A Spliced Isoform of Interleukin 6 mRNA Produced by Renal Cell Carcinoma Encodes for an Interleukin 6 Inhibitor

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Abstract

Interleukin 6 (IL-6) is a multifunctional and pleiotropic cytokine and in renal cell carcinoma (RCC), this cytokine exerts proinflammatory, immunosuppressive and growth-stimulating properties. A spliced isoform of IL-6 mRNA has been described in peripheral blood mononuclear cells and encodes for a potential protein lacking IL-6 activity. In the present study, a novel spliced form of IL-6 mRNA was found detectable in RCC cell lines, normal renal cells, but not in other tumor cells. This splicing resulted in a frameshift and the generation of multiple stop codon in the spliced IL-6 mRNA. However, two ATG of the third IL-6 exon were identified as translation initiation sites and two truncated IL-6 (tIL-6) with the expected molecular weight were recovered from transfected cell supernatant. The cDNA of a spliced form of IL-6 mRNA detected in RCC lines was cloned and expressed in a baculovirus expression vector. The functional properties of the tIL-6 were investigated and the protein encoded by the spliced IL-6 mRNA, including mitogenic activity on tumor cells. In conclusion, this spliced form of IL-6 mRNA detected in RCC encodes for a truncated IL-6 with IL-6 antagonist properties. (Cancer Res 2005; 65(1): 2-5)

Introduction

Interleukin 6 (IL-6) is a multifunctional and pleiotropic cytokine involved in the pathophysiology of several inflammatory and neoplastic diseases. In renal cell carcinoma (RCC), IL-6 exerts proinflammatory, immunosuppressive, and growth-stimulating properties in vitro and in vivo (1–3). High IL-6 serum levels have been found correlated to a poor survival, response to immunotherapy, and a paraneoplastic inflammatory syndrome in vivo in patients with advanced RCC; serum levels of IL-6 above 35 pg/mL were linked with a more than 3-fold increased probability of death (1, 4–6). The inhibition of IL-6 activity in vivo has been proposed as a therapeutic strategy in IL-6-dependent neoplastic diseases, in particular in myeloma, lymphomas, and RCCs (7–9). A spliced isoform of IL-6 mRNA has been described in peripheral blood mononuclear cells (PBMC; ref. 10). This spliced mRNA encodes for a protein in which only the amino acids encoded by the second exon of IL-6, i.e., those involved in the binding on the gp130 chains, were missing (11) and the resulting protein lacked IL-6 activity (11). The presence and potential role of spliced IL-6 mRNA in RCC was not known.

In the present report, a novel spliced form of IL-6 mRNA was found specifically produced in RCC cell lines, benign renal tumors, and normal proximal tubular epithelial cell line, and found to encode for a truncated IL-6 (tIL-6) with IL-6 inhibitory activity.

Materials and Methods

Cell Lines and Culture

A714, ACHN, CAKI1, CAKI2, U266, B9, and MOLT4 were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Four RCC cell lines were established in the laboratory from primary tumors (CLB-TUT, CLB-VER, CLB-MET) or node metastases (CLB-TUG) of patients with RCC. All cell lines were cultured in RPMI with 2 mmol/L L-glutamine, 100 IU penicillin, 100 mg/mL streptomycin and 10% FCS.

Construction of the Vectors

IL-6 mRNAs were extracted by 106 cells with Trizol LS method (Invitrogen, Cergy Pontoise, France). cDNA were obtained with 30 pmol of DT18 primer and Expand reverse transcriptase kit (Roche, Meylan, France), cloned using the Expand polymerase kit (Roche) with 300 mmol of primers (P5 gAgAgCTTTATgCggCCgCCAgACCCAgCT, p3 gAAATgCCgAgAAACTCTAgAg(Agg)CC) followed a ligation in Blue-script II SK’ vector (Stratagene, Ozyme, Montigny, France). The resulting vector was named E15. For the IL-6 cDNA starting from the first ATG of exon 3 (blue arrow, Fig. 1C), the same method was used in the 5’ end and 5’ end primer (gCAgAAgCcCCgCAgCatCCATCCACCATgCcTg). The resulting vector was named PCRM2.E15.6.7. The different expressions vectors were constructed by digestions of E15 or PCRM2.E.15.6.7 with NotI and XhoI, and ligating in KA107 vector (12).

Sequences were analyzed using Applied Biosystems 377 gene analyzer (Applied Biosystems, Bois Colombes, France) and Vector NTI software (Invitrogen).

Production of Proteins Using a Baculovirus Expression Vector

Spliced IL-6 cDNA in E15 vector was modified to insert a restriction site (BamHI) in 5’ end and a 5-His tag as well as an EcoRI site in 3’ end of the cDNA, using the Expand polymerase kit (Roche) and 300 mmol of primers (P5 CCTTtgATCCATggCGtaAAAAGATgg p3 ggggAAATCTAgAg(Agg)TggTgATggTgATggTgATggTgATgCCgAaCCgC) then cloned in the pAcGP67-B provirus (Invitrogen). The virus was produced after cotransfection in High Five cells with Baculogold linear virus and pAcGP67-B provirus, according to the manufacturer’s instructions (Invitrogen), which were then used to infect High Five cells.

Purification

The produced proteins were dialyzed in a 15-kDa membrane in buffer 1 [20 mmol, phosphate buffer (pH 7.4), 1 mol/L NaCl, 10 mmol imidazole], and purified on a 5 mL chelating Ni-NTA column charged with cupric (Amersham, Gif sur Yvette, France) using fast protein liquid chromatography (Bio-Rad, Marne la coquette, France). Briefly, the column was activated by 0.5 volume of CuSO4 0.1 mol/L and stabilized by 10 volumes of buffer 1. After injection of the protein, the column was washed using 5 volumes of buffer 1 and 6 volumes of buffer 2 [20 mmol phosphate buffer (pH 7.4), 1 mol/L NaH2PO4]. The proteins were then eluted using a gradient from 0% to 60% of elution buffer [20 mmol phosphate buffer (pH 7.4), NaCl 0.5 mol/L, EDTA 50 mmol]. The flow rate and the pressure of the column...
were 1 mL min^{-1} and 100 PSI. The resulting protein preparations were then dialyzed in 30 volumes of PBS.

**Evaluation of IL-6 Bioactivity**

Triated thymidine incorporation in B9 cells and U266 cells was measured as previously reported (3).

**Statistics**

All statistical analyses were done using the procedures of the Statistical Package for the Social Sciences 10.0 program (SPSS, Inc., Chicago).

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**Results and Discussion**

A spliced form of IL-6 mRNA was detected in eight RCC cell lines tested (Fig. 1A), in the oncocytoma cell line and in the benign proximal normal tubular renal cells, but not in any of the lymphoma, myeloma, neuroblastoma, and lung cancer cell lines tested (data not shown). Sequence analysis of the cDNA obtained from the RCC cell lines was consistent and distinct from that of spliced IL-6 mRNA previously described in activated PBMC (Fig. 1B; refs. 10, 13). This splicing generated a frameshift from the third

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**Figure 1.** An alternatively spliced isoform of IL-6 mRNA in RCC cell line. A, RT-PCR analysis of IL-6 transcripts from RCC cell line. Lanes 1-4, four ATCC RCC cell lines (lane 1, A704; lane 2, ACHN; lane 3, Caki1; lane 4, Caki2). Lanes 5-8, four RCC cell lines established in the laboratory (lane 5, CLB-TUT; lane 6, CLG-TUG; lane 7, CLB-MET; lane 8, CLB-VER). B, this partial sequence chromatogram showing the junction of the exon 1 and exon 3 in the RCC spliced IL-6 mRNA sequence. Sequence analysis of PCR product show a different IL-6 mRNA spliced isoform in RCC cell lines as compared with PBMC, in which an adenosine was deleted upstream of exon 3, preventing the frameshift observed in RCC-specific sliced IL-6 mRNA (10). C, RCC spliced IL-6 mRNA is translated into two proteins encoded by the 3' terminal part of the exon 3 as well as exons 4 and 5. Pink, ATg in the first exon; blue, the stop codon generated after splicing; green, the start codon in the third exon; yellow, Kozak sequence; blue arrow, cloning for baculovirus and expression vector.

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exon of IL-6, resulting in the generation of four stop codons not present in the full-length IL-6 mRNA, although it can be found in the spliced IL-6 mRNA in PBMC (Fig. 1B). Although the splice variant was observed only in cell lines from renal tissues, further investigations on tissue cDNA banks are needed to determine whether this variant is actually specific for kidney tissues.

The cell lysates of MOLT4 transfected with expression vector encoding for the spliced RCC IL-6 mRNA labeled with a poly-His tag contained two proteins with molecular weights of 17 and 18 kDa, respectively (Fig. 2A). We therefore asked whether the two codons encoding for methionine found in exon 3 of the normal IL-6 mRNA (229-231 and 283-285 bp; Fig. 1C) could act as translation initiation sites. Notably, a Kozak-like sequence (14) was observed close to the second ATG of exon 3 (Fig. 1C). The predicted proteins encoded by the COOH-terminal part of the third exon as well as exons 4 and 5 had an expected molecular weight consistent with that of the two proteins produced by the transfected MOLT4 cells (Fig. 2A). Interestingly, MOLT4 cells transfected with expression vectors of either the full-length spliced IL-6 cDNA, or the tIL-6 cDNA starting from the first ATG of exon 3 produced soluble factors inhibiting IL-6 bioactivity (Fig. 2B). The spliced IL-6 mRNA construct starting from the first ATG in the third exon was labeled with a poly-His tag on the 3’ end and was inserted in a baculovirus. The protein tIL-6 produced by infected High Five cells and purified on a Ni-NTA column was found to block the proliferation of the IL-6-dependent B9 cell line, and of the IL-6-dependent U266 myeloma cell line (Fig. 2C), and of the CLB-TUG renal carcinoma cell line (data not shown). The effect of tIL-6 on IL-6 induced inhibition of DC differentiation from CD34 blood progenitors cultured in the presence of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor was then tested (2, 15). In the absence of IL-6 or tIL-6, CD34 progenitors differentiated into a dendritic (CD14-CD11a+) cell population representing 72.6%, of recovered cells, as compared with 27% with IL-6 alone, 68% with tIL-6 alone, and 61% with IL-6 and tIL-6, showing that tIL-6 also reverts IL-6-induced inhibition of DC differentiation (data not shown).

The tIL-6 described in the present report lacks the sequence encoded by the second exon, which is responsible for the binding on the second chain of gp130 and is requested for IL-6 signal transduction (11). tIL-6 may then be unable to transduce a signal because the second IL-6 receptor gp130 chain cannot bind to the truncated molecule; IL-6 is therefore acting as an IL-6 receptor antagonist. The protein translated from the second ATG of the third exon of IL-6 as well as from RCC spliced IL-6 mRNA was found to exert IL-6 inhibitory properties on proliferation and differentiation, in contrast with the tIL-6 protein detected in PBMC in the previous study (16). Several reason may account for this discrepancy: (1) tIL-6 described in

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**Figure 2.** RCC spliced mRNA is translated into two protein with IL-6 inhibitory activities. A, cell lysates transfected by an expression vector for the spliced isoform of IL-6 mRNA were subjected to Western blot analysis using anti-HIS tag antibodies. B, analysis of biological activity transfected cell supernatant. The proteins translated by this spliced mRNA exerted IL-6 inhibitory activity as shown in the B9 proliferation assay. The proteins translated by the first ATG codon in the third exon have the same inhibitory activity. C, IL-6 proteins produced by baculovirus exert IL-6 inhibitory activity in two proliferation assays using the B9 and U266 cell lines.
the present study has been produced in eukaryotic cells versus bacteria for the previous report, (2) the protein preparation was denatured and renatured in the previous study (16), (3) finally, the T7DI mouse cell line was used for quantification of IL-6 bioactivity in the previous study and this cell line has a different sensitivity for species IL-6 (17). Whether tIL-6 protein is produced in vivo in RCC remains unclear; the protein is not recognized by the anti-IL-6 antibody available (which mostly recognize the amino acids encoded by the second exon) nor by the polyclonal antibody used in the previous study (not shown). The establishment of antibody directed against tIL-6 is under way. Of note, important discrepancies between IL-6 bioactivity and IL-6 concentration as measured by ELISA were observed in RCC culture supernatant as well as in the serum of patients with metastatic disease, suggesting the presence of an IL-6 antagonist in addition to soluble receptors (3). Other potential physiologic roles for this RCC-spliced IL-6 mRNA remain to be investigated: spliced forms of cytokine mRNA have been reported for IL-5 and IL-18, and also play a regulatory role for cytokine activity (18–20).

This natural tIL-6 antagonist may play an important role in IL-6 biology. In addition, because tIL-6 is a natural molecule, it may be a useful tool to inhibit IL-6 bioactivity in vitro and in vivo in IL-6-dependent diseases.

Acknowledgments


Grant support: L’Association pour le Recherche sur le Cancer.

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