Advances in Brief

Tumor Necrosis Factor Acts as a Tumor Promoter in BALB/3T3 Cell Transformation

Atsumasa Komori, Jun Yatsunami, Masami Suganuma, Sachiko Okabe, Saori Abe, Ayako Sakai, Kiyoshi Sasaki, and Hirotu Fujiwara

Abstract

Tumor necrosis factor (TNF), a cytokine, and okadaic acid, a tumor promoter, strongly phosphorylated the same proteins, vimentin and heat shock protein 27, although their time courses were different. Human TNF-α at a concentration of 0.6 nM markedly stimulated transformation of BALB/3T3 cells initiated with 3-methylcholanthrene. The human TNF-α was about 1000 times more effective than the chemical tumor promoters, okadaic acid and 12-O-tetradecanoylphorbol-13-acetate. TNF induced growth of v-Ha-ras transfected BALB/3T3 cells (Bhas 42 cells), whereas it did not induce growth of nontransfected BALB/3T3 cells. Okadaic acid induced mouse TNF-α from Bhas 42 and BALB/3T3 cells. The results suggest that a chemical tumor promoter induces the secretion of TNF-α from various cells. The TNF then acts as an endogenous tumor promoter in vivo.

Introduction

Tumor necrosis factor, a protein with a molecular weight of 17,000, is produced mainly by activated macrophages and was originally identified as a serum factor inducing hemorrhagic necrosis of transplanted solid tumors in mice (1). TNF is also implicated through a cytokine network in various biological processes, such as inflammation, immunoregulation, cachexia, and mitogenesis (1). TNF and IL-1 induce similar cellular responses, although they are different proteins and bind to different receptors. A recent paper reporting that okadaic acid mimics TNF/IL-1 in inducing protein phosphorylation and expression of early response genes, such as c-jun and egr-1 (2), attracted our attention to a possible link between TNF and tumor promoters. Okadaic acid is a polyether compound of a C38 fatty acid isolated from a black sponge, Halichondria okadai, and is a potent inhibitor of protein phosphatases 1 and 2A. We have reported that okadaic acid induced mouse TNF-α from Bhas 42 and BALB/3T3 cells. Thus, the study with the okadaic acid class tumor promoters led us to hypothesize that the effects induced by inhibition of protein phosphatase 1 and 2A activities are involved in tumor promotion of human cancer development (6). If okadaic acid and TNF/IL-1 induce the same effects on initiated cells, TNF/IL-1 would be endogenous tumor promoters. On the basis of this, we first found that human TNF-α induced hyperphosphorylation of vimentin and HSP 27 in primary human fibroblasts, as does okadaic acid (2, 7). Human TNF-α, like okadaic acid, dose-dependently induced transformation of BALB/3T3 cells initiated with MCA. In addition, okadaic acid induced mouse TNF-α release from mouse BALB/3T3 cells and v-Ha-ras transfected BALB/3T3 cells (Bhas 42 cells). Thus TNF-α was acting as a tumor promoter. This prompted us to extend the concept to include its action as a general mediator of tumor promotion; i.e., TNF is released from the initiated cells as well as from various neighboring cells, including lymphocytes. TPA also induces TNF production in human monocytes (8). The released TNF-α stimulates clonal growth of the initiated cells via an autocrine and/or paracrine mechanism. This paper proposes a general mechanism of tumor promotion in human tissues, based on the recent results.

Materials and Methods

Chemicals and Cells. hTNF-α (3 × 10⁷ units/mg), mTNF-α (4 × 10⁷ units/mg), and an enzyme linked immunosorbent assay kit for determination of mouse TNF-α were purchased from Genzyme Co., Cambridge, MA. Anti-vimentin antibody was purchased from Labsystem, Helsinki, Finland. A reagent, MTT for MTT assay, was purchased from Sigma Chemical Co., St. Louis, MO. Okadaic acid was isolated from the black sponge, H. okadai, as reported previously (3). Primary human fibroblasts were used as described previously (7). BALB/3T3 clone A31-1-1 cells (BALB/3T3) (JCRB0601), and v-Ha-ras transfected BALB/3T3 clone (Bhas 42) (9) were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Phosphorylation of Vimentin and HSP 27 in Primary Human Fibroblasts Treated with hTNF-α. The cells (1 × 10⁶), which had been grown in a 60-mm culture dish in 4 ml of phosphate deficient Dulbecco's modified Eagle's medium containing 10% FCS, as described previously, were labeled with ³²P, to a final concentration of 3.7 MBq/ml and incubated with hTNF-α either at 25 ng/ml (1.47 nM) up to 60 min or at various concentrations (2.5 to 250 ng/ml, 0.14 to 14.7 nM) for 15 min. After the reaction, the cells were solubilized in lysis buffer. The supernatant fluids were precipitated with anti-vimentin antibody and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Identification of HSP 27 was determined by molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as reported previously (10). The radioactivity was determined by a BAS-2000 Image Analyzer (Fuji Film Co., Tokyo, Japan). Similar results were obtained by twice repeated experiments.

Effects of hTNF-α and mTNF-α on Growth of Bhas 42 and BALB/3T3 Cells. Both cell lines (1 × 10⁵ cells), which were seeded on a 96-well plate in MEM containing 10% FCS, were maintained in a humidified incubator with 5% CO₂ atmosphere in air at 37°C. The cells were treated with various concentrations of hTNF-α and mTNF-α (0.1 μg/ml to 100 ng/ml, 0.006 μM to 5.9 μM) for 72 h. The number of cells was determined by MTT assay. Each of the values was the mean of quadruplicate analysis.

Cell Transformation with hTNF-α. The two-stage transformation experiment was carried out by the same protocol described previously (11). The BALB/3T3 cells were initiated with 1.8 μM MCA (0.1 μg/ml) for 72 h. The
cells were further treated with various concentrations of hTNF-α (0.3 to 10.0 ng/ml, 0.02 to 0.6 μM) or 0.5 μM TPA (300 ng/ml) as a positive control for 2 weeks. The cells were subsequently cultured in a normal medium for 3 weeks and stained with Giemsa. Foci larger than 3 mm in diameter were identified as transformed foci, according to the reported criteria of Sakai and Fujiki (11).

Induction of mTNF-α Release from Mouse Bhas 42 and BALB/3T3 Cells by Okadaic Acid. Both cell lines (2 × 10^5 cells), which were grown in 1.0 ml of MEM containing 10% FCS/well on a 12-well plate, were treated with okadaic acid either at a concentration of 0.2 μM, dissolved in 0.5 ml of MEM containing 0.1% FCS, up to 48 h or at various concentrations (0.05 μM to 0.5 μM) for 24 h. After centrifugation, the supernatant fluids were stored at -20°C before the assay. Aliquots (0.2 ml) of the supernatant fluids were used for determination of mTNF-α with an enzyme linked immunosorbent assay kit (12), which contained a hamster monoclonal antibody specific for mTNF-α and a goat polyclonal anti-mTNF-α antibody. The method was reliable to 50 pg/ml (2.9 pm) of mTNF-α. Each of the values was the mean of quadruplicate analysis.

Results and Discussion

Phosphorylation of Vimentin and HSP 27 in Primary Human Fibroblasts Treated with hTNF-α. One of the most significant effects of okadaic acid on primary human fibroblasts is hyperphosphorylation of vimentin and HSP 27, induced by inhibition of protein phosphatases 1 and 2A (7). Vimentin phosphorylation is slightly stronger than that of HSP 27, the latter of which is also induced by TPA (10). Since hyperphosphorylation of these proteins is a suitable marker for detecting tumor promoters like okadaic acid, we first studied whether hTNF-α induces hyperphosphorylation of these two proteins in primary human fibroblasts. Treatment with hTNF-α at various concentrations (2.5 to 250 ng/ml, 0.14 to 14.7 nm) for 15 min significantly induced hyperphosphorylation of vimentin in the cells (data not shown). hTNF-α at a concentration of 25 ng/ml (1.47 nm) increased the maximum level of phosphorylation of vimentin in the cells 15 min after treatment to twice that of its basal level (Fig. 1). Okadaic acid at a concentration of 100 nm increased the maximum level of phosphorylation of vimentin 2 h after treatment 10 times over that of its basal level (7). Okadaic acid below concentrations of 1 nm did not significantly induce hyperphosphorylation of vimentin by autoradiography (data not shown). The difference between the time course of the hyperphosphorylation of okadaic acid and hTNF-α is due to the different mechanisms of action; okadaic acid is an inhibitor of protein phosphatases 1 and 2A and TNF-α is thought to activate protein kinases (2, 7).

The hyperphosphorylation of HSP 27 in primary human fibroblasts treated with hTNF-α followed almost the same time course as that of vimentin (data not shown). These results strongly indicated that hTNF-α induces phosphorylation of the same proteins as does the tumor promoter, okadaic acid, but the time course of their phosphorylation is different.

Effects of hTNF-α and mTNF-α on Growth of Bhas 42 and BALB/3T3 Cells. Fig. 2A shows that hTNF-α at concentrations from 0.1 pg/ml to 10 ng/ml (0.006 pm to 0.6 nm) specifically induced growth of Bhas 42 cells measured after 72 h of incubation, whereas hTNF-α was not significantly effective in BALB/3T3 cells. The fractional absorbance of the MTT assay was 140% for Bhas 42 cells and 115% for BALB/3T3 cells, at a concentration of 10 ng/ml (0.6 nm) hTNF-α. The effects of various concentrations of hTNF-α and mTNF-α on the growth of Bhas 42 cells are shown in Fig. 2B. hTNF-α and mTNF-α similarly induced cell growth at concentrations of around 0.1 to 100 pg/ml (0.006 pm to 0.006 nm). The fractional absorbance of the MTT assay was 139% by hTNF-α and 140% by mTNF-α at a concentration of 100 pg/ml. Although TNF is reported to stimulate the growth of cells (13, 14), this experiment showed specific growth of Bhas 42 cells by TNF-α but not that of BALB/3T3 cells.

Cell Transformation with hTNF-α. Table 1 shows the results of two-stage BALB/3T3 cell transformation. In the absence of a tumor promoter, MCA at a concentration of 0.1 μg/ml (5.9 nm) induced 1 focus from among 12 dishes and many foci at 5.0 μg/ml (29.5 nm).
Table 1  Tumor promoting activity of TNF in two-stage BALB/3T3 cell transformation

<table>
<thead>
<tr>
<th>Initiator (µg/ml)</th>
<th>Treatment (ng/ml)</th>
<th>CFEa (%)</th>
<th>No. of dishes examined</th>
<th>Total no. of foci</th>
<th>Av. no. of foci/dish</th>
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<tr>
<td>DMSO</td>
<td>PBS</td>
<td>52</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>TPA</td>
<td>30</td>
<td>5/36</td>
<td>2</td>
<td>0.25</td>
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<tr>
<td>MCA 0.1</td>
<td>PBS</td>
<td>60</td>
<td>11/12</td>
<td>44</td>
<td>3.67</td>
</tr>
<tr>
<td>MCA 0.1</td>
<td>TPA</td>
<td>30</td>
<td>11/12</td>
<td>23</td>
<td>1.92</td>
</tr>
<tr>
<td>DMSO</td>
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<td>1/2</td>
<td>2</td>
<td>0.17</td>
</tr>
<tr>
<td>MCA 0.1</td>
<td>TNF</td>
<td>3.0</td>
<td>4/12</td>
<td>4</td>
<td>0.33</td>
</tr>
<tr>
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<tr>
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<td>MCA 0.1</td>
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a CFE, colony forming efficiency; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline.

b 0.5% of medium.

c 0.2% of medium.

d Levels of significance calculated by the one-tailed Fisher exact test; d, P < 0.001 versus 0.1 µg/ml MCA/PBS; e, P < 0.002 versus 0.1 µg/ml MCA/PBS.

Fig. 3. Induction of mTNF-a release from Bhas 42 and BALB/3T3 cells by okadaic acid. Bhas 42 (A) and BALB/3T3 (B) cells were treated with 0.2 µM okadaic acid, up to 48 h. mTNF-a released into the medium was determined with an enzyme linked immunosorbent assay kit.

hTNF-a markedly stimulated the transformation dose dependently, as judged by the following three parameters: number of dishes with foci/number of dishes examined; total number of foci; and average numbers of foci/dish. hTNF-a at a concentration of 10 ng/ml (0.6 µM) induced 1.83 foci/dish, whereas TPA at a concentration of 300 ng/ml (0.5 µM) as a positive control induced 1.92 foci/dish. Based on their numbers of foci/dish. hTNF-a at a concentration of 10 ng/ml (0.6 nM) markedly stimulated the transformation dose dependently, as do okadaic acid and TPA (2, 15). Thus, instead of repeated applications of okadaic acid or TPA, TNF present in its milieu can sustain tumor promoting events in various tissues (16). TNF also acts as an endogenous tumor promoter when it allows the clonal growth of initiated cells. Fernandez et al. (17) recently reported that the Ha-ras gene-induced progressor tumor variants are resistant to cytolysis of TNF. In addition, TNF is found in fresh human tumor tissues taken from patients and can enhance metastasis (18, 19). The processes of tumor promotion and tumor progression are associated with inflammation and tissue damage (19, 20) and are compatible with our postulated role for TNF. An endogenous tumor promoter, i.e., TNF, can be induced and secreted. The evidence strongly suggests that a chemical tumor promoter is an inducer of TNF-α, via an autocrine or paracrine mechanism. TNF-α can stimulate further TNF-α secretion from various cells. Thus, we think that TNF-α is a central mediator (tumor promoter) for cancer development in vivo.

Acknowledgments

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References

14. Vökel, J., Palombella, V. J., Henriksson-Destefano, D., Swenson, C., Feinman, R., Hira, I., and Tsujimoto, M. Fibroblast growth-enhancing activity of tumor necrosis factor on various cell lines depending on various conditions, although it was originally thought to be a cytotoxic agent (13, 14). Combination with other cytokines amplifies the effects of TNF as a growth factor.

TNF at concentrations up to 1 nm does not inhibit protein phosphatases 1 and 2A (data not shown) but induces protein phosphorylation through activation of various protein kinases, resulting in expression of early response genes, as do okadaic acid and TPA (2, 15). Thus, instead of repeated applications of okadaic acid or TPA, TNF present in its milieu can sustain tumor promoting events in various tissues (16). TNF also acts as an endogenous tumor promoter when it allows the clonal growth of initiated cells. Fernandez et al. (17) recently reported that the Ha-ras gene-induced progressor tumor variants are resistant to cytolysis of TNF. In addition, TNF is found in fresh human tumor tissues taken from patients and can enhance metastasis (18, 19). The processes of tumor promotion and tumor progression are associated with inflammation and tissue damage (19, 20) and are compatible with our postulated role for TNF. An endogenous tumor promoter, i.e., TNF, can be induced and secreted. The evidence strongly suggests that a chemical tumor promoter is an inducer of TNF-α, via an autocrine or paracrine mechanism. TNF-α can stimulate further TNF-α secretion from various cells. Thus, we think that TNF-α is a central mediator (tumor promoter) for cancer development in vivo.
TUMOR PROMOTION BY HUMAN TNF-α


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