CYTOTOXIC EFFECTS OF HUMAN ALPHA INTERFERON AGAINST HUMAN RENAL CANCER CELL LINE (KU-2)

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ABSTRACT

Reactivity of natural killer (NK) cells to an established human renal cancer cell line (KU-2) was determined by cellular immunoadsorbant technique, utilizing human leukemia cell line (CCRF-CEM) which is highly sensitive to NK cells. The influence of human alpha interferon (HuIFN-α) on NK activity against KU-2 cells was also determined by microcytotoxicity assay. Percent cytotoxicity increased 84%, 52% and 59% in non E-rosette forming (non ERF) lymphocytes, ERF lymphocytes and unseparated peripheral blood lymphocytes (PBL), respectively, under effector cells/target cells ratio being 25/1 and preincubation with 150 IU/ml HuIFN-α. The results indicated that more marked influence of HuIFN-α upon non ERF lymphocytes was expected than ERF lymphocytes.

Identification of the effector lymphocytes under influence of HuIFN-α was undertaken by scanning electron microscopic observation. Lymphocytes characterized by relatively smooth surface and short microvilli were observed to be adherent to the surface of the KU-2 cells showing destruction of their morphological integrity.

To determine the direct cytotoxic effects, the KU-2 cells were exposed to HuIFN-α. When exposed to 50 IU/ml HuIFN-α, cellular doubling time extended from 23 hrs to 38 hrs. With more than 200 IU/ml, the growth was acceptably inhibited and no dose dependent effect on the growth curves was observed in large dose series.

From the results of in vitro studies, it was determined that both NK activation and direct inhibitory mechanisms were involved in the growth inhibitory effect of HuIFN-α against KU-2 cells. HuIFN-α was suggested to play an important role in a multimodal treatment of human renal cell carcinoma.

INTRODUCTION

The anticancer effects of interferon (IFN) have been shown by i) anti-
viral inhibition of the synthesis of viral nucleic acid and cell transformation,\(^1\) ii) inhibition of cell proliferation,\(^2\) and iii) activation of the immune mechanism,\(^2\) i.e. by enhancing activity of natural killer (NK) cells. NK cells have been considered to play a role in immunesurveillance. Research groups in Urology at School of Medicine, Keio University have studied to prove whether an established human renal cancer cell line (KU-2) is sensitive to NK cells by cellular immunoabsorbant technique and to find out whether activity of NK cells is enhanced by human alpha interferon (HuIFN-\(\alpha\)), based on microcytotoxicity assay and identification of effector lymphocytes by scanning electron microscope (SEM).

Direct inhibitory effect of HuIFN-\(\alpha\) on the growth of KU-2 cells was also studied.

**MATERIALS AND METHODS**

**Target cells**

KU-2, an established human renal cancer cell line, was used as the target cells. KU-2 was originated from a pulmonary metastatic lesion of hypernephroma capable of producing erythropoietin. After having been transplanted to nude mice, it was established as a cell line in the September, 1975.\(^3,4\)

**Lymphocyte preparation**

Peripheral blood lymphocytes (PBL) were donated from healthy adult volunteers (seven males and one female, age ranging from 20 to 38). Heparinized whole blood was treated with carbonyl iron powder and agitated for 30 min in 37\(^\circ\)C water bath in order to eliminate phagocytic cells. Blood obtained under the above conditions was centrifuged over Ficoll-Conray according to the method of Boyum (PBL-1).\(^5\) PBL were divided into fractions of non E-rosette forming (non ERF) cells and ERF cells using neuraminidase treated sheep red blood cells (SRBC) at room temperature.

**Interferon**

HuIFN-\(\alpha\) was obtained from Dr. S. Kohno in the Japanese National Institute of Health. Specific activity of the HuIFN-\(\alpha\) was \(1\times10^8\) IU/mg protein.

**Cytotoxicity test**

The microcytotoxicity assay, developed by Takasugi and Klein\(^6\) was used. Five-hundred KU-2 cells suspended in 10 \(\mu\)l MEM with 10% FCS were seeded on wells in Terasaki tissue culture plate (Falcon, Oxnard, Ca. No. 3034) and 3 hrs later lymphocytes were placed on the KU-2 cells at EC/TC ratio of 25/1 and incubated in humidified 5% CO\(_2\) at 37\(^\circ\)C for 48 hrs. Non viable KU-
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2 cells and lymphocytes were washed off with normal saline solution. Fixation of the attached cells was performed with methanol, and Giemsa stain was carried out. The number of attached cells was counted.

Percent cytotoxicity was calculated by the formula written below.

\[
\% \text{ cytotoxicity} = \frac{A - B}{A} \times 100
\]

where A: Attached KU-2 cells incubated without lymphocytes.
B: Attached KU-2 cells incubated with lymphocytes.

Cellular immunoadsorbant technique

CCRF-CEM, human leukemia cell line known to be highly sensitive to NK cells, was washed in PBS, left in PBS with 0.2\% formaldehyde at 4°C at 5×10^6 cells/ml, and washed in PBS 6 times. One ml of poly-L-lysine (Sigma Chemical Co. St. Louis, Mo., No. p1865, m.w. 70,000) in the concentration of 50 µg/ml was put in 60 mm polystyrene tissue culture plates, and left at room temperature for 60 min, and washed 3 times in PBS. In the poly-L-lysine coated plates thus prepared 2 ml of CCRF-CEM cells at 6×10^6 cells/ml was put and was left at room temperature for 30 min. Then 2 ml of PBS with 0.2\% formaldehyde was added, and left at 4°C for 1 hr. Then the plates were washed in PBS gently to prevent the CCRF-CEM cells from being detached off the plates, and left at room temperature for 60 min. Then PBL of 2 ml at 6×10^6 cells/ml in MEM with 10\% FCS were added to the plates. The plates were left for 60 min at 37°C and then they were gently agitated. The supernatant was decanted and the floating cells in the supernatant were washed by MEM with 10\% FCS (PBL-2). Two kinds of PBL (PBL-1 and PBL-2) were put on KU-2 cells seeded on the wells of tissue culture plates at EC/TC ratio of 25/1, and cytotoxicity was determined by microcytotoxicity assay.

Effect of HuIFN-α on NK cells

After adding HuIFN-α to the unseparated PBL and two PBL subpopulations namely, ERF cells and non ERF cells, they were kept still at 37°C for 60 min, and washed in PBS 3 times. Comparative determination of cytotoxicity by PBL and two different subpopulations of lymphocytes against KU-2 cells was carried out by microcytotoxicity assay with EC/TC ratio adjusted to 25/1, at three different concentrations of HuIFN-α; i) 10 IU/ml, ii) 50 IU/ml, iii) 150 IU/ml and controls.

Identification of effector lymphocytes by scanning electron microscope

Ten mm square cover glasses were placed at the bottom of a Lab-Tek tissue culture chamber slide (Miles, Naperville, Ill., No. 4902), where KU-2
cells were incubated for 48 hrs. PBL treated with HuIFN-α 150 IU/ml for 60 min or untreated PBL were then put on the KU-2 cells. Twenty-four hrs later, observation under light microscope was made and MEM was removed very slowly through a #23 gauge needle, then fixed for 2 hrs with 2.5% glutaraldehyde. Then observation under SEM was made.

Effect of HuIFN-α on growth of KU-2 cells

KU-2 cells were grown in 24-well tissue culture plate (Nunc, Kamstrupvej, 90, Kamstrup, DK-40000, Roskilde, Denmark, No. 1-68357). Forty-eight hrs after seeding KU-2 cells (1×10⁴ cells) in each well with 1 ml MEM with 10% FCS, HuIFN-α was added at different concentration of i) 10 IU/ml, ii) 50 IU/ml, iii) 200 IU/ml, iv) 500 IU/ml and v) 5,000 IU/ml, and at each concentration a growth curve was determined.

RESULT

Cytotoxicity of PBL and CCRF-CEM non adherent PBL against KU-2 cells

Monolayers of CCRF-CEM cells fixed with formaldehyde partially reduced NK activity of human lymphocytes against KU-2 cells. As shown in Table 1, percent cytotoxicity against KU-2 cells of PBL, non adherent to the monolayer, was 25.0% (±10.9%). On the other hand percent cytotoxicity of unseparated control PBL was 46.7% (±6.7%).

This result indicates that the non adherent fraction of PBL had only a 53.5% cytotoxic activity against KU-2 cells when compared to the unseparated PBL.

Table 1

<table>
<thead>
<tr>
<th>% cytotoxicity at EC/TC cell ratio of 25:1 and PBL/CCRF-CEM ratio of 1:1</th>
<th>Reduced cell count</th>
<th>Reduction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>210.7±22.8*</td>
<td>46.7±6.7**</td>
</tr>
<tr>
<td>Non adherent PBL</td>
<td>108.4±29.2*</td>
<td>25.0±10.9**</td>
</tr>
</tbody>
</table>

Number of KU-2 cells as control: 462.4 ± 125.6
* p < 0.01 ** p < 0.05

Effect of HuIFN-α on NK cells

As shown in Table 2, without HuIFN-α treatment, the cytotoxicity rates
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Table 2
% cytotoxicity of ERF-cells and non ERF-cells treated with HuIFN-α at EC/TC of 25/1

<table>
<thead>
<tr>
<th>Lymphocyte</th>
<th>HuIFN-α</th>
<th>Reduction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERF-cells</td>
<td>0</td>
<td>29.3±14.3</td>
</tr>
<tr>
<td></td>
<td>10 IU/ml</td>
<td>29.0±9.6</td>
</tr>
<tr>
<td></td>
<td>50 IU/ml</td>
<td>36.4±14.6</td>
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<tr>
<td></td>
<td>150 IU/ml</td>
<td>44.8±8.7</td>
</tr>
<tr>
<td>Non ERF-cells</td>
<td>0</td>
<td>35.9±13.8</td>
</tr>
<tr>
<td></td>
<td>10 IU/ml</td>
<td>39.4±9.6</td>
</tr>
<tr>
<td></td>
<td>50 IU/ml</td>
<td>62.2±11.9</td>
</tr>
<tr>
<td></td>
<td>150 IU/ml</td>
<td>66.0±10.2</td>
</tr>
<tr>
<td>PBL</td>
<td>0</td>
<td>41.2±7.9</td>
</tr>
<tr>
<td></td>
<td>10 IU/ml</td>
<td>43.2±8.1</td>
</tr>
<tr>
<td></td>
<td>50 IU/ml</td>
<td>63.8±11.6</td>
</tr>
<tr>
<td></td>
<td>150 IU/ml</td>
<td>65.4±10.3</td>
</tr>
</tbody>
</table>

Fig. 1 Phasecontrast microscopic observation of cytotoxic changes in KU-2 cells by lymphocytes. Most of KU-2 cells show shrinkage and destruction but some others tend to form sheet attached to the cover glass.
were 35.9% (± 13.8%), 29.3% (± 14.3%) and 41.2% (± 7.9%) in the non ERF cells, ERF cells and unseparated PBL, respectively. On the other hand, with HuIFN-α treatment, at a concentration of 150 IU/ml, the reduction rate increased to 66.0% (± 10.2%) in non ERF cells, 44.8% (± 8.7%) in ERF cells and 65.4% (± 10.3%) in PBL, which were an 84%, 52%, and 59% increase, respectively when compared with the non HuIFN-α treated series. At IFN levels of 10 IU/ml, marked NK activation was not observed, but at IFN levels of 50 IU/ml NK activity was increased 73.3%, 24.2% and 54.9% in non ERF cells, ERF cells and unseparated PBL, respectively. From these results optimal NK activation was observed at HuIFN-α concentrations of more than 50 IU/ml. A greater increase in NK activation by HuIFN-α was noted in non ERF cells when compared with ERF cells and unseparated PBL. From phasecontrast microscopic observation most of KU-2 cells incubated with PBL which were not treated with HuIFN-α showed shrinkage and destruction but some others tended to form sheets attached to the cover glass (Fig. 1). On the other hand, KU-2 cells incubated with HuIFN-α treated PBL became spherical, losing signs of viable cells and to be detached from the cover glass (Fig. 2).

Under scanning electron microscopic observation, morphological integrity

Fig. 2 Phasecontrast microscopic observation of cytotoxic changes in KU-2 cells by lymphocytes preincubated with HuIFN-α 150 IU/ml for 60 min. Most of KU-2 cells are spherical with attaching lymphocytes and to be detached from the cover glass.
Fig. 3 Scanning electron microscopic demonstration of KU-2 cells and lymphocytes. Morphological integrity of KU-2 cells is well preserved.

Fig. 4 Scanning electron microscopic demonstration of KU-2 cells and effector lymphocytes preincubated with HuIFN-α 150 IU/ml for 60 min. Morphological integrity of KU-2 cells is disturbed.
of KU-2 cells was lost when PBL was treated with HuIFN-α, whereas it was well preserved when PBL was not treated with HuIFN-α. Furthermore these lymphocytes were characterized by relatively smooth surface and short microvilli (Fig. 3, 4).

**Effect of HuIFN-α on the growth of KU-2 cells**

The effect of HuIFN-α on the growth of KU-2 cells was determined by counting the number of attached cells daily. Table 3 and Fig. 5 show the growth of KU-2 cells in the presence of different concentrations of HuIFN-α. Growth inhibition of HuIFN-α on KU-2 cells depended on the concentrations of HuIFN-α, at low concentrations (from 10 IU/ml to 200 IU/ml) (P<0.002). Doubling time of the cells was lengthened from 23 hrs, in the control culture, to 38 hrs at 50 IU/ml of HuIFN-α. At 200 IU/ml, growth of the KU-2 cells was virtually arrested. Concentrations of HuIFN-α at 500 IU/ml slowed the growth rate of KU-2 cells somewhat more but this change was not statistically significant.

When KU-2 cells were exposed to HuIFN-α (from 50 IU/ml to 200 IU/ml), cellular growth was inhibited but degenerative changes in morphology under light microscope were not recognized and the cells were maintained as cell sheets.
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Fig. 5 Effect of HuIFN-α on the growth of KU-2 cells. Cells were seeded at $10^4$ for each well (1.7 cm in diameter), and 48 hrs later fresh medium was added with HuIFN-α at the concentrations indicated. Medium with HuIFN-α was changed every 2 days.

DISCUSSION

There have been many reports suggesting the presence of an immunological relationship between the host and tumor as biological characteristics of renal cell carcinoma such as spontaneous regression of metastatic sites after removal of primary lesion and extremely late recurrence following radical nephrectomy. These studies have provided the impetus for continued trials of immunotherapy, and several agents are being studied. IFN has been expected three anticancer mechanisms, namely antiviral effects, activation of the immune mechanisms and direct inhibitory effect of cellular proliferation. Furthermore, many evidences have been accumulated showing that there is augmentation of NK activity by IFN. The previous report indicated that KU-2 cells were susceptible to the cytotoxic effect of NK cells, and this was reconfirmed by means of immunoadsorbant technique in this study. The study revealed PBL that were non-adherent to a monolayer of highly NK sensitive
CCRFC-CEM cells, had only a 53.5% cytotoxic activity when compared to the unseparated PBL which acts as the control. This reduction in cytotoxicity can be explained by a hypothesis that NK recognition sites on the surface of effector lymphocytes could interact with a NK target antigen on both CCRF-CEM cells and KU-2 cells. If both CCRF-CEM cells and KU-2 cells possessed one identical NK target antigen, this would result in selective depletion of NK effector cells from the PBL population by preincubation with CCRF-CEM cells.

Further studies were undertaken to see if there were effects of IFN upon NK activity with the hypothesis that KU-2 cells had NK target antigen. The results showed that pretreatment of effector lymphocytes with HuIFN-α increased cytotoxicity in each fraction of non ERF cells, ERF cells and unseparated PBL, most in non ERF fraction which potentially included NK cells. Controversial results in augmented spontaneous cytotoxicity due to the effect of IFN on the effector cells and/or the target cells have been reported by some investigators. Among these studies, recent report by Einhorn showed that induced enhancement of spontaneous cytotoxicity was due to effects on the effector cells rather than on the target cells since preincubation of the lymphocytes with IFN augmented spontaneous cytotoxicity, whereas preincubation of the target cells with IFN did not increase their susceptibility to spontaneous cytotoxicity. His research group also reported the highest cytotoxicity in non ERF cells. The results from present studies are consistent with Einhorn’s reports, however, characterization of effector cells in human spontaneous cytotoxicity is still a matter of controversy and it seems to be valid that a population of NK cells express low avidity receptor for sheep erythrocytes under optimal thermal condition in contrast with the hitherto reported observation that a population of NK cells lack conventional T and B markers. Observed characteristics of effector cells by SEM in this study resembled to T cells, and from recent report that NK cells are prethymic T cells and further differentiation under the influence of the thymus results in thymic and post thymic T cells in which NK activity is no longer expressed or expressed only low level, characteristics of the effector cells observed under SEM coincide with the results based on microcytotoxicity assay.

On the other hand it has been reported that cell multiplication of mouse L-cell was inhibited by an IFN preparation and more recently that IFN inhibits the multiplication of a variety of malignant and non malignant cells, while the sensitivity is varied. In the present study, the cell doubling time of KU-2 cells was extended by incubation with 50 IU/ml of HuIFN-α, while at the concentration from 200 IU/ml to 5,000 IU/ml, no significant dose dependency was observed. Furthermore no remarkable change, morphological-
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ly under light microscope, was observed even at 5,000 IU/ml HuIFN-α. Little
is known about the mechanisms of inhibition of cell multiplication, but it has
been known that IFN lengthen the cell cycle time. Killander and his group found
that IFN lengthened all phases of the cell cycle in asynchronously dividing
mouse leukemia cell lines, however, Balkwill and Taylor-Papadimitriou found
that the G1 and S+G2 phases are elongated in quiescent fibroblasts stimulated to divide. Mataress and Rossi found that the G1 and G2 phases were lengthened in IFN-treated Friend leukemia cells. Also Fuse and Kuwata, Sokawa and his group reported that IFN only affected the G1 phase in human and mouse leukemia cells stimulated from quiescence. In this study, the mechanisms of direct effect of HuIFN-α to KU-2 cells was not confirmed, however, it was found that HuIFN-α elongated the cell doubling time at 50 IU/ml or more of HuIFN-α and the effect was cytostatic.

From this study two different ways of cytotoxic effect of HuIFN-α against KU-2 cells were determined by an in vitro model of human renal cell carcinoma. Anticancer effects of IFN can be expected in the patients with renal cell carcinoma, when the cancer cells are NK sensitive. However, even if NK cells are activated or recruited by IFN, only limited number of cancer cells will be killed because of limited number of NK cells in the host. On the other hand, the direct effect of HuIFN-α to KU-2 cells was cytostatic effect while the most of the conventional anticancer agents had cytocidal effect so that a rapid response by IFN would not be expected. Furthermore, the result from the present study that the growth of KU-2 cells were significantly inhibited by 50 IU/ml HuIFN-α and the fact that the serum IFN levels more than 50 IU/ml was retained for 12 hrs or more after single intramuscular injection of $3 \times 10^6$ IU might indicate an optimal dosis in patients with renal cell carcinoma. Referring these sluggish cytotoxic effects by two different mechanisms, cell reduction surgery is recommended before initiation of HuIFN-α therapy. Irradiation and chemotherapy should be studied as combination with IFN, not only on the aspect of growth kinetics but also broadening anticancer spectrum, biological synergism and reduction of additive toxic effects.

CONCLUSION

Using established human renal cancer cell line KU-2 as a target, cytotoxic effects of HuIFN-α was studied from two different mechanisms, namely NK activation and direct inhibition of cell multiplication, and following results were obtained.

1) From immunoadsorbant technique, it was suggested that KU-2 and highly NK sensitive human leukemia cell line CCRF-CEM possessed identical
NK target antigen.

ii) NK activity against KU-2 cells was increased 84%, 52% and 59% in non ERF cells, ERF cells and unseparated PBL, respectively, by preincubation of effector cells with 150 IU/ml HuIFN-α, under EC/TC ratio being 25/1. The NK activity of non ERF cells was significantly higher than that of ERF cells, at each concentration of 50 IU/ml and 150 IU/ml HuIFN-α.

iii) Observed characteristics of effector lymphocytes under influence of HuIFN-α was relatively smooth surface and short microvilli, which resembled to T cell.

iv) Growth inhibitory effect of HuIFN-α on KU-2 cells were observed at low concentrations up to 200 IU/ml as dose dependent. At higher dosis of HuIFN-α over 500 IU/ml, there was cytostatic.

v) HuIFN-α was suspected to play an important role in a multimodal treatment of human renal cell carcinoma.

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