Expression of the interleukin 6 receptor in primary renal cell carcinoma

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Abstract

Aims—Interleukin 6 (IL-6) is expressed in the majority of renal cell carcinomas and has an important role in the proliferation of some renal cell carcinoma cell lines. This action is mediated by two membrane proteins, gp80 (the IL-6 receptor; IL-6R), which binds IL-6, and gp130, which transduces the signal. The soluble form of gp80 (sIL-6R) is able to activate gp130 when complexed to the IL-6 molecule. These considerations prompted an investigation of IL-6R expression in this malignancy. IL-6, C reactive protein (CRP), and sIL-6R were also measured in serum and correlated to clinical and pathological features.

Methods—Immunostaining was performed on cryostat sections from renal cell carcinoma tumours with M91, an anti-IL-6R monoclonal antibody, using the alkaline phosphatase antialkaline phosphatase technique. The proliferation index was measured using the Ki-67 monoclonal antibody. CRP, IL-6, and sIL-6R were measured in serum before nephrectomy, using an immunoenzymatic or immunoradiometric assay.

Results—There were significant differences in survival in patients with tumours larger than 8 cm, metastasis at diagnosis, high nuclear grade tumours, detectable serum concentrations of IL-6 (correlated to CRP serum concentration), more than 4% proliferating cells, and the presence of the IL-6R in situ. Furthermore, the serum IL-6 concentration correlated with tumour size and stage. The mean serum sIL-6R concentration was not significantly different from that observed in 40 normal subjects. Tumour IL-6R expression was present in 10 samples. There was a significant association between the presence of the IL-6 receptor in tumours and tumour stage, nuclear grade, proliferation index, and serum IL-6.

Conclusions—This study revealed the importance of IL-6/CRP and IL-6R expression in situ as potential new prognostic factors and opens the way to new therapeutic strategies in renal cell carcinoma.

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Keywords: renal cell carcinoma; interleukin 6; IL-6 receptor; C reactive protein

Renal cell carcinoma arising from renal tubular epithelial cells is the third most common adult malignancy.1 Recent studies highlighted the presence of chromosomal abnormalities including allelic loss of chromosome 3p21 and possible in vitro tumour induction after v-src oncogene transfection of normal tubular cells.3 Growth factors implicated in renal cell carcinoma progression have not been defined in vivo. Interleukin 6 (IL-6) is a multifunctional cytokine that regulates haematopoiesis, the production of acute phase proteins, and the immune response; it plays a critical role in the differentiation of B cells into plasma cells. IL-6 is produced by a variety of cells including macrophages, T cells, B cells, fibroblasts, endothelial cells, and epithelial cells, particularly renal mesangial and tubular cells.5 IL-6 pleiotropic functions are mediated by two membrane proteins, an 80 kDa binding receptor (IL-6R) and the gp130 protein that transduces the signal.6 Soluble gp80 (sIL-6R) binds to IL-6 and the IL-6/sIL-6R complex binds and activates the gp130 transducer chain.7 8 In addition to its physiological functions, IL-6 exerts growth stimulatory activities in a variety of malignancies, particularly B cell neoplasias including multiple myeloma.9 In this disease, the serum IL-6 and sIL-6R concentrations have been found to be powerful prognostic factors.10-12 Serum concentrations of IL-6 have been detected in some patients with renal cell carcinoma and IL-6 accounted for most of the paraneoplastic symptoms observed in these patients.13 14 Moreover, high serum IL-6 concentrations have been correlated with poor survival.13 15 IL-6 is known to induce autocrine growth in renal cell carcinoma cell lines in vitro,16 although this has not been found regularly.17 Takenawa et al analysed IL-6 and IL-6R expression in normal and tumorous renal tissues from patients after nephrectomy. IL-6 gene expression was detected in more than 50% of renal cell carcinoma cases and the presence of the IL-6 cytoplasmic protein was confirmed by immunohistochemistry. Using the complementary DNA polymerase chain reaction, they detected IL-6R transcripts in all specimens.18 These data provide evidence that IL-6 and its receptor may play a crucial role in proliferation of renal cell carcinoma and in the development of symptoms.

Using an anti-IL-6R monoclonal antibody and immunohistochemistry, we analysed the expression of this protein in 38 primary renal cell carcinoma patients at diagnosis; tissues were obtained after nephrectomy and
evaluated for proliferative status by KI-67 labelling. IL-6 and sIL-6R serum concentrations were also measured and correlated to clinical and pathological features.

**Materials and methods**

**Patients and samples**

Tissue samples of primary renal cell carcinoma were obtained surgically from 38 patients at diagnosis. Definitive staging was determined according to Robson's classification. Histological diagnosis was based on examination of haematoxylin and eosin stained slides from paraffin embedded tissue sections. For all patients, a tumour sample was frozen at -20°C in OCT compound (Miles, Elkhart, Indiana, USA). Serum and plasma samples were obtained from all untreated patients just before surgical nephrectomy and stored at -20°C until assay. Serum and plasma samples were also obtained from a control population including 40 healthy donors with comparable sex and age distributions.

**Determination of IL-6 serum concentration**

Serum IL-6 concentrations were measured in samples from 25 patients using a commercially available enzyme immunoassay (Immunotech, Marseille, France). The test detection limit was 10 pg/ml, and lower concentrations were considered to be undetectable.

**Determination of serum C reactive protein concentration**

Serum C reactive protein (CRP) concentrations were measured using a fluorescence polarization immunoenzymatic assay (Abbott Laboratories, Chicago, Illinois, USA). The detection limit of the CRP assay was 2.5 mg/l.

**Determination of the sIL-6R serum concentration**

An immunoradiometric assay was performed with the PM-1 monoclonal antibody. Flat bottom microtitration plates with breakaway wells (Nunc, Roskilde, Denmark) were coated with PM-1 monoclonal antibody (1 µg/ml) in 100 µl of 0.1 M Tris-HCl (pH 9) for 16 hours at 4°C and washed three times with phosphate buffered saline (PBS) Tween 20. Each well was then saturated with 200 µl 3% bovine serum albumin (BSA) in PBS for four hours at room temperature. After three washings in PBS/Tween 20, 50 µl of recombinant human sIL-6R or patient serum, diluted in PBS/0.2% BSA, was added along with 50 µl of iodinated MT18 monoclonal antibody (3 × 10^6 counts/min/ml) in PBS/0.2% BSA in the presence of purified mouse immunoglobulins. Plates were stirred during overnight incubation at 4°C, then washed three times with PBS/Tween 20 and the radioactivity in each well was measured in a γ counter. A standard curve was plotted with percentages of maximal bound radioactivity as a function of recombinant human sIL-6R concentrations in the different patient sera assayed. With purified recombinant human sIL-6R, concentrations of up to 100 ng/ml could be measured accurately. Sensitivity, defined as the minimal recombinant human sIL-6 concentration giving a signal difference with background standard deviation, was determined to be 1 ng/ml. An IL-6 concentration below 20 ng/ml did not interfere with this assay. PM-1 and MT18 anti-IL-6R monoclonal antibodies and recombinant human sIL-6R have been described previously.

**Immunohistochemistry**

Immunostaining was performed on 5 µm frozen sections fixed in cold ethanol/acetone using a Techmate 500 automated (Dako A/S, Glostrup, Denmark). As previously shown, seven monoclonal antibodies produced in INSERM Unit 291 (Montpellier, France) were tested on a myeloma cell line (XG7) which is known to express IL-6R strongly. Using flow cytometry (Facsan, Becton Dickinson, Mountain view, California, USA) and immunohistochemistry, the M91 monoclonal antibody constantly gave positive labelling within the 0.05-0.5 mg/ml range without false positive staining.

The MIB-1 mouse IgG, monoclonal antibody (Immunotech) was used for cell proliferation analysis. It reacts with the KI-67 nuclear antigen associated with cell proliferation. This antigen is present throughout the cell cycle (G1, S, G2, and M phases) and absent in resting (G0) cells. Mouse immunoglobulins of the same isotype were used as negative controls. After rehydration in PBS, slides were incubated with the primary monoclonal antibody for 30 minutes at room temperature. Sections were washed in PBS and then incubated with diluted (1/25) rabbit anti-mouse immunoglobulin (Z 259; Dako) in PBS for 30 minutes at room temperature. Finally, sections were incubated with alkaline phosphatase and mouse monoclonal antialkaline phosphatase (D 651; Dako) at a 1/25 dilution. The immunohistochemical reaction was then revealed in the dark using fast red (Sigma, St Louis, Michigan, USA) as chromogenic substrate and counterstained with haematoxylin. Double staining was performed using fast red and fast blue (Sigma) chromogens in succession. Positive and negative controls were obtained for each reaction.

IL-6R cytoplasmic positive cells and KI-67 positive nuclei were counted at ×25 magnification on a Leitz laborlux 25 microscope (Leica, Rueil Malmaison, France). Microscope fields in the sections were randomly chosen from left to right and up to down. In each case, the immunostained slides were evaluated by counting at least 1000 adenocarcinoma renal cells in four different fields; lymphocytes and endothelial cells were carefully avoided. Fields were accepted for evaluation if they did not contain areas of extensive necrosis, non-specific background staining or sectioning artefacts. The results are expressed as the percentage of positive cells.

**Statistical analysis**

Data were first assessed by examining the relation between each predictor factor. For categorical variables, χ² analysis was used; Fisher's exact test was used if χ² analysis was not valid. The continuous variable correlations were...
Results

PATIENT POPULATION

Thirty-eight untreated patients were included in this study, 27 men and 11 women. The mean age was 61.2 years (range 39–83 years). Clinical and pathological data are summarised in Table 1. There were seven patients with stage I tumours (18.4%), 19 with stage II tumours (50%), six with stage III tumours (15.8%), and six with stage IV tumours (15.8%). Nineteen patients (50%) had high local tumour burden (> 6 cm). Most tumours (17; 44.7%) were intermediate nuclear grade, according to Furnham’s system. The mean follow up was 14 months (range 1–28 months). The overall survival curves indicated a two year survival rate of 81% with a standard error of 6.4%.

There were significant survival differences when tumours were larger than 8 cm (56% vs 86%), there were metastases at diagnosis (33% vs 90%), high nuclear grade tumours (35% vs 86%), detectable serum IL-6 activity (70% vs 85%), more than 4% Kl-67 positive nuclei (63% vs 88%), and presence of IL-6R in situ (60% vs 89%) (fig 1). Survival was not correlated with sex, age, or serum sIL-6R concentration.

SERUM IL-6 AND CRP CONCENTRATIONS

Eleven of the 38 patients (28.9%) including four of six patients with metastatic disease (66.6%) had detectable IL-6 in their serum with a mean concentration of 62 pg/ml (range 17.5–240). IL-6 was not detected (< 10 pg/ml) in normal controls. CRP serum concentrations correlated significantly with serum concentrations of IL-6 (p = 0.0001, r = 0.69). Patients with undetectable concentrations of IL-6 in their serum had a mean CRP concentration of 2.8 mg/l, while patients with detectable IL-6 concentrations had a mean CRP concentration of 54.1 mg/l. Serum IL-6 concentrations were correlated with tumour size (p = 0.0087,
Figure 3  Immunostaining with an anti-IL-6R monoclonal antibody in the same renal cell carcinoma case as shown in fig 2: six positive cells are seen around a blood vessel (Haematoxylin and eosin, fast red stained; original magnification ×250.)

\[ r = 0.34 \text{ and } p = 0.0035, \quad r = 0.41 \text{ but not with cytological grade.} \]

**SERUM sIL-6R CONCENTRATION**

The mean serum sIL-6R concentration in patients with renal carcinoma was 83.9 ng/ml, (mean 80.5; range 38-150). There was no significant difference between these figures and those obtained for controls (mean 95.7; range 39-171). Moreover, there was no correlation with tumour size, nuclear grade, tumour stage, expression of IL-6R or KI-67 in situ, or IL-6 serum and plasma concentrations.

**KI-67 IMMUNOSTAINING IN RENAL CELL CARCINOMA TISSUES**

The percentage of positive nuclei was measured in the peripheral zone of the tumour, outside of necrotic zones (fig 2). It ranged between < 1 and 15%, while the growth fraction only peaked at 1% in normal renal tissue. Using a 4% cut off point, we observed a good correlation between KI-67 labelling and tumour stage (p = 1.54, 10-4, Fisher’s exact test), nuclear grade (p = 8.48, 10-3, Fisher’s exact test), and the presence of the IL-6 receptor in situ (p = 1.26, 10-4, Fisher’s exact test). No correlation between individual growth fractions and tumour size was found.

**Table 2  IL-6 receptor immunostaining in renal cell carcinoma tissues**

<table>
<thead>
<tr>
<th>Tumour size (cm)</th>
<th>IL-6R -ve</th>
<th>IL-6R +ve</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2-12</td>
<td>mean 5.9</td>
<td>mean 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>Tumour stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>7 (100%)</td>
<td>0</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>II</td>
<td>15 (79%)</td>
<td>4 (21%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>5 (83.3%)</td>
<td>1 (16.6%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1 (16.6%)</td>
<td>5 (83.3%)</td>
<td></td>
</tr>
<tr>
<td>Nuclear grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14 (100%)</td>
<td>0</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>2</td>
<td>12 (70.6%)</td>
<td>5 (29.4%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2 (28.6%)</td>
<td>5 (71.4%)</td>
<td></td>
</tr>
<tr>
<td>KI-67 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4%</td>
<td>25 (92.6%)</td>
<td>2 (17.4%)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>&gt;4%</td>
<td>3 (27.3%)</td>
<td>8 (72.7%)</td>
<td></td>
</tr>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>mean 8.32</td>
<td>range 0-100</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Serum soluble IL-6R (ng/ml)</td>
<td>mean 87.43</td>
<td>range 49-150</td>
<td>NS</td>
</tr>
</tbody>
</table>

Correlation with tumour stage, nuclear grade, percentage of KI-67 positive cells by Fisher’s exact test; tumour size, serum IL-6 and serum sIL-6R by Mann-Whitney test.

**Discussion**

Many reports have provided evidence of a role for IL-6 in the proliferation of renal cell carcinoma tumours. IL-6 is one of the major cytokines involved in inflammatory processes and, thus, it is responsible for a major proportion of the paraneoplastic symptoms seen in patients with renal cell carcinoma. However, the clinical importance of IL-6, sIL-6R, and IL-6R in tumour cells has not yet been established clearly.

In our study, 28.9% of patients with renal cell carcinoma and 66.7% of those with metastatic disease had detectable serum IL-6 activity and we noted a good correlation with the
IL-6 receptor expression in primary renal cell carcinoma

CRP serum concentration, which reflects the whole production of IL-6 in vivo. As previously demonstrated, CRP is produced by hepatocytes under the control of IL-6 and the CRP serum concentration correlates with bioactive circulating IL-6 concentrations. Tsukamoto et al reported that 25% of their 71 patients with renal cell carcinoma had significant serum IL-6 bioactivity. These raised serum concentrations were found in parallel with an increase in the carcinoma grade and progression of the tumour stage. In our study, there was a good correlation between IL-6 concentration, histological grade, proliferation index, and tumour stage. The clinical impact was demonstrated by the difference in overall survival of patients with or without detectable serum IL-6. Furthermore, Blay et al found that the two linked markers, CRP and IL-6 serum concentration, were the most powerful prognosis markers present in 48% of metastatic patients with renal cell carcinoma. In their study, the group of patients with the highest IL-6 and CRP serum concentrations showed the worst response to immunotherapy. Tumour stage, histological grade, and recently, proliferation index were shown to be powerful prognostic factors in independent studies. Therefore, serum CRP measurements would seem to be a more practical prognostic index than IL-6 in renal cell carcinoma as demonstrated in different diseases including multiple myeloma and non-Hodgkin’s lymphoma. However, these data were only analysed retrospectively and would have to be confirmed in a prospective therapeutic study before being valid for treatment selection.

The exact role of IL-6 in tumour cell growth is not yet clear. It was shown that some renal cell carcinoma tumour cells exhibited tumour growth under IL-6 exposure, a situation that was reversed when using anti-IL-6 in vitro. However, this response was not observed in all tumour cell lines or in fresh cells. In vivo, IL-6 accounts for a major part of the paraneoplastic syndrome, including inflammatory signs and hypercalcaemia, as previously demonstrated by our group, with reversion of these signs when anti-IL-6 monoclonal antibody is used in vivo (unpublished data). Furthermore, in this study some patients had slight objective tumour reduction which correlated with CRP inhibition.

The soluble form of the IL-6 receptor (55 kDa), generated through alternative splicing, has an agonist role: it is able to bind IL-6 and the IL-6/sIL-6R complex can bind and activate the gp130 transducer chain. We did not note any increased serum sIL-6R concentrations in patients with renal carcinoma compared to controls, although this phenomenon has been observed in multiple myeloma. Soluble IL-6R (sIL-6R) is now considered to be a major growth factor for malignant plasma cells and in large series studies of patients with multiple myeloma both have been shown to be powerful prognostic factors. However, in our study sIL-6R was not of any prognostic value, possibly because of the relatively low number of patients.

To characterise the role of IL-6 in this disease, it is important to assess the presence of IL-6R on tumour cells. Tanekawa et al showed that the IL-6 receptor (gp80) was present in 26% of patients by northern blot analysis and in all samples when using a sensitive polymerase chain reaction technique. These results suggested the presence of an autocrine process and of IL-6R in renal carcinoma patients. However, the recent production of monoclonal antibodies specific to IL-6R offers new potential for exploring its role at the protein level in renal cell carcinoma. Different monoclonal antibodies have been developed by several groups including ours. The antibody used in this study recognises different epitopes, including the gp130 linkage. Using immunohistochemistry, we noted IL-6R positive cytoplasm in 10 out of 38 patients (26%), but the mean percentage of positive cells was only 4.14%. Possibly, IL-6R expression is detected in some cells because of a variable expression level and/or because the biochemical technique used is not a very sensitive method. Non-tumour cells from surrounding tissue (endothelial and inflammatory cells) were negative. Our subgroup of patients expressing IL-6R had the highest aggressive disease with high tumour burden, advanced histological grade, high proliferation rate and CRP/IL-6 serum concentration, as well as the worst prognosis as shown on the survival curve (60% survival at two years vs 89%). These data allowed us to identify an aggressive subgroup of patients with a clear role of IL-6 in tumour progression and/or proliferation. New therapeutic approaches could be used to treat such patients including anti-IL-6 strategies, combined anti-IL-6/therapy and immunotherapy (such as, interferon α and/or IL-2), or new techniques involving disruption of autocrine loops by other anti-IL-6R drugs7 that interfere with the binding of gp80 and gp130.

11 Grepp PR, Gaillard JP, Kalish LA, Oken MM, Miller AM, Kyle RA, et al. Independent prognostic value for serum soluble interleukin-6 receptor (sIL-6R) in Eastern


