Tumor Degeneration by Human Embryonic Fibroblasts and Its Enhancement by Interferon

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ABSTRACT

KB cells from a human nasopharyngeal tumor were cocultivated with human embryonic fibroblasts (HF 8101 cells); 7 to 14 days after incubation, "spongy degeneration"-like changes developed in the target cell-growing area. These changes developed in other target cells [HeLa cells from human cervical cancer, human hepatoma cells (PLC/PRF/5), and human amnion FL cells] cocultured with several kinds of human embryonic fibroblasts [HF 8101 cells, HF 8103 cells, and HEL cells]; however, HF 8101 cells did not cause degenerative changes in murine L929 cells.

The degenerative changes were enhanced by treatment with human leukocyte interferon or human fibroblast interferon at a dose of 1,000 or 10,000 IU/ml, but there was no significant difference in the enhancing effect between human leukocyte and human fibroblast interferons. Mouse L929 interferon did not enhance the degenerative changes in KB cells caused by HF 8101 cells.

It was concluded that human fibroblasts caused the degenerative changes in the human tumor cells and the continuous cell line and that the changes were enhanced by treatment with either human leukocyte interferon or human fibroblast interferon.

INTRODUCTION

It is well known that IFN2 has antitumor activity. There are 2 manners in which IFN acts on tumor cells. One is a direct action by which IFN inhibits the cell growth (16, 18) or directly destroys the tumor cells (1, 10, 15). The other is an indirect action by which lymphocytes, including NK cells (7, 14, 19) and macrophages (11, 13, 17) activated by IFN, lyse the tumor cells or suppress their proliferation. It is possible that tumor cells are affected also by nonlymphoid cells, e.g., the fibroblasts which constitute an interstitial tissue or a capsule surrounding the tumor.

It is important to examine the interaction of tumor cells with fibroblasts to clarify the mechanism of the development of extension and the invasiveness of the tumor, because in general the well-encapsulated tumor is benign or less malignant.

We developed a method to determine the interaction of fibroblasts with tumor cells. The present study is intended to examine the influence of fibroblasts on tumor cells in vitro and the enhancing effect of IFN on the changes caused by the fibroblasts.

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: IFN, interferon; MEM, Eagle's minimum essential medium; HuIFN-α, human leukocyte interferon; HuIFN-β, human fibroblast interferon; MuIFN-α, β, mouse L929 interferon.

Received August 24, 1982; accepted June 8, 1983.

MATERIALS AND METHODS

Cells. KB cells from a human nasopharyngeal tumor, HeLa cells from human cervical cancer, hepatitis B virus surface antigen-producing human hepatoma cells (PLC/PRF/5), FL cells from human amnion cells, and murine L929 cells, which were grown and maintained with MEM (Nissui Co., Ltd., Tokyo, Japan) containing 5% newborn calf serum (M. A. Bioproducts, Maryland), were used for target cells. FL cells and L929 cells were also used for IFN assay and the propagation of vesicular stomatitis virus. Human embryonic fibroblasts (HF 8101 and HF 8103 cells) were grown and maintained with MEM containing 10% fetal bovine serum (M. A. Bioproducts).

IFN. HuIFN-α, which was prepared in human peripheral leukocytes infected with Sendai virus and purified by the method of Cantell and Hirvonen (3), was kindly supplied by Dr. Nita, Kyoto Red Cross Blood Center, Kyoto, Japan. Its specific activity was about 3 × 106 IU/mg of protein.

HuIFN-β, which was prepared in human foreskin fibroblasts treated with polyinosinic acid-polycytidylic acid and purified by the method of Carter and Horoszewicz (4), was kindly supplied by Dr. Nobuhara, Research laboratory of Cell Science, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan. Its specific activity was more than 1 × 107 IU/mg of protein.

MuIFN-α, β was prepared in murine L929 cells infected with Newcastle disease virus (Miyadera strain) and purified by polyuridylicate-agarose column chromatography. Its specific activity was more than 107 IU/mg of protein.

IFN Assay Method. Human IFN activity was assayed by the modified dye uptake method using FL cells and vesicular stomatitis virus (6). Murine IFN activity was assayed by the microassay method using L929 cells and vesicular stomatitis virus. The details were described elsewhere (8).

Method for Assay of Tumor Cell Degeneration by Fibroblasts. About 100 μl of tumor cell suspension of the concentration of 2 × 105 cells/ml was put into the stainless steel cup (4 mm inner diameter, 8 mm high) which was placed in the center of a plastic Petri dish (3 cm in diameter). Six or 24 hr after the incubation at 37°C in a 5% CO2 atmosphere, 2 ml of human fibroblast suspension were added to the target cell culture at a density of 2 to 4 × 106 cells/ml after the removal of the stainless steel cup, and this medium was incubated at 37°C in a 5% CO2 atmosphere. The cocultivated target cells and fibroblasts were grown and maintained with MEM containing 10% fetal bovine serum together with the appropriately diluted IFN solution or MEM. The medium was replaced at an interval of 3 days for 10 to 14 days.

After the incubation, the cells in the Petri dish were stained with Giemsa's solution, and the area of the target cells was photographed. The ratio (in percentage) of the destroyed area to the target cell-growing area in the IFN treatment and in control groups were evaluated by f test, and the difference in the ratios was considered to be significant when p < 0.05.

SEPTEMBER 1983

4323

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RESULTS

Degenerative Changes of Target Cells by Human Embryonic Fibroblasts. KB cells were put into the metal cup placed in the center of the plastic dish, and 6 or 24 hr later human embryonic fibroblasts (HF 8101 cells) were added to the KB cell culture. At an interval of 3 days, this medium was replaced by the fresh medium. About 7 days later, cell-free spotty areas developed where the target cell was destroyed. These areas appeared in the target KB cell-growing area but not in the surrounding fibroblast area at all. About 10 to 14 days after the coculture, the number and the size of the cell-free spotty areas increased and these changes were "spongy degeneration"-like (Fig. 1). Clustering, pyknotic changes of nucleus and the narrowing of cytoplasm were found in KB cells cocultured with human fibroblasts. Similar changes were found in the other human target cells: HeLa cells; human hepatoma cells; and FL cells. Also, there were no significant differences in the ratio of the destroyed areas to the target cell-growing area among KB, HeLa, and human hepatoma cells (Table 1). However, the target cells including KB cells, HeLa cells, human hepatoma cells, and FL cells cultivated in the central area or over the whole area of the plastic Petri dish without human embryonic fibroblasts did not show the same changes even if the overgrowth of KB cells developed. When murine L929 cells were used as target cells and cocultured with human fibroblast cells, no degenerative changes were observed.

The KB cells became degenerative also when cocultured with the other human embryonic fibroblasts (HF 8103 cells) or human embryonic lung cells (HEL cells) in place of HF 8101 cells (Table 2). Human fibroblasts ($1 \times 10^5$, $4 \times 10^5$, and $8 \times 10^5$/ml) were cocultured with KB cells growing in the center of plastic dishes, both with and without HuIFN-α (1,000 IU/ml). There was no significant difference in the ratio of the destroyed area to the target cell-growing area among the 3 different initial cell densities. HuIFN-α increased the ratio, but no difference in the ratio was found among different initial cell densities in the treatment with HuIFN-α.

Enhancing Effect of HuIFN-α on the Degenerative Changes of Target Cells by Human Fibroblasts. The IFN was added to the coculture of KB cells with human embryonic fibroblasts (HF 8101 cells) and incubated for 10 to 14 days, including the replacement of the medium containing IFN at an interval of 3 days. The ratio of the destroyed areas to the KB cell-growing area increased significantly by treatment with 1,000 or 10,000 IU of HuIFN-α per ml (Table 1). This enhancement was found also in HeLa cells, HEL cells, and human hepatoma cells cocultured with human embryonic fibroblasts treated with 1,000 or 10,000 IU of HuIFN-α per ml (Table 1). There seemed to be no significant differences in the enhancement of the degeneration among various human target cells. However, the enhancement of the degeneration by HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.

Various human embryonic fibroblasts (HF 8101 cells, HF 8103 cells) and HEL cells were cocultured with KB cells in the presence of various IFN concentrations (Table 1). The enhancement of the degeneration of L929 cells by the HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.

Table 1

<table>
<thead>
<tr>
<th>Target cell</th>
<th>IFN titer (IU/ml)</th>
<th>Ratio (%)</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>0</td>
<td>2.70 ± 1.45$^a$</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>8.13 ± 3.52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>11.10 ± 0.85</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HeLa</td>
<td>0</td>
<td>3.00 ± 1.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>5.13 ± 1.67</td>
<td>NS$^b$</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>9.48 ± 2.77</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FL</td>
<td>0</td>
<td>1.60 ± 0.14</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>5.90 ± 1.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>8.35 ± 1.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Human hepatoma</td>
<td>0</td>
<td>1.25 ± 0.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>0.93 ± 0.40</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>7.95 ± 5.63</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>17.08 ± 2.23</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>L929</td>
<td>0</td>
<td>0.70 ± 0.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>1.15 ± 0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>0.90 ± 0.59</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D.

$^b$ NS, not significant.

Table 2

<table>
<thead>
<tr>
<th>Human fibroblast</th>
<th>IFN (IU/ml)</th>
<th>Ratio (%)</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF 8103</td>
<td>0</td>
<td>10.70 ± 1.58$^a$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>17.30 ± 2.70</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>47.48 ± 3.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HEL</td>
<td>0</td>
<td>2.23 ± 1.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>2.93 ± 0.88</td>
<td>NS$^b$</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>9.58 ± 0.88</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D.

$^b$ NS, not significant.

Fig. 1. Degenerative changes in the KB cells cocultured with human embryonic fibroblasts (HF 8101 cells) and incubated for 10 to 14 days, including the replacement of the medium containing IFN at an interval of 3 days. The ratio of the destroyed areas to the KB cell-growing area increased significantly by treatment with 1,000 or 10,000 IU of HuIFN-α per ml (Table 1). This enhancement was found also in HeLa cells, HEL cells, and human hepatoma cells cocultured with human embryonic fibroblasts treated with 1,000 or 10,000 IU of HuIFN-α per ml (Table 1). There seemed to be no significant differences in the enhancement of the degeneration among various human target cells. However, the enhancement of the degeneration by HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.

Various human embryonic fibroblasts (HF 8101 cells, HF 8103 cells) and HEL cells were cocultured with KB cells in the presence of various IFN concentrations (Table 1). The enhancement of the degeneration of L929 cells by the HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.

 Various human embryonic fibroblasts (HF 8101 cells, HF 8103 cells) and HEL cells were cocultured with KB cells in the presence of various IFN concentrations (Table 1). The enhancement of the degeneration of L929 cells by the HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.

Various human embryonic fibroblasts (HF 8101 cells, HF 8103 cells) and HEL cells were cocultured with KB cells in the presence of various IFN concentrations (Table 1). The enhancement of the degeneration of L929 cells by the HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.

Various human embryonic fibroblasts (HF 8101 cells, HF 8103 cells) and HEL cells were cocultured with KB cells in the presence of various IFN concentrations (Table 1). The enhancement of the degeneration of L929 cells by the HuIFN-α was not found even in the long-term culture (over 2 weeks) of the target cells, including KB cells, HeLa cells, human hepatoma cells, and FL cells, in the absence of the human embryonic fibroblasts; and HuIFN-α did not enhance the degeneration of L929 cells by human fibroblasts.
of HulFN-α. HulFN-α enhanced the degenerative changes in KB cells by HF 8101 cells, HF 8103 cells, or HEL cells at a dose of 1,000 or 10,000 IU/ml (Tables 1 and 2). Thus, HulFN-α enhanced the degenerative changes in the human target cells, irrespective of the kinds of target cells and human fibroblasts used. KB cells were grown in the center of the Petri dish using a metal cup, and then only the supernatant of human fibroblast culture was added to the KB cell culture both with or without HulFN-α. Seven to 10 days later, the degenerative changes developed in KB cells, and HulFN-α enhanced these changes significantly at 1,000 and 10,000 IU/ml (data not shown).

Enhancing Effect of Several Kinds of IFNs in the Degeneration of KB Cells by Human Fibroblasts (HF 8101 Cells). KB cells and human embryonic fibroblasts (HF 8101 cells) were cocultured together with HulFN-α, HulFN-β, or MulFN-α,β. HulFN-α increased significantly the ratio of the destroyed areas to the KB cell-growing area at a dose of 1,000 or 10,000 IU/ml (p < 0.05 or < 0.001). HulFN-β also increased the ratios at a dose of 1,000 or 10,000 IU/ml (p < 0.001 or < 0.0001). There was no significant difference in the increase in the ratios between HulFN-α and HulFN-β. MulFN-α,β, however, did not increase the ratio at a dose of 100 or 1,000 IU/ml, but did effect a slight but significant increase in the ratio at a dose of 10,000 IU/ml (p < 0.05) (Table 3).

DISCUSSION

The role of fibroblasts, which are closely related to interstitial tissue or to a capsule surrounding a tumor, in tumor development is not yet well known. It seems probable that the fibroblasts or the interstitial cells can suppress the tumor cells because a benign tumor or less malignant tumor is often well encapsulated. Therefore, it is important to examine the effect of fibroblasts on tumor cells. It was recognized in this study that human embryonic fibroblasts induced degenerative changes in tumor cells. These "spongy degeneration"-like changes were caused by several kinds of human embryonic fibroblasts, which may mean that these changes were due to one of the general properties of fibroblasts. They were species specific because human embryonic fibroblasts did not cause degenerative changes in murine L929 cells.

In addition, these changes were enhanced by treatment with HulFN-α or HulFN-β at a dose of more than 1,000 IU/ml. There was no difference in the enhancing effect between HulFN-α and HulFN-β. However, murine IFN hardly increased the degenerative changes caused by human fibroblasts in tumor cells or a continuous cell line. Although 10,000 IU of murine IFN per ml enhanced significantly the degeneration of the target cells, this is in accordance with antiviral activity because human cells (KB cells in this case) have a slight sensitivity to murine IFN (9). This means that the enhancing effect of IFN on the degeneration of target cells by fibroblasts was also species specific. It is suggested that IFN itself enhanced the degenerative changes in target cells because HulFN-α and HulFN-β enhanced the degenerative changes to the same degree, but MulFN-α,β did not, although there is no clear dose relationship among all the experiments. Currently, we are trying to use more highly purified HulFN in order to confirm that IFN itself enhanced the degenerative changes.

There was no difference in the ratio of the destroyed area to the target cell-growing area even when human embryonic fibroblasts were dispersed at different cell densities (1, 4, and 8 × 10⁵/ml). The number of human embryonic fibroblasts reached the plateau in almost as short a time (2 to 4 days) in spite of the difference in the cell densities, and it took a relatively long time (10 to 14 days) to cause the degenerative changes in KB cells. Therefore, the cell densities at the dispersion did not affect the ratio of the destroyed area to the target cell-growing area.

Thus, it was very interesting that the fibroblasts could affect the tumor cells and that this effect was enhanced by IFN. Eagle and Levine (5) reported that in mixed culture of euploid human fibroblasts and amnion cells the 2 cell types did not inhibit each other and neither inhibited the growth of aneuploid epithelial cells. Barski and Belehradek (2) also reported that malignant cells had a tendency to infiltrate the normal cell colony when the normal and malignant outgrowth met each other in the mixed culture. Also, rather than no cell growth inhibition, we observed the invasion of tumor cells when the dissociated tumor cells and human fibroblasts were mixed and cocultured. However, when tumor cells were seeded only in the center of the plastic dish at a high density and human fibroblasts were added, the degenerative changes in tumor cells were recognized (data not shown). The reason for this phenomenon may be due to the following fact. Since the outgrowth of tumor cells seeded only in the center of the dish at a high density was very slow, the fibroblasts could be maintained long enough to produce the degenerative changes in the tumor cells. However, in the mixed culture, the fibroblasts had been invaded by tumor cells before they caused the degenerative changes.

We have evidence that the degenerative changes caused by fibroblasts in the tumor cells or the continuous cell line were intermediated by the factor(s) produced from fibroblasts, because the supernatant of human fibroblast culture caused the same degenerative changes in KB cells as the coculture with human fibroblasts did. Khan et al. (12) reported that human fibroblasts produced bone-resorbing factor, lymphotoxin, and proliferation inhibition factor. We now have a study in progress about the relationship of our tumor-degenerating factor(s) to the 3 factors mentioned above.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Nita, Kyoto Red Cross Blood Center, Kyoto.

Table 3

Enhancing effect of several kinds of IFNs on the target cell degenerative changes by human fibroblasts

<table>
<thead>
<tr>
<th>IFN (IU/ml)</th>
<th>Ratio (%)</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HulFN-α</td>
<td>0</td>
<td>3.05 ± 1.61*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.15 ± 1.52 NS</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>8.40 ± 5.30 &lt;0.05</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>21.98 ± 12.69 &lt;0.001</td>
</tr>
<tr>
<td>HulFN-β</td>
<td>0</td>
<td>2.98 ± 0.56 NS</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>14.08 ± 6.50 &lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>26.15 ± 3.14 &lt;0.0001</td>
</tr>
<tr>
<td>MulFN-α,β</td>
<td>0</td>
<td>1.30 ± 0.89 NS</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.13 ± 1.41 NS</td>
</tr>
<tr>
<td></td>
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<td>2.31 ± 1.61 NS</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>2.97 ± 2.27 &lt;0.05</td>
</tr>
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</table>

* Mean ± S.D. 
† NS, not significant.

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