Interleukin 4 Potentiates the Antiproliferative Effects of Tumor Necrosis Factor on Various Tumor Cell Lines

Klara Totpal and Bharat B. Aggarwal

Cytokine Research Laboratory, Department of Clinical Immunology and Biological Therapy, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

In response to a given stimulus, usually a number of cytokines are secreted simultaneously by the immune system. Whether these cytokines are meant to function as a single agent or in combination with others is not understood. Tumor necrosis factor (TNF) has been shown to exhibit antiproliferative effects against a wide variety of tumor cell lines in vitro. In the present report, we investigated the effects of a T-cell-derived cytokine, interleukin 4 (IL-4), on the antiproliferative effects of TNF against different tumor cell lines. The growth characteristics of human breast cancer cells (MDA-MB-330) were minimally affected when the cells were exposed to either TNF or IL-4 alone. However, together these 2 cytokines inhibited cell growth in a dose-dependent manner. The enhancement of the cytotoxic effects of TNF by IL-4 were not just limited to breast tumor cells, but were also observed with human epidermoid carcinoma cells (A-431) and human histiocytic lymphoma cells (U-937). The enhancement of the cytotoxic effect of TNF by IL-4 against various tumor cell lines was found comparable with that by γ-interferon (IFN-γ). Interestingly, for certain tumor cell types, IL-4 alone was found to enhance cell proliferation. IL-4 had no effect on the growth-stimulatory activity of TNF on normal human foreskin fibroblasts. Pre-exposure of U-937 cells to IFN-γ led to a greater than 2-fold induction in TNF receptors, but no modulation of TNF receptors by IL-4 was observed. Moreover, the presence of IFN-γ was found to further potentiate the antiproliferative effects of TNF and IL-4. These results clearly suggest that IL-4 potentiates the antiproliferative responses of TNF by a mechanism different from that of IFN-γ. Although it is well known that IL-4 can modulate the production of TNF from macrophages, this is the first report to suggest that IL-4 can also modulate TNF-dependent antiproliferative responses.

INTRODUCTION

IL-4 is a cytokine produced by activated T-lymphocytes, and it modulates the immune system. It was described originally as a B-cell growth factor because of its ability to costimulate B-lymphocyte proliferation (1). Besides regulating B-cell growth, IL-4 has now been shown to affect a wide variety of cells including T-cells, mast cells, macrophages, and hematopoietic progenitor cells (2-4). IL-4 enhances the growth of T-cells and mast cells (5-8), induces major histocompatibility complex class II antigen expression on B-cells and monocytes (9), augments antigen-specific cytotoxic T-cells, and enhances the macrophage-mediated cytotoxicity (10-12). The production of several cytokines including IL-1α, IL-β, IFN-γ, and TNF-α has been shown to be down-regulated by IL-4, but it also up-regulates the production of IL-6 from B-cells (13-15). IL-2-induced lymphokine-activated killer cell and natural killer cell activity is down-modulated by IL-4 (16, 17). Besides proliferative effects, IL-4 has also recently been shown to inhibit the growth in vitro of human lymphoid and plasma cell neoplasms (18).

TNF is another cytokine that is primarily a product of activated monocytes and is highly pleiotropic in nature (19, 20). TNF inhibits the growth of a wide variety of tumor cells in culture and stimulates the growth of fibroblasts, B-cells, and thymocytes. The activation of the immune system with a given stimulus usually leads to the production of several cytokines, both growth stimulatory and growth inhibitory in nature. Whether these cytokines are meant to function as a single agent or in combination with others is not completely understood. However, it has been shown that the antiproliferative effects of TNF on tumor cells can be potentiated by interferons and interleukin 1 (21-30). In this report, we investigated the effects of another T-cell-derived cytokine, IL-4, on the antiproliferative effects of TNF on several different cell lines are potentiated by IL-4. The latter alone has no significant growth-inhibitory effect on these cell lines. For some cell lines, IL-4 was found slightly growth stimulatory. Even though the degree of enhancement of the cytotoxic effects of TNF by IL-4 was comparable with that of IFN-γ, the mechanism of potentiation appears to be different.

MATERIALS AND METHODS

Materials. Tumor cell lines ZR-75-1 (CRL 1500), MDA-MB-468 (HTB 132), HS-0578T (HTB 126), BT-20 (HTB 19), MCF-7 (HTB 22), A-375 (CRL 1619), MDA-MB-330 (HTB 127), A-431 (CRL 1555), and U-937 (CRL 1593) were obtained from American Type Cell Culture Collection (Rockville, MD). Cells were tested for Mycoplasma contamination using the DNA-based assay kit purchased from GenProbe (San Diego, CA). RPMI 1640 and fetal bovine serum were obtained from Grant Island Biological Company (Grand Island, NY). Recombinant TNF and IFN-γ were obtained from Genentech, Inc. (South San Francisco, CA). Recombinant IL-4 was a kind gift from Dr. T. L. Nagabhushan of Schering-Plough, Inc. (Bloomfield, NJ).

Cell Culture. All cell cultures were maintained in continuous exponential growth by weekly passage of cells. Cells were routinely grown in RPMI 1640 supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal bovine serum (10%) in a humidifier incubator in 5% CO₂ in air.

Cytotoxicity Assays. Cells (5 x 10³) in 0.1 ml of the medium were plated in 96-well Costar plates. After overnight incubation, the medium was removed and a serial dilution of the test sample was layered in 0.1 ml of the volume. After 72 h of incubation at 37°C, the medium was removed and viable cells were monitored by crystal violet staining according to the procedure described previously (22). The percentage of relative cell viability was calculated as absorbance in the presence of the test sample divided by absorbance in the absence of the test sample (medium) multiplied by 100.

Radioreceptor Assay. Receptor binding assays were carried out essentially as described previously (23). Briefly, 1 x 10⁶ cells were incubated in 12 x 75-mm polystyrene tubes in 0.2 ml of fresh ice-cold medium.
SYNERGISTIC ANTIPROLIFERATIVE EFFECTS OF TNF AND IL-4

(RPMI 1640 + 10% fetal bovine serum) containing 0.65 × 10^5 counts per min of ^125^I-labeled TNF, either in the presence (nonspecific binding) or absence (total binding) of a 100-fold excess of unlabeled TNF. After 45-min incubation at 4°C, medium was removed, cells were washed 3 times by centrifugation, and cell-bound radioactivity was determined. All determinations were made in triplicate.

RESULTS

The effect of human interleukin 4 on the antiproliferative effects of TNF against different tumor cell lines was investigated. We first examined the effect of different concentrations of TNF alone and in combination with a fixed concentration of IL-4 (100 ng/ml) on a breast tumor cell line (MDA-MB 330), vulvar carcinoma cell line (A431), and a histiocytic lymphoma cell line (U-937). The results shown in Figs. 1 and 2 indicate that TNF alone in amounts up to 200 ng/ml had a minimal effect on these cells. However, the addition of IL-4 inhibited the growth of these cells in a dose-dependent manner. For instance, in the case of MDA-MB 330, 10% cell growth inhibition was observed with 200 ng/ml of TNF, but when 100 ng/ml of IL-4 was also added, growth inhibition increased to almost 60% (Fig. 1). Similar potentiation of the cytotoxic effects of TNF by IL-4 was also observed with the epithelial and lymphoma cell lines (Fig. 2).

The effect of variable concentrations of IL-4 on a fixed concentration of TNF (20 ng/ml) was also investigated. The results shown in Fig. 3 indicate that A-431 cells (Fig. 3, right) were inhibited in a dose-dependent manner in the presence of IL-4 and TNF. Even at a concentration of 1 μg/ml, IL-4 alone was ineffective (Tables 1 and 2). A similar dose-dependent response of IL-4 was observed with breast tumor cells (Fig. 3 left). TNF has been shown to stimulate the proliferation of fibroblasts. Therefore, we tested the effect of IL-4 on the growth-stimulatory activity of TNF on normal human foreskin fibroblasts. It was found that IL-4 had no effect on the TNF-dependent proliferation of fibroblasts (data not shown).

Previously, it has been shown that IFN-γ also enhances the cytotoxic effects of TNF on a wide variety of tumor cell lines (21–28). Therefore, we compared the potentiation of the antiproliferative response of TNF by IL-4 with that of IFN-γ. The results shown in Fig. 4 indicate that on breast tumor cells both IL-4 and IFN-γ at equal concentrations (100 ng/ml) enhance the growth-inhibitory effects of TNF. IFN-γ, however, was found to be approximately 10-fold more effective than IL-4. Approximately 60–70% inhibition of cell growth was observed. Similar results were obtained with A-431 and U-937 cells (Table 1). A-431 cells were found to be highly sensitive to the combination of TNF and IFN-γ. To observe synergistic effects, 100-fold less interferon was needed for these cells as compared to other cell types. IL-4 also appears to slightly enhance the antiproliferative effects of IFN-γ, although not to the same extent as TNF (Table 1). Furthermore, it appears that the combination of all 3 cytokines together is maximally effective.
SYNERGISTIC ANTIPROLIFERATIVE EFFECTS OF TNF AND IL-4

Fig. 3. Effect of different concentrations of IL-4 on the TNF (20 ng/ml)-dependent cytotoxicity against A-431 (right panel) and MDA-MB-330 (left panel) cell lines. The percent relative cell viability with TNF (20 ng/ml) alone was 102% for A-431 cells and 112% for MDA-MB-330 cells. Cells (5 x 10^5) were incubated with the indicated concentrations of the cytokines in 0.1 ml of the media for 72 h. Then cell viability was determined as indicated in "Materials and Methods." All determinations were made in triplicate. Bars, SD.

Table 1 Effects of interleukin 4 and γ-interferon on the TNF-dependent antiproliferative effects against various human tumor cell lines

Cells (5 x 10^5) were incubated in a 0.1-ml volume of media containing either TNF (200 ng/ml), IL-4 (100 ng/ml), or IFN-γ (100 ng/ml), or a combination of cytokines as indicated above. In the case of A-431 cells, concentrations of all cytokines were the same except for IFN-γ (1 ng/ml). After 72 h of incubation with cytokines, the cell viability was determined by crystal violet staining as indicated in "Materials and Methods."

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>MDA-MB-330 relative cell viability (%)</th>
<th>A-431 relative cell viability (%)</th>
<th>U-937 relative cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 3.2</td>
<td>100 ± 5.5</td>
<td>100 ± 9.3</td>
</tr>
<tr>
<td>TNF</td>
<td>80 ± 1.7</td>
<td>71 ± 3.1</td>
<td>89 ± 3.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>103 ± 6.55</td>
<td>86 ± 2.2</td>
<td>104 ± 5.0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>82 ± 5.47</td>
<td>69 ± 1.7</td>
<td>104 ± 6.1</td>
</tr>
<tr>
<td>TNF + IFN-γ</td>
<td>42 ± 3.51</td>
<td>33 ± 5.03</td>
<td>50 ± 1.1</td>
</tr>
<tr>
<td>TNF + IL-4</td>
<td>51 ± 0.58</td>
<td>45 ± 1.0</td>
<td>64 ± 3.5</td>
</tr>
<tr>
<td>IFN-γ + IL-4</td>
<td>60 ± 2.89</td>
<td>48 ± 5.1</td>
<td>89 ± 5.7</td>
</tr>
<tr>
<td>TNF + IFN-γ + IL-4</td>
<td>38 ± 2.65</td>
<td>20 ± 1.0</td>
<td>41 ± 1.0</td>
</tr>
</tbody>
</table>

*Mean ± SD.

For instance, in the case of A431 cells, only 30% growth inhibition is observed with TNF alone, 55% with IL-4 and TNF, and 80% with IFN-γ, IL-4, and TNF present (Table 1). These results also imply that perhaps the mechanism by which IFN-γ enhances the anticellular effects of TNF is different from that of IL-4.

It is known that IFN-γ induces the receptors for TNF. Therefore, we investigated the possibility of TNF receptor induction by IL-4. The results shown in Table 3 clearly indicate that overnight preexposure of U-937 cells with IFN-γ leads to more than a 2-fold induction of TNF receptors, but the exposure of cells to similar concentration of IL-4 under identical conditions does not lead to an increase in the specific binding of TNF, suggesting no increase in the synthesis of new receptors. Thus, IL-4 appears to work in this system by a different mechanism than does IFN-γ.

Interestingly, during the course of these studies, we noted that for certain cell types, IL-4 alone causes the proliferation of cells. As shown in Table 3, in the case of the breast tumor cell line (ZR-75-1), IL-4 enhanced the growth of these cells by almost 40% over the control. Increased proliferation of cells was also observed with some of the other cell lines, but to a lesser degree. The growth-stimulatory effects of IL-4 were found to be dose-dependent (data not shown).

Table 2 Modulation of growth of various human tumor cell lines by interleukin 4

Cells (5 x 10^5) were incubated with interleukin 4 (1 ng/ml) for 72 h and then the relative cell viability was determined by crystal violet staining as indicated in "Materials and Methods." All determinations were made in triplicate.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Relative cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1 (breast tumor)</td>
<td>140 ± 6.2*</td>
</tr>
<tr>
<td>MDA-MB-468 (breast tumor)</td>
<td>130 ± 3.4</td>
</tr>
<tr>
<td>HS-0578T (breast tumor)</td>
<td>132 ± 8.1</td>
</tr>
<tr>
<td>BT-20 (breast tumor)</td>
<td>110 ± 5.5</td>
</tr>
<tr>
<td>MCF-7 (breast tumor)</td>
<td>125 ± 2.0</td>
</tr>
<tr>
<td>A-375 (melanoma)</td>
<td>120 ± 6.2</td>
</tr>
<tr>
<td>MDA-MB-330 (breast tumor)</td>
<td>110 ± 7.0</td>
</tr>
<tr>
<td>A-431 (vulvar carcinoma)</td>
<td>86 ± 2.48</td>
</tr>
<tr>
<td>U-937 (histiocytic lymphoma)</td>
<td>110 ± 7.4</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Fig. 4. Comparison of the effect of IL-4 and IFN-γ on the TNF-dependent cytotoxicity against MDA-MB-330 cells. Cells (5 x 10^5) were incubated in 0.1 ml of the medium containing different concentrations of TNF and either IL-4 (100 ng/ml) or IFN-γ (100 ng/ml) for 72 h. Then cell viability was determined as indicated in "Materials and Methods." All determinations were made in triplicate. Bars, SD.

For instance, in the case of A431 cells, only 30% growth inhibition is observed with TNF alone, 55% with IL-4 and TNF, and 80% with IFN-γ, IL-4, and TNF present (Table 1). These results also imply that perhaps the mechanism by which IFN-γ enhances the anticellular effects of TNF is different from that of IL-4.

It is known that IFN-γ induces the receptors for TNF. Therefore, we investigated the possibility of TNF receptor induction by IL-4. The results shown in Table 3 clearly indicate that overnight preexposure of U-937 cells with IFN-γ leads to more than a 2-fold induction of TNF receptors, but the exposure of cells to similar concentration of IL-4 under identical conditions does not lead to an increase in the specific binding of TNF, suggesting no increase in the synthesis of new receptors. Thus, IL-4 appears to work in this system by a different mechanism than does IFN-γ.

Interestingly, during the course of these studies, we noted that for certain cell types, IL-4 alone causes the proliferation of cells. As shown in Table 3, in the case of the breast tumor cell line (ZR-75-1), IL-4 enhanced the growth of these cells by almost 40% over the control. Increased proliferation of cells was also observed with some of the other cell lines, but to a lesser degree. The growth-stimulatory effects of IL-4 were found to be dose-dependent (data not shown).

For instance, in the case of A431 cells, only 30% growth inhibition is observed with TNF alone, 55% with IL-4 and TNF, and 80% with IFN-γ, IL-4, and TNF present (Table 1). These results also imply that perhaps the mechanism by which IFN-γ enhances the anticellular effects of TNF is different from that of IL-4.

It is known that IFN-γ induces the receptors for TNF. Therefore, we investigated the possibility of TNF receptor induction by IL-4. The results shown in Table 3 clearly indicate that overnight preexposure of U-937 cells with IFN-γ leads to more than a 2-fold induction of TNF receptors, but the exposure of cells to similar concentration of IL-4 under identical conditions does not lead to an increase in the specific binding of TNF, suggesting no increase in the synthesis of new receptors. Thus, IL-4 appears to work in this system by a different mechanism than does IFN-γ.

Interestingly, during the course of these studies, we noted that for certain cell types, IL-4 alone causes the proliferation of cells. As shown in Table 3, in the case of the breast tumor cell line (ZR-75-1), IL-4 enhanced the growth of these cells by almost 40% over the control. Increased proliferation of cells was also observed with some of the other cell lines, but to a lesser degree. The growth-stimulatory effects of IL-4 were found to be dose-dependent (data not shown).
Table 3 Effect of interleukin 4 and γ-interferon on the specific binding of tumor necrosis factor to U-937 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total binding (counts per min)</th>
<th>Non-specific binding (counts per min)</th>
<th>Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3310 ± 87</td>
<td>290 ± 104</td>
<td>3020</td>
</tr>
<tr>
<td>Interleukin-4</td>
<td>3281 ± 126</td>
<td>235 ± 66</td>
<td>3046</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>7370 ± 291</td>
<td>383 ± 162</td>
<td>6987</td>
</tr>
</tbody>
</table>

DISCUSSION

Our results indicate that interleukin 4 can enhance the antiproliferative effects of TNF against a number of different tumor cell lines in a dose-dependent manner. However, IL-4 has no effect on the fibroblast growth-stimulatory property of TNF. Interestingly, the proliferation of some cell lines was significantly stimulated by IL-4 alone. Several groups have reported that the antiproliferative effects of TNF are potentiated by IFN-γ (21-28). The enhancement of the cytotoxic effects of TNF by IL-4 were found comparable with that of IFN-γ. The mechanism by which IFN-γ potentiates TNF response is not clear. It is known that the up-regulation of cytotoxic effects of TNF by IFN-γ accompanies the up-regulation of TNF receptors (23, 24). The results presented here clearly confirm the up-regulation of TNF receptors by IFN-γ, but no increase in the receptors by IL-4 was observed, suggesting that the mechanism by which IFN-γ works is different from that of IL-4. Since the synergistic antiproliferative effects of TNF and IFN-γ can be further enhanced by IL-4, it also suggests that the mechanisms by which IFN-γ and IL-4 function in this system are different. The enhancement of TNF-dependent proliferation of fibroblasts has been shown to be suppressed by IFN-γ (22). Since IL-4 was found to have no effect on the growth of fibroblasts caused by TNF, it also indicates that IFN-γ and IL-4 function by independent mechanisms.

Besides IFN-γ and IL-4, the antiproliferative effects of TNF against a human melanoma cell line, A375, have also been reported to be synergistically potentiated by interleukin 1 (30). It is not known whether IL-1 causes the induction of TNF receptors in this system. In another study, however, a down-regulation of TNF receptors by IL-1 on human fibroblastoid cell line SV-80 was reported (31). The latter workers also demonstrated that IL-1 induces resistance to the cytotoxic effects when applied before the application of TNF, it is found to potentiate the cytotoxicity when applied together with TNF. The mechanism of this kind of opposing effects of TNF remains unclear. In the studies described, however, we found that IL-4 potentiates the TNF effects, whether applied simultaneously or with IL-4 pretreatment of cells followed by TNF (data not shown).

Since inhibitors of protein synthesis are also known to potentiate the antiproliferative effects of TNF (32), therefore, it is less likely that the mechanism of synergism between TNF and IL-4 is due to new protein synthesis. Exposure of cells to TNF at elevated temperatures is also known to enhance the antiproliferative effects, but its mechanism is not understood (32, 33). It is possible that the mechanism by which various cytokines and other agents potentiate the antiproliferative effects of TNF is through inhibition rather than induction of new protein synthesis.

**REFERENCES**


Interleukin 4 Potentiates the Antiproliferative Effects of Tumor Necrosis Factor on Various Tumor Cell Lines

Klara Totpal and Bharat B. Aggarwal