Direct and indirect effects of human interferon α on renal cell carcinoma: a new in vitro assay system for evaluating cytokine-mediated antitumor effects

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Abstract. A highly purified natural α-interferon (nIFNα) was tested in vitro for direct and indirect antiproliferative activity against renal cell carcinoma (RCC), using a modified human tumor clonogenic assay and clinically achievable concentrations. In preclinical experiments, the indirect (cytokine-mediated) antiproliferative activity of nIFNα was investigated using ACHN cells (established human RCC cell line). Continuous exposure to nIFNα at concentrations of more than 5 IU/ml in the presence of feeder cells (a mixture of 5 × 10⁴ monocytes/dish and 5 × 10⁵ lymphocytes/dish, obtained from healthy donors) significantly inhibited colony formation of ACHN cells in comparison with growth inhibition in the absence of feeder cells (P < 0.05). Various cytokines were measured in the supernatants lying over the medium on the feeder-layer agarose containing the same conditioned feeder cells. With IFNα at 500 IU/ml, tumor necrosis factor α (TNFα) and IFNγ were detected at markedly high levels for 2–24 h. Neutralizing anti-TNFα monoclonal antibody significantly reduced the indirect antiproliferative activity. Using our modified human tumor clonogenic assay technique, sufficient numbers of colonies for drug testing were observed in 19 of 31 surgical specimens (61.3%). In these clinical materials, nIFNα at a clinically achievable concentration (50 IU/ml) significantly inhibited colony growth in the presence of feeder cells consisting of 5 × 10⁴ monocytes/dish and 5 × 10⁵ lymphocytes/dish, obtained from the patient whose tumor was examined (P < 0.05). In colony-forming cases, a significant correlation between the percentage colony survival and TNFα concentration in the supernatant was observed (r = −0.95, P < 0.01). These results suggest that this assay system may be an appropriate technique for evaluating the antiproliferative activities of nIFNα involving cytokine-mediated action, and that TNFα may play an important role in this cytokine-mediated activity.

Key words: Interferon α – TNF α – Human tumor clonogenic assay – Renal cell carcinoma

Introduction

Approximately 50% of patients with renal cell carcinoma (RCC) have metastatic disease at the time of presentation [7]. Fewer than 10% of patients with metastatic RCC survive for 2 years [7]. Systemic chemotherapy for metastatic RCC remains ineffective, with no demonstrated survival advantage [5]. Interferons (IFN) have been shown to have antiproliferative activities in RCC to some extent. However, the results of clinical studies show that only about 18% of patients with metastatic RCC benefit from IFNα treatment [13]. A reliable means of testing sensitivity to IFNα would enable us to select appropriate candidates for IFNα therapy.

It is very difficult, however, to evaluate the antiproliferative activities of IFNα because this cytokine has both direct growth-inhibitory effects [19] and immune-mediated indirect antiproliferative effects [14] on tumor cells. Recently, a soft-agar human tumor clonogenic assay (HTCA) [9, 24] has been shown to be useful for evaluating drug sensitivity of fresh human tumors to cytotoxic agents and biological response modifiers [3, 6, 15, 22, 23]. Although many papers have been published concerning the evaluation of either the direct [6, 15] or indirect effects [22, 23] of IFNα using HTCA, there have been few papers in which both effects were correctly evaluated [22, 23].

In this study, we established a new IFN assay system, which was modified from the HTCA technique by adding monocytes and lymphocytes, obtained from patients’ peripheral blood, into the feeder layers. Using this assay system, we investigated whether indirect (cytokine-mediated) antiproliferative activity could be induced by IFNα at clinically concentrations and what kinds of cytokines were induced.
Materials and methods

Human tumor clonogenic assay. The method originally described by Hamburger and Salmon [9, 24] was used in this study with slight modification. Briefly, feeder layers were prepared in 35-mm petri dishes (Falcon 3801, Becton Dickinson, Sunnyvale, Calif.) using RPMI-1640 culture medium (Flow Laboratories, McLean, Va.) supplemented with 25% heat-inactivated fetal calf serum (lot no. 0130103, Flow Laboratories) and 0.006% penicillin/streptomycin (Gibco, Grand Island, N.Y.) in 0.6% agarose. Tumor cells were suspended in the upper layer with 0.3% agarose prepared with the same medium as the feeder layer. Agarose double-layer cultures of tumor cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 7–21 days. In order to distinguish non-colony cell aggregates, three plates were fixed with 10% formaldehyde before cultivation as positive controls. Each experiment was conducted in triplicate and six plates were prepared as controls. At the end of incubation, colonies were counted under inverted phase-contrast microscopy (Nikon, Tokyo). Colonies were defined as cell aggregates at least 60 μm in diameter and 30 cells in number. The total number of colonies was calculated as the number of aggregates on the positive control subtracted from the number of colonies on the experimental plate. Results were expressed as a percentage of the control value.

Preparation of target cells. In preclinical experiments of this study, the ACHN cell line (CRL 1611, American Tissue Culture Collection), derived from human renal cell carcinoma, was used as target cells in the colony-forming assay. The plating density was 5 x 10⁵ cells/dish in order to achieve at least 200 tumor colonies/dish.

Preparation of feeder cells. Samples of 40 ml heparinized human peripheral blood were obtained from healthy donors for preclinical experiments and from patients whose surgical specimens were used as target cells for the clinical investigations. Blood samples were layered on 40 ml Ficoll-Paque (Pharmacia, Uppsala) and centrifuged at 400 g for 20 min at room temperature. The mononuclear cell layer was collected by pipette. After a wash with phosphate-buffered saline (PBS) without calcium and magnesium (DPBS, Whittaker, Walerville), the cells were resuspended and decanted into a 75 cm² polystyrene tissue-culture flask (Corning 25110, Corning, N.Y.) After 1 h of incubation at 37°C with 5% CO₂ to allow monocyte attachment to the flask, non-adherent cells were separated and the flask was washed twice with PBS. The adherent cells were removed from the flask by a cell scraper (Falcon 3086, Becton Dickinson) and plated in the feeder layer at a density of 5 x 10⁴ cells/dish. More than 90% of this cell population was monocytes as determined by morphological examination using Giemsa-stained preparations. Mononuclear cells were also plated in the feeder layer at a density of 5 x 10⁴ cells/dish.

Measurement of cytokines in the supernatant. In order to measure the various kinds of cytokines such as interleukin-1α, β (IL-1α, β), tumor necrosis factor α (TNFα) and IFNγ in the supernatants, 1 ml RPMI-1640 medium that contained nIFNα at a concentration of 500 IU/ml and 10% fetal calf serum was overlayed on feeder-layer agarose that contained feeder cells (5 x 10⁶ monocytes/dish and 5 x 10⁵ lymphocytes/dish). In preclinical experiments, mononuclear cells obtained from healthy donors were used as the feeder cells and these dishes were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 2, 4, 8 and 24 h to investigate the kinetics of the cytokines. In the clinical study, mononuclear cells from patients with RCC were used as the feeder cells and culture dishes were incubated with IFNα for 24 h. Supernatants were harvested at each indicated time after incubation and stored at -20°C until immediately before a measurement of cytokine titers.

Measurement of each cytokine was performed by the sandwich enzyme immunoassay using solid-phase reaction methods and coated monoclonal antibodies to each cytokine [26]. The minimum measurable level of each cytokine was 10 pg/ml for IL-1α, 20 pg/ml for IL-1β and 100 pg/ml for IFNγ. All measurements of cytokines were performed by one of the co-authors. (Y. Ohimoto).

Interferon sensitivity testing. Experiments comprising continuous exposure of tumor cells to nIFNα were performed by adding 0.1 ml nIFNα solution at a final concentration ranging from 5 IU/ml to 500 IU/ml in both the upper and feeder layer agarose. Control cultures were incubated with addition of 0.1 ml PBS alone. The resulting colony growth was expressed as a percentage of that in control cultures. In order to distinguish the direct and indirect antiproliferative activities of nIFNα, direct activity was defined as the percentage of colony growth in the absence of feeder cells, while combined direct and indirect activity was defined as the percentage of colony growth in the presence of feeder cells. All experiments were done in triplicate.

Table 1. Effect of feeder cells on antiproliferative activity of natural interferon α (nIFNα)

<table>
<thead>
<tr>
<th>nIFNα (IU/ml)</th>
<th>Colony survival (%) with feeder cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Monocytes#</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>95 ± 10</td>
</tr>
<tr>
<td>50</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>500</td>
<td>34 ± 4</td>
</tr>
</tbody>
</table>

# Applied as a continuous exposure to both layers of cultures

* Mean ± SD of three separate experiments done in triplicate

* Obtained from healthy donors

* Significant inhibition (P < 0.05) of colony growth rate compared to that without feeder cells

Source of interferon. IFN used in this study was highly purified natural IFNα (nIFNα, specific activity 1 x 10¹⁵ IU/mg protein) produced by stimulating human lymphoblast cells (BALL-1) with Sendai virus and consisting of three components or subtypes (73%, α2, 2% α7 and 25% α8). This material was kindly supplied by Ohtsuka Pharmaceutical Co., (Tokushima, Japan), and was free of endotoxin.

Anti-TNFα monoclonal antibody. Neutralizing monoclonal antibody against human recombinant TNFα was kindly supplied by Cellular Technology Institutes (Tokushima, Japan). The neutralizing titer of anti-TNFα monoclonal antibody was more than 3 x 10⁴ units/mg (1.5 x 10⁶ pg/mg). The purity as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis is more than 90%. No detectable reactivity was observed with human IL-1, TNFβ, IL-2, IL-3, IL-4, IL-6, granulocyte/macrophage-colony-stimulating factor (GM-CSF), G-CSF, M-CSF, INFα and INFγ by immunoblot.

Results

The influence of feeder cells on colony growth was investigated first (Table 1). nIFNα inhibited colony growth of ACHN cells in a dose-dependent manner without feeder cells at concentrations ranging from 5 IU/ml to 500 IU/ml. If either monocytes or lymphocytes were added as feeder cells, nIFNα exposure at concentrations ranging from 5 IU/ml to 500 IU/ml did not significantly reduce colony growth compared with that observed in non-feeder-cell cultures, except that colony numbers were reduced signifi-