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Humanised antihuman IL-6R antibody with interferon inhibits renal cell carcinoma cell growth *in vitro* and *in vivo* through suppressed SOCS3 expression

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Abstract Interleukin-6 (IL-6), one of the proinflammatory cytokines, is considered to be one of the factors associated with poor prognosis of patients with renal cell carcinoma (RCC). Suppressor of cytokine signalling-3 (SOCS3) is rapidly up-regulated by IL-6 and a negative regulator of cytokine signalling. SOCS3 not only suppresses cytokine-mediated JAK/STAT signalling, but also sustains MAPK pathways. In our study, among the RCC cell lines, IL-6 mRNA expression was the highest in the 786-O cells, which also showed the highest level of SOCS3 mRNA expression under the condition of interferon stimulation. In contrast, ACHN cells had the lowest expression of both IL-6 and SOCS3 mRNA under the same condition. Our study is undertaken to evaluate the effect of humanised antihuman IL-6 receptor (IL-6R) antibody, which completely neutralises IL-6 activity, in RCC cell proliferation and its effect on signalling pathways. IL-6R antibody, tocilizumab, significantly suppressed cell proliferation in 786-O cells with interferon stimulation. Western blot analysis revealed that the tocilizumab enhanced the interferon-induced phosphorylation of STAT1 and inhibited SOCS3 expression and the phosphorylation of both STAT3 and ERK. In contrast, the IL-6 inhibited STAT1 phosphorylation, enhanced STAT3 phosphorylation and accelerated cell proliferation in ACHN cells. The *in vivo* effects of combination therapy with tocilizumab and interferon showed significant suppression of 786-O tumour growth in a xenograft model. Morphological observation of the tumours revealed the apoptosis, invasion of inflammatory

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cells and fibrosis. These findings suggest that combination therapy using an antihuman IL-6R antibody with interferon may represent a novel therapeutic approach for the treatment of RCC.

1. Introduction

Renal cell carcinoma (RCC) is the most prevalent malignancy arising within the kidney[1] and 20% of patients present with advanced diseases which are often difficult to treat.[2,3] Recently, novel targeted agents have been used for the treatment of advanced RCC[4,5] and have shown efficacy against metastatic RCC.[6,5] However, their effects were still limited and were shown not to be curative.[7]

Although immunotherapy seems to have a minimal role in the management of advanced RCC, the combination of immunotherapy with molecular targeted agents is of great interest as a potential first line therapy.[8–10] Interferon (IFN)-α is one of the most frequently used agents in immunotherapy against metastatic or recurrent RCC, however, drug resistance needs to be overcome to achieve a sufficiently positive effect.

We have previously reported that suppressor of cytokine signalling (SOCS) 3 protein plays an important role in IFN-α resistance in RCC through inactivation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.[11] The silencing of SOCS3 expression is one possible strategy to restore sensitivity to IFN-α-resistant cells. SOCS3 was found to be rapidly up-regulated by interleukin (IL)-6 and acts as a classical feedback inhibitor of cytokine signalling.[12,13]

Monoclonal antibodies to IL-6 receptor (IL-6R) antagonists are currently available as an approach to selectively block IL-6 signalling. Tocilizumab is a humanised antihuman IL-6R antibody that binds to the IL-6-binding site of human IL-6R and competitively inhibits IL-6 signalling. Tocilizumab is therapeutically effective against rheumatoid arthritis, juvenile idiopathic arthritis, Castleman's disease and Crohn's disease.[14–17]

In this study, we show that the autocrine secretion of IL-6 induced by IFN-stimulation causes the expression of SOCS3 in RCC cells. We also show that combination therapy with the humanised antihuman IL-6R antibody, tocilizumab and IFN-α can suppress IFN-α resistant RCC tumour growth.

2. Materials and methods

2.1. Cell lines/IFN-α/recombinant human IL-6/tocilizumab

The human RCC cell lines ACHN, TUHR3TKB, TUHR4TKB, Caki 1 and 786-O were cultured at 37 °C in 5% CO_2 using minimum essential medium (MEM) with 0.1 mmol/L non-essential amino acids, 10% foetal bovine serum for ACHN, TUHR3TKB and TUHR4TKB or McCoy's medium with 10% foetal bovine serum for Caki 1 and RPMI 1640 with 10% foetal bovine serum for 786-O. The human RCC cell lines ACHN, Caki 1 and 786-O were obtained from the American Tissue Culture Collection (ATCC). TUHR3TKB and TUHR4TKB were obtained from RIKEN BioResource Center (Tsukuba, Japan). Natural type IFN-α (Sumiferon; Dainippon Sumitomo Pharma Co., Japan) was used in this experiment. Recombinant human IL-6 was purchased from STEMCELL Technologies, Inc. (Vancouver, Canada). Fifty picograms per millilitre of recombinant human IL-6 was used for the experiment according to the median concentration of RCC patients.[18] Tocilizumab, a humanised antihuman IL-6R antibody, was purchased from Chugai Pharmaceutical Co., Tokyo, Japan. A pharmacokinetic analysis of rheumatoid arthritis patients treated with tocilizumab showed that the maximum concentration was 183 ± 86 μg/ml and minimum concentration was 9.7 ± 11 μg/ml[19] and we found that tocilizumab at a concentration of 50 μg/ml reduced SOCS3 mRNA levels by approximately 80% (data not shown). Thus, the optimum concentration of tocilizumab was determined to be 50 μg/ml.

2.2. RNA isolation and real-time quantitative polymerase chain reaction (PCR)

RNA isolation and cDNA construction were performed using a Cells-to-C Kit (Life Technologies, CA) according to the manufacturer's instructions. TaqMan PCR reagents for SOCS3 (Hs00269575), IL-6 (Hs00985639) and IL-6R (Hs01075666) were purchased from ABI (Applied Biosystems, CA). Quantitative real-time PCR was carried out using TaqMan Master Mix Reagents Kit protocol with a StepOne real-time PCR System (Applied Biosystems, CA). The data were standardised against beta-actin gene expression using Pre-Developed TaqMan Assay reagents (Applied Biosystems, CA).

2.3. Measurement of IL-6 using an enzyme-linked immunosorbent assay (ELISA)

IL-6 concentration in the supernatant of RCC cells was measured using a Human IL-6 Quantikine ELISA Kit (R&D systems, MN) according to the manufacturer's instructions. Each cultured RCC cell line was given IFN-α at a final concentration of 1000 IU/mL to