface of non-lymphocytic cells.

Pre- and post-treatment immunologic data were analyzed separately by multiple logistic regression analyses, and two different statistical models were made to assess which parameter(s) could predict clinical response, or vice versa, treatment failure. The analysis showed that, within the pre-treatment model, a large eosinophil number predicts the failure of rhuIL-2 treatment.

In contrast, within the post-treatment model, high C4 serum titers and, again, a large number of circulating eosinophils predict neoplastic disease progression, that is immunotherapy failure.

As far as C4 is concerned, its negative predictive value could be related to the fact that it is considered as an indirect index of macrophage activation;17 thus, even though macrophages certainly release substances with antitumor activity such as TNF-α, IL-1,
The value of eosinophils as effector cells in antitumor immune response remains highly controversial. Thus, eosinophils are considered beneficial when present within tumors, while they are often interpreted as indicators of poor prognosis when present in high concentrations in the peripheral blood of patients with neoplasia.11–14 Direct15 or antibody-dependent16 cancer lysis by eosinophils has been demonstrated in vitro models and eosinophilic cationic proteins have been proposed as possible mediators of this antitumor activity of eosinophils,43 even though their behavior in cancer patients is far from being well defined or understood.44 However, we have recently demonstrated that eosinophils are unlikely to play a direct cytotoxic role on tumor cells through the release of their cationic proteins ECP and EPX, at least in vitro.18 Moreover, in our study, blood eosinophilia predicts a poor response to low-dose rhuIL-2 + IFN immunotherapy in patients with advanced RCC.

The conflicting results reported in the literature, taken together with our findings, clearly indicate that the precise role of eosinophils in IL-2-treated cancer patients is far from understood and deserves further thorough investigations. In particular, since it is not absolutely obvious that the blood parameters observed after the first cycle of treatment remain substantially unmodified in subsequent cycles, a longitudinal study is warranted to clarify the relationship existing between duration of response and modifications of immunologic parameters.

Contributions and Acknowledgments

CP and MM had the original idea of the study and wrote the paper. MDA and MAC performed all laboratory investigations; SQ and MDA performed the statistical analyses; CP and CB enrolled patients into the study. Finally, CB reviewed the manuscript and, as Senior Author, is cited last.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- On the basis of the results of the above study, biological predictors of failure to respond to very low dose s.c. IL-2 and interferon-α therapy are now available, at least in patients with renal cell carcinoma; indeed, hypereosinophilia and the activation of macrophages are negative prognostic factors.

- Thus, new immunologic approaches to the treatment of these neoplasms should also consider the modulation of both eosinophil and macrophage activity. The role of these cells and their relationship with the tumor on the one hand, and with the effector cells of the immune system on the other, deserves further studies.

References


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