The Synergistic Effect of Human Recombinant Interferon-α2a in Combination with Interferon-γ and the Induction of Interferon-α2a Receptor by Interferon-γ

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(Received December 6, 1988)

An in vitro examination was made of the synergistic antiproliferative effect of recombinant interferon-γ (IFN-γ) with interferon-α2a (IFN-α2a) on human tumor cell lines, including lung small cell carcinoma QG-90, amelanotic melanoma HMV-1, renal carcinoma ACHN and Burkitt lymphoma Daudi. Sixty-four percent of HMV-1 cells and 75% of ACHN cells seeded were killed by treatment with 10 pm and 1000 pm of IFN-γ, respectively. Furthermore, 69% of HMV-1 cells and 59% of ACHN cells were killed by 1000 pm of IFN-α2a. When HMV-1 was treated first with 10 pm of IFN-γ, and then with 1000 pm of IFN-α2a, 81% of cells seeded were killed (p < 0.001). Similarly, 89% of ACHN cells were killed by the same sequential treatment with 1000 pm IFN-γ, and then of IFN-α2a (p < 0.001). However, adverse sequence of treatment could not produce such a synergistic result. On the surface of these susceptible cells, HMV-1 and ACHN, to both IFNs, the number of receptors for IFN-α2a increased significantly after treatment with IFN-γ without any serious change of the dissociation constant, suggesting that increase in the number of receptors for IFN-α2a may be the major mechanism of the synergistic effects of IFN-γ with IFN-α2a.

Keywords — interferon-γ; interferon-α2a; synergistic effect; interferon receptor; human tumor

Introduction

Interferons (IFNs) have multiple functions on various types of mammalian cells.1–6 Some biological and/or biochemical interactions between IFNs and cells are triggered by binding of IFNs to their specific receptors on the cell membrane,9,10 though the precise mechanisms of expression of receptors for IFNs remain still unclear. Many investigators are now progressively studying the character of IFN-receptors and biochemical mechanisms of cell-killing after the binding of IFNS to the receptors. We also know that IFNs induce common sets of cellular proteins: in particular, the inducible activity of IFN-γ is stronger than those of IFN-α and -β.11–13 Furthermore, IFN-γ exhibits synergistic effects with several other lymphokines, including IFN-α, IFN-β, interleukin-2, TNF-α and -β.14–17 It is said that increase in the number of TNF-receptors on the cell membrane primed with IFN-γ is the major mechanism of the synergistic antiproliferative effect of IFN-γ and TNF-α.18,19

The present paper deals with the synergistic antiproliferative effects of IFN-γ and IFN-α2a on human tumor cell lines, and discusses the priming effect of IFN-γ on expression of IFN-α2a receptors on the surface of tumor cells.

Materials and Methods

Cell Cultures — Four human tumor cell lines, including lung small cell carcinoma QG-90, amelanotic melanoma HMV-1, renal adenocarcinoma ACHN and Burkitt lymphoma Daudi were used in this study. QG-90 was kindly supplied by Ida, Basic Research Laboratories, Toray Ind., Inc., Kamakura, Japan and HMV-1 was generously given by Sekiguchi, Department of Clinical Oncology, Institute of Medical Science, University of Tokyo, Japan. ACHN and Daudi were obtained from Flow Laboratories Inc., Virginia, USA. The doubling times of QG-90, HMV-1, ACHN and Daudi are about 31.2, 25.8, 27.6. and 30 h respectively. These cells were cultivated at 37 °C in a 5% CO2-95% air humidified incubator with RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 100 μg/ml of kanamycin sulfate (Banyu Pharmaceutical Co., Ltd., Tokyo), 2 mM L-glutamine (Nissui
Seiyaku Co., Ltd.) and 10% (v/v) heatinactivated fetal calf serum (Mitubisi Chemical Ind., Ltd., Tokyo): this medium was designated as the culture medium.

**IFNs** — Preparations of highly purified recombinant human IFN-γ\(^{20}\) and IFN-α\(_{2a}\)\(^{21}\) were supplied by Genentech, Inc., Calif., USA and Nippon Roshe Co., Ltd., Kamakura, Japan, respectively. Their antiviral activities were measured by the plaque reduction test with FL cell and Sindbis Virus: the specific antiviral activities of IFN-γ and IFN-α\(_{2a}\) were calculated as 41 and 200 units/ng protein, respectively.

**Growth Inhibition Test** — A cell suspension of 1 × 10\(^4\) cells per ml of culture medium of ACHN, HMV-1, QG-90 or Daudi were seeded into 24 well plates (Nunc Inc., Roskilde, Denmark) and cultivated at 37 °C in a humidified atmosphere of 5% CO\(_2\) in air. Twenty-four hours later, ACHN and Daudi were treated with 1 ml of 1 nM IFN-γ or IFN-α\(_{2a}\), HMV-1 and QG-90 were treated with 1 ml of 10 pM IFN-γ or 1 nM IFN-α\(_{2a}\). As controls, these cells were cultivated with the culture medium without any IFN. Two days after the start of incubation in culture, medium with or without IFNs, the cells in each well were rinsed three times with phosphate-buffered saline (PBS), and then cultivated with medium supplemented with the same amount of IFN-γ or IFN-α\(_{2a}\) for a further 4 d. On days 1, 3, 5 and 6 post treatment, the number of cells were measured using a Coulter Counter Model ZBI (Coulter Electronics Inc., Hialeah, FL) (the test was triplicated). The inhibition ratio was calculated by the following formula:

\[
\text{% inhibition} = \left(1 - \frac{\text{cell number of the treated group}}{\text{cell number of the control group}}\right) \times 100
\]

**Radioiodination of IFN-α\(_{2a}\)** — IFN-α\(_{2a}\) was radioiodinated with \(^{125}\)I-labeled Bolton-Hunter reagent (New England Nuclear, Boston, MA, USA).\(^{22}\) Fifty μl of IFN-α\(_{2a}\) solution [1 × 10\(^7\) units (50 μg)] was allowed to react on ice with 1 mCi of Bolton-Hunter reagent (4400 Ci/mmol) for 1 h, and 0.5 ml of 0.2 M glycine in 0.1 M borate buffer (pH 8.5) was then added to the reaction mixture. The mixture was then fractionated by gel filtration with Sephadex G-25 column (0.8 × 25 cm; Vo, 0.25 ml/min; 4.5 ml) using PBS containing 1% (w/v) bovine serum albumin, and 1.0 ml quantities of effluent were collected. After appropriate dilution of each fraction with PBS, the radio-activity of each dilution was measured using a gamma counter Model DP5500 (Beckman Instruments, Inc., USA): the counting efficiency for \(^{125}\)I was about 100%. The antiviral activity of the diluted fractions were also assayed by the above procedure: the recovery yield of radioactivity IFN-α\(_{2a}\) was approximately 100%. The initial specific activities of radioactive preparation of IFN-α\(_{2a}\) was 8800 cpm/ng, and this preparation was stored at 4 °C until use.

**Binding Assay** — Confluent monolayers of ACHN, HMV-1 and QG-90 (1.4 to 4.6 × 10\(^6\) cells/35 mm dish) and suspension culture of Daudi (2.0 × 10\(^6\) cells/35 mm dish) were pre-treated with 1 nM (ACHN and Daudi) or 10 pM (HMV-1 and QG-90) of IFN-γ for 2 d, and then cultivated with culture medium supplemented with \(^{125}\)I-IFN-α\(_{2a}\) (1.5635—50 ng/ml) for a further 120 min at 4 °C. The monolayer cultures were then scraped with a “rubber policeman” and pipetted. Each cell suspension was layered onto 2 ml of a cold sucrose solution (0.25 M sucrose, 10 mM tris-HCl, pH 7.5, 5.2 mM MgCl\(_2\)) and centrifuged at 500 × g at 4 °C. After the supernatant fluid was removed with a pipet, the radioactivity of cells was counted using the gamma counter. The number of binding sites of IFN-α\(_{2a}\) to each cell line and their apparent dissociation constants (K\(_d\)) were estimated by Scatchard plotting analysis.\(^{23}\) The specific binding was defined as the difference between the total binding and nonspecific binding occurred in the presence of a 100-fold excess of unlabeled IFN-α\(_{2a}\).

**Results**

**Synergistic Effect**

The antiproliferative effects of IFN-γ and IFN-α\(_{2a}\) on four cell lines are summarized in Table I. Percent inhibition of growth with 10 or 1000 pM of IFN-γ or with 1000 pM of IFN-α\(_{2a}\) was different from cell to cell. Thirty-seven to
TABLE I. Antiproliferative or Killing Activities of Combination of IFN-α2a with IFN-γ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACHN</td>
</tr>
<tr>
<td>Medium → Medium</td>
<td>0</td>
</tr>
<tr>
<td>Medium → IFN-α2a</td>
<td>27</td>
</tr>
<tr>
<td>Medium → IFN-γ</td>
<td>27</td>
</tr>
<tr>
<td>IFN-α2a → Medium</td>
<td>54</td>
</tr>
<tr>
<td>IFN-γ → Medium</td>
<td>54</td>
</tr>
<tr>
<td>IFN-α2a → IFN-α2a</td>
<td>59</td>
</tr>
<tr>
<td>IFN-α2a → IFN-γ</td>
<td>73</td>
</tr>
<tr>
<td>IFN-γ → IFN-α2a</td>
<td>89</td>
</tr>
<tr>
<td>IFN-γ → IFN-γ</td>
<td>75</td>
</tr>
</tbody>
</table>

a) Sequential treatment was performed as described in the text. ACHN and Daudi were treated with 1000 pm of IFN-γ and 1000 pm of IFN-α2a. HMV-1 and QG-90 were treated with 10 pm of IFN-γ and 1000 pm of IFN-α2a.

b) Significant difference from all other groups at the level of 0.1%.

58% of ACHN and HMV-1 cells susceptible to both IFNs were killed by each IFN alone, and much more cells were killed by the second treatment with IFN-γ or IFN-α2a (resulted in 59 to 73% inhibition). In case of sequential treatment with IFN-γ and IFN-α2a (IFN-γ → IFN-α2a), 89% of ACHN cells were killed (p < 0.001), against 54% and 73% was killed by IFN-α2a and IFN-γ alone, respectively. This synergistic effect of the sequential treatment (IFN-γ → IFN-α2a) was statistically significant at the level of 0.1% against all other combinations (Table I). The reverse sequence of treatment (IFN-α2a → IFN-γ) showed no such a synergistic effect (73% inhibition; significantly less active than IFN-γ → IFN-α2a). Against HMV-1 cells, the

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Fig. 1. Effects of IFN-γ on the Binding of 125I-IFN-α2a to Tumor Cell Lines

Cells were incubated in the presence (O) or absence (●) of 10 pm (HMV-1 and QG-90) or 1 nm (ACHN and Daudi) of IFN-γ for 48 h at 37 °C. 125I-IFN-α2a binding to the cells was measured by gammacounter as described in the text.
TABLE II. Priming Effect of IFN-γ on the Expression of IFN-α2a Receptors on the Surface of Tumor Cell Lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Priming with IFN-γ</th>
<th>IFN-α2a receptors (Scatchard analysis)</th>
<th>( K_d ) (10(^{-10}) M)</th>
<th>Sites/cell</th>
<th>Ratio (^a)\</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACHN</td>
<td>—</td>
<td>4.4</td>
<td>840</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.5</td>
<td>1,700</td>
<td>202</td>
<td>—</td>
</tr>
<tr>
<td>HMV-1</td>
<td>—</td>
<td>2.0</td>
<td>300</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.4</td>
<td>600</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
<td>Daudi</td>
<td>—</td>
<td>3.4</td>
<td>800</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.9</td>
<td>780</td>
<td>98</td>
<td>—</td>
</tr>
<tr>
<td>QG-90</td>
<td>—</td>
<td>9.3</td>
<td>240</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9.3</td>
<td>160</td>
<td>67</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Percent of the number of receptors on the cells without priming with IFN-γ.

sequential treatment of IFN-γ and IFN-α2a (IFN-γ → IFN-α2a) showed the same effect: 81% of cells seeded were killed. Against QG-90 susceptible to IFN-γ, but resistant to IFN-α2a, or against Daudi susceptible to IFN-α2a but not IFN-γ, no such combined (synergistic) effect of the sequen
tial treatment (IFN-γ → IFN-α2a) was observed.

Increase in the Number of Binding Sites of 125I-IFN-α2a by Priming with IFN-γ

The priming effect of IFN-γ on the expression of binding sites for 125I-IFN-α2a in four cell lines are shown in Fig. 1. The intensity of specific binding of 125I-IFN-α2a (radio-counts) to ACHN cell increased with increase in priming dose of IFN-γ and reached saturation at 25 ng/ml. On the cells without priming with IFN-γ, the Scatchard analysis (Table II) of ACHN cells revealed that approximately 840 molecules per cell of binding sites for 125I-IFN-α2a were expressed with apparent \( K_d \) values of 4.4 × 10\(^{-10}\) M. On the cells primed with IFN-γ, on the other hand, the number of specific binding sites for 125I-IFN-α2a increased to 1700 with \( K_d \) a value of 7.5 × 10\(^{-10}\) M. Similarly, the number of binding sites for 125I-IFN-α2a on HMV-1 cells increased from 300 to about 600 molecules by priming with IFN-γ. In contrast, on both QG-90 and Daudi cells to which little synergistic antiproliferative effect of the sequen
tial treatment (IFN-γ → IFN-α2a) was observed, no increase in the number of binding sites for 125I-IFN-α2a was found by priming of these cell lines with IFN-γ. The number of binding molecules of 125I-IFN-α2a on Daudi cell was about 800 irrespective of priming with IFN-γ. On QG-90 cells, in contrast, the number of binding molecules of 125I-IFN-α2a decreased from 240 to 160 by pretreatment with IFN-γ.

Discussion

It is well known that IFN-γ exhibits antiproliferative or killing activity against tumor cells in combination with IFN-α, IFN-β, TNF-α or with TNF-β,\(^{14-17}\) and it regulates the expression of cell surface molecules, including receptors for TNF,\(^{18,19}\) Fc\(^\gamma\),\(^{24}\) and for the third component of complement (C3b).\(^{25}\) In the present study, an in vitro synergistic antiproliferative or killing effect of IFN-γ with IFN-α2a on two out of four tumor cell lines was found. ACHN and HMV-1 susceptible to both IFNs were killed significantly by sequential treatment with IFN-γ and then with IFN-α2a, and the number of receptors for IFN-α2a was increased by priming them with TFN-γ. In regard to both Daudi and QG-90, nonsusceptible to IFN-γ or IFN-α2a, no synergistic effect of the sequential treatment was observed. Susceptible cells to IFN-α2a have much more receptors for IFN-α2a\(^{29}\) Thus, these results suggest that the expression of IFN-α2a receptors on HMV-1 and ACHN cells may be regulated positively by treatment with IFN-γ. Little change in the dissociation constant (\( K_d \)) of IFN-α2a in HMV-1 and
ACHN cells were found, indicating no qualitative difference between former receptors and newly expressed receptors. On Daudi and QG-90 cells on which no synergistic killing effect of these IFNs was observed, little decrease in the number of IFN-α<sub>2a</sub> receptor was found. Baglioni et al. and Fuse et al. also made clear that the binding activity of IFN to cells which respond poorly to antiproliferative activity of IFN was less than that to highly susceptible cell lines. Tujimoto et al. observed that antiproliferative activity of the TNF was augmented by IFN-γ in accord with the increase in the number of TNF-receptors. Thus, we supposed that the synergistic antiproliferative effect of IFN-γ and IFN-α<sub>2a</sub> on ACHN and HMV-1 cells might be attributed to the augmentation of expression of IFN-α<sub>2a</sub> receptors on these cells by priming with IFN-γ. A decrease in the number of IFN-α<sub>2a</sub> receptors on QG-90 cells by priming with IFN-γ may be attributed to a certain damage of the cell surface by treatment with IFN-γ, because this cell line is highly sensitive to IFN-γ.

The intracellular biochemical or biomolecular reactions follow on the binding of IFNs to receptor molecules remain to be studied.

References

22) A. E. Bolton and W. M. Hunter: The labeling of proteins to high specific radioactivities by conjugation to a

