Analysis of Murine Cellular Receptors for Tumor-killing Factor

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ABSTRACT

Receptors for tumor-killing factor (TKF) on the surface of murine cells were analyzed using radioiodinated TKF. Not only sensitive cells but also insensitive cells were found to have specific receptors. Among the sensitive cells, no clear relation was observed between the number of receptors on the cell surface and sensitivity to TKF. Compounds affecting microfilaments (cytochalasin B and D) and microtubules (colchicine and Colcemid) significantly inhibited cytolyosis of sensitive cells induced by receptor-bound TKF. It is concluded that internalization of receptor-bound TKF is a prerequisite for triggering cytolyosis.

INTRODUCTION

Previously we reported purification of a tumor-specific cytotoxic protein termed TKF to homogeneity from the culture medium of a murine macrophage-like cell line, J774.1 (1). Although J774.1 cells do not produce TKF under usual culture conditions, they start to produce it when Sarcophaga lectin was added to the culture medium. Sarcophaga lectin is a galactose-binding animal lectin purified from the hemolymph of Sarcophaga peregrina (flesh fly) larvae (2).

Recently, Sarcophaga lectin itself was found to have a significant antitumor effect on both transplanted syngeneic and allogeneic murine tumors (3). During treatment of tumor-bearing mice with Sarcophaga lectin, we found that significant cytotoxic activity transiently appeared in the serum of tumor-bearing mice treated with Sarcophaga lectin (3). From inhibition experiments with antibody against TKF, we concluded that this cytotoxic activity is due to TKF (4). Therefore, it is conceivable that TKF produced in situ is partly responsible for the antitumor effect of Sarcophaga lectin, since TKF was shown to have significant antitumor activity both to solid and ascites tumors (5).

TKF is likely to be a protein similar to TNF judging from its molecular weight and subunit structure. However, nothing is known about the molecular mechanism of the cytotoxicity of these factors. To obtain more direct insight into the cytotoxic activity of TKF, we analyzed the receptors for this protein on the surface of target cells. Although no significant number of receptors was found on the surface of J774.1 cells, a producer of TKF, many sensitive and insensitive cells were shown to have TKF receptors. From the results we suggest that the internalization of receptor-bound TKF and the fusion of the resulting endosomes with lysosomes are prerequisites for the expression of the cytotoxic activity of TKF.

MATERIALS AND METHODS

Sarcophaga Lectin and TKF. Sarcophaga lectin was purified from the hemolymph of third instar larvae of S. peregrina (flesh fly) collected 48 h after their body wall had been injured with a hypodermic needle, as reported before (2). TKF was purified from the culture medium of J774.1 cells stimulated with Sarcophaga lectin, as described before (1).

Cells. Mouse macrophage-like line J774.1 cells and tumorigenic fibroblast L929 cells were routinely grown in RPMI 1640 medium and Eagle's minimal essential medium, respectively, supplemented with 10% fetal calf serum at 37°C under a humidified atmosphere of 5% CO2 in air. Mouse nontumorigenic fibroblast 3T3 and SV40-transformed 3T3 (SV3T3) were obtained from Dr. Kitagawa (National Institute of Health of Japan). Mouse melanoma B16 cells and LP3 fibroblasts were obtained from Dr. Osawa (University of Tokyo). Mouse lymphoma EL4 cells were obtained from Dr. Inoue (University of Tokyo). A primary culture of mouse embryonic fibroblasts was established from an ICR mouse embryo.

Chemicals. Cytochalasin B, cytochalasin D, colchicine and Colcemid were purchased from Sigma.

Radioiodination of TKF. TKF was radioiodinated by a modification of the method of Hunter and Greenwood (6). To 50 µl of TKF solution (200 µg/ml in PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, and 8 mM Na2HPO4, pH 7.4) were added 0.5 mCi of 125I (13.8 mCi/µg; Amersham/Searle) and 50 µl of chloramine-T (1 mg/ml in 0.5 M phosphate buffer, pH 7.5). The mixture was incubated for 1 min at room temperature; the reaction was stopped by adding 100 µl of sodium bisulfite (2 mg/ml) and 10 µl of 2 M NaI, and the mixture was applied to a column of Sephadex G-25 (0.6 x 24 cm) equilibrated with PBS to remove free 125I. The specific activity of radioiodinated TKF was usually 3 x 105 cpm/µg, and the recovery of the biological activity of TKF throughout radioiodination was 61%. The preparation was stored at −80°C in the presence of bovine serum albumin (1 mg/ml).

Binding Assays. For testing the binding of TKF to adherent cells (J774.1, L929, 3T3, SV3T3 and B16), cells grown in a large culture dish (6 cm diameter) were treated with 0.05% trypsin in PBS containing 0.02% EDTA for 3 min at 37°C, and the resulting dissociated cells were washed and seeded in wells of 24-well flat bottomed culture plates at a density of 5 x 105 cells/well. The culture plates were incubated for 4 h at 37°C in a CO2 incubator. Then the culture medium was removed, 200 µl of fresh medium containing various concentrations of 125I-labeled TKF and 10% fetal calf serum was added, and incubation was continued at 4°C for 2 h with gentle shaking. At the end of the incubation, the medium was removed, and the monolayers of cells were washed twice with 500 µl of ice-cold medium containing 10% fetal calf serum. Then the cells were solubilized by adding 500 µl of 1% SDS solution and the radioactivity in the resulting lysate was counted in a gamma counter. For testing the binding of TKF to nonadherent EL4 cells, the preincubation step was omitted and cells were collected by centrifugation for determination of radioactivity. Nonspecific binding of 125I-labeled TKF and 10% fetal calf serum was added, and incubation was continued at 4°C for 2 h with gentle shaking. At the end of the incubation, the medium was removed, and the monolayers of cells were washed twice with 500 µl of ice-cold medium containing 10% fetal calf serum. Then the cells were solubilized by adding 500 µl of 1% SDS solution and the radioactivity in the resulting lysate was counted in a gamma counter.

Cytotoxicity Assay. Two methods were used for cytotoxicity assay. One method was measurement of release of radioactivity from target cells labeled with [3H]thymidine when incubated in the presence of TKF, as described before (5). Wells contained 2-2.5 x 104 H-labeled cells, 200 µl of culture medium containing 10% fetal calf serum, and serially diluted TKF. After incubation for 42 h at 37°C, the percentage of cytolyosis was calculated from the equation

\[
\text{% cytolyosis} = \frac{(\text{Experimental count} - \text{control count})}{(\text{Total count} - \text{control count})} \times 100
\]

where total count is the radioactivity in the supernatant after treating cells with 1% SDS, and control count is the radioactivity released in the absence of a test sample. The other method was essentially that of Drysdale et al. (7). TKF was diluted serially with culture medium...
containing 10% fetal calf serum. Then 0.1 ml of a suspension of target cells (5 × 10^4/0.1 ml) containing actinomycin D (2 μg/ml) was added to 0.1 ml of diluted TKF in 96-well microtiter plates, and the plates were incubated at 37°C for 18 h in a CO_2 incubator. The supernatant was then discarded, and adherent viable cells were stained with crystal violet solution (0.2% in 2% ethanol) for 10 min at 37°C. The plates were then rinsed well, and 0.2 ml of 1% SDS solution was added to each well to solubilize stained cells. The absorbance at 570 nm of the resulting lysates was measured. The percentage of cytotoxicity was calculated from the absorbances of the sample and control (medium treated in the same way) wells according to the equation

\[
\% \text{ cytotoxicity} = \left(1 - \frac{\text{Absorbance with sample}}{\text{Control absorbance}}\right) \times 100
\]

RESULTS

Specific Binding of TKF to L929 Cells. As reported before, L929 cells were extensively lysed when treated with TKF purified from the culture medium of J774.1 cells stimulated with Sarcophaga lectin (1), suggesting the presence of specific receptors for TKF on their surface. To demonstrate the presence of such receptors, we tested the specific binding of radioiodinated TKF to L929 cells. A single peak of labeled protein was detected when radioiodinated TKF was analyzed by SDS-polyacrylamide gel electrophoresis. This peak coincided in position with unlabeled TKF with a molecular mass of 15,000 subjected to electrophoresis simultaneously, as is shown in Fig. 1, and no appreciable radioactivity due to protein contaminating the preparation of TKF was detected. Since this ^125I-labeled TKF retained 61% of the original cytotoxic activity, we concluded that it could be used for analysis of TKF receptors.

First, we examined the specific binding of ^125I-labeled TKF to L929 cells, which was defined as competitive binding with unlabeled TKF. Binding experiments were carried out at 4°C to prevent internalization of bound TKF. The time course of binding is shown in Fig. 2A. The binding reached a plateau after 2 h, so we used these conditions in subsequent experiments. As shown in Fig. 2B, binding of ^125I-labeled TKF to L929 cells was competitively inhibited by TKF up to 95% with a 200-fold excess of TKF. This result indicated that TKF bound to specific cell surface receptors. These receptors seemed to be a protein, because cells immediately after treatment with trypsin had no ability to bind ^125I-labeled TKF. As described in "Materials and Methods," we collected target cells from master dishes by trypsin treatment and reseeded a fixed number of cells into wells of culture plates at a density of 5 × 10^3 cells/well. After preincubation for the indicated times at 37°C, the medium was removed and specific binding of ^125I-labeled TKF was measured by incubating the cells with ^125I-labeled TKF for 2 h at 4°C. The cells all adhered to the wells under these conditions. Bars, SD.

![Fig. 1. SDS-polyacrylamide gel electrophoresis of radioiodinated TKF. ^125I-labeled TKF (1 × 10^4 cpm) was subjected to electrophoresis under denaturing conditions. After electrophoresis, the gel was sliced into 20 sections and the radioactivity in each section was counted. The recovery of radioactivity from the gel was 85%. Unlabeled TKF (1 μg) was electrophoresed simultaneously with labeled TKF and located by staining the gel.](image)

![Fig. 2. Time course of binding and competitive inhibition of binding by unlabeled TKF. A, binding of ^125I-labeled TKF (1 × 10^4 cpm; 0.5 ng) to L929 cells (5 × 10^5) with time at 4°C was measured in the presence and absence of 100-fold excess of unlabeled TKF. Values are for specific binding; B, ^125I-labeled TKF was added to L929 cells simultaneously with increasing amounts of unlabeled TKF. After incubation for 2 h at 4°C, the radioactivity bound to the cells was measured. Bars, SD.](image)

![Fig. 3. Effect of trypsin on receptors of L929 cells. L929 cells were detached from the bottom of culture dishes by treatment with 0.05% trypsin solution, and the resulting dissociated cells were reseeded in a multiwell culture plate at a density of 5 × 10^3 cells/well. After preincubation for the indicated times at 37°C, the medium was removed and specific binding of ^125I-labeled TKF was measured by incubating the cells with ^125I-labeled TKF for 2 h at 4°C. The cells all adhered to the wells under these conditions. Bars, SD.](image)

![Fig. 4. A, dose-response curve of ^125I-labeled TKF binding to a fixed number of L929 cells reached a saturation level, as is shown in Fig. 4A. From the double reciprocal plot shown in Fig. 4B, the number of binding sites and the apparent K_d, for TKF binding were calculated to be 3560/cell and 4.9 × 10^-11 M, respectively. These values were comparable to those obtained with human TNF-α and L929 cells (8).](image)
TKF receptors of various cells to determine whether there was any relation between sensitivity and receptor number. As summarized in Table 1, four murine fibroblast cell lines tested were all sensitive to 4.5–840 pM of TKF and had receptors on their surface. The number of receptors varied from 2190–8760/cell, but highly sensitive LP3 cells did not have more receptors than less sensitive 3T3 cells. Normal embryonic fibroblasts were insensitive to TKF, although they bound a significant amount of 125I-labeled TKF and their number of receptors was calculated to be 1200/cell. This number was clearly less than those of sensitive cells. Murine melanoma (B16) and lymphoma (EL4) cells were insensitive to TKF, and their number of receptors was much lower than those of sensitive fibroblasts. However, since no clear relationship was found between sensitivity and receptor number in sensitive fibroblasts, it seems unlikely that TKF sensitivity depends upon the number of receptors; probably, there is no meaning in comparing the receptor numbers of these insensitive cells with those of fibroblasts, since the origins and characteristics of these cells are totally different.

The receptors of EL4 cells were found to have a very high affinity to TKF, and their apparent \( K_d \) of TKF binding was calculated to be \( 6.2 \times 10^{-12} \) M. Possibly these receptors are qualitatively different from those of other cells. J774.1 cells, a producer of TKF, showed no appreciable binding of 125I-labeled TKF. Their receptor sites may have been completely occupied by TKF produced in situ, and so no exogenously added 125I-labeled TKF could bind, since J774.1 cells are known to produce TKF receptors of various cells to determine whether there was any relation between sensitivity and receptor number. As summarized in Table 1, four murine fibroblast cell lines tested were all sensitive to 4.5–840 pM of TKF and had receptors on their surface. The number of receptors varied from 2190–8760/cell, but highly sensitive LP3 cells did not have more receptors than less sensitive 3T3 cells. Normal embryonic fibroblasts were insensitive to TKF, although they bound a significant amount of 125I-labeled TKF and their number of receptors was calculated to be 1200/cell. This number was clearly less than those of sensitive cells. Murine melanoma (B16) and lymphoma (EL4) cells were insensitive to TKF, and their number of receptors was much lower than those of sensitive fibroblasts. However, since no clear relationship was found between sensitivity and receptor number in sensitive fibroblasts, it seems unlikely that TKF sensitivity depends upon the number of receptors; probably, there is no meaning in comparing the receptor numbers of these insensitive cells with those of fibroblasts, since the origins and characteristics of these cells are totally different.

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**Table 1. Binding of TKF to various cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Specific binding (cpm x 10^6 cells)</th>
<th>( LC_{50} ) (pm)</th>
<th>Binding sites/cell</th>
<th>( K_d ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L929</td>
<td>1,596 ± 34</td>
<td>90</td>
<td>3,560</td>
<td>4.9 x 10^{-11}</td>
</tr>
<tr>
<td></td>
<td>3T3</td>
<td>2.028 ± 42</td>
<td>370</td>
<td>8,760</td>
<td>1.1 x 10^{-10}</td>
</tr>
<tr>
<td></td>
<td>SV3T3</td>
<td>463 ± 2</td>
<td>840</td>
<td>2,190</td>
<td>1.3 x 10^{-10}</td>
</tr>
<tr>
<td></td>
<td>LP3</td>
<td>704 ± 47</td>
<td>4.5</td>
<td>2,680</td>
<td>6.5 x 10^{-11}</td>
</tr>
<tr>
<td>2</td>
<td>L929</td>
<td>1,878 ± 54</td>
<td>&gt;11,700</td>
<td>800</td>
<td>1.1 x 10^{-10}</td>
</tr>
<tr>
<td></td>
<td>B16</td>
<td>229 ± 24</td>
<td>&gt;11,700</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>J774.1</td>
<td>−3 ± 9</td>
<td>&gt;11,700</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EL4</td>
<td>298 ± 2</td>
<td>&gt;11,700</td>
<td>520</td>
<td>6.2 x 10^{-12}</td>
</tr>
<tr>
<td>3</td>
<td>L929</td>
<td>1,383 ± 60</td>
<td>&gt;117,000</td>
<td>1,200</td>
<td>1.2 x 10^{-10}</td>
</tr>
<tr>
<td></td>
<td>Murine embryo fibroblasts</td>
<td>280 ± 0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD.

† Determined with crystal violet stain.

It is clear that there are both sensitive and insensitive cells with significant numbers of receptors for TKF. Then is the binding of TKF a prerequisite for subsequent cytolysis of the sensitive cells? The following experiment was made to answer this question. A fixed number of L929 cells and various amounts of 125I-labeled TKF were incubated at 4°C for 2 h to allow complete binding. The cells were then washed well with fresh medium to remove nonspecifically bound TKF, and culture was continued at 37°C for 18 h in TKF-free medium in the presence of actinomycin D, 1 µg/ml. Cytotoxicity was measured at the end of incubation, and the extent of cytolysis was plotted against the amount of TKF bound to the cells at the start of incubation. As is shown in Fig. 5, the extent of cytolysis depended upon the amount of TKF bound to the receptors, and 2–3 fmol of receptor-bound TKF was sufficient to achieve more than 90% cytolysis of 5 x 10^5 cells, indicating that binding of TKF was a prerequisite for subsequent cytolysis.

Effects of Compounds That Affect Microfilaments and Microtubules on TKF Activity. The mechanism of cytolysis by TKF is unknown, but at least two possible mechanisms may be considered. One is that TKF remains on the cell surface and some stimulus that triggers cytolysis is transmitted from the TKF-bound receptor to the cytoplasm. The other is that TKF is internalized via its receptor, and that TKF plays a role in cytolysis when it is incorporated. We examined the second possibility by testing the effects on TKF activity of substances that affect microfilaments and microtubules, assuming that these substances inhibit internalization of receptor-bound TKF and fusion of the resulting endosomes with lysosomes, respectively. We used the experimental conditions described in Fig. 5, with a dose of TKF giving 73% cytolysis, and added test substances to the culture medium at the start of incubation to see if they inhibited cytolysis.

As is evident from Table 2, reagents disrupting microfilaments (cytochalasins B and D) and microtubules (colchicine and Colcemid) significantly inhibited cytolysis. It is generally believed that reagents disrupting microfilaments inhibit endocytosis, and those disrupting microtubules inhibit the movement of endosomes, and thus the fusion of endosomes with lysosomes (9–11). Therefore, conceivably internalization of receptor-bound TKF and the subsequent fusion of the resulting endosomes with lysosomes are essential for the cytolysis. Cytolysis was inhibited dose dependently by the inhibitors tested at up to 20 µM, but higher doses were toxic to the cells.

Macromolecular synthesis was apparently not directly related to the expression of the cytotoxicity of TKF, because assays...
insensitive cells are likely to mediate other functions of TKF. We found that EL4 cells, which are insensitive lymphoma cells, have a receptor with very high affinity to TKF on their surface; namely, the $K_d$ for TKF binding to EL4 receptors was one order of magnitude lower than the values for other cells.

Substances affecting microfilaments and microtubules were found to inhibit the cytotoxicity of TKF. This finding strongly suggests that the movement of receptor-bound TKF is essential for the induction of cytolysis. Cytochalasins B and D are known to inhibit endocytosis (9), while colchicine and Colcemid inhibit the fusion of the resulting endosomes with lysosomes by inhibiting the movement of endosomes (10, 11). Therefore, we assume that internalization of receptor-bound TKF occurs, resulting in the fusion of endosomes containing receptor-bound TKF with lysosomes and that these processes are essential for expression of the cytotoxic activity of TKF. Conceivably, some degradation product of TKF produced during these processes plays a role in cytolysis, because in the case of TNF, simple microinjection of intact molecules into sensitive cells was found not to induce cytolysis (21). The present results are preliminary and indirect. Demonstration that receptor-bound TKF is actually incorporated into cells and studies on the fate of receptor-bound TKF seem important.

Recently, we found that Sarcophaga lectin is induced transiently in the early embryonic stage of Sarcophaga (22). Since this lectin is a potent inducer of TKF, a similar lectin may play a role in mammalian embryos, enhancing the production of TKF. From this point of view, the fact that embryonic fibroblasts have TKF receptors is especially interesting. It seems to be important to elucidate the other pleiotropic functions of TKF besides induction of cytolysis.

ACKNOWLEDGMENTS

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REFERENCES

1. Itoh, A., Ohsawa, F., and Natori, S. Purification of a cytotoxic protein produced by the murine macrophage-like cell line J774.1 in response to Sarcophaga lectin (5). From studies on the binding of $^{125}$I-labeled TKF to various cells, we concluded that there are specific receptors for TKF on the surface of cells, as found for TNF (8, 12−15). This conclusion was drawn because $^{125}$I-labeled TKF retained sufficient biological activity to assess specific binding and cytotoxicity. Using this TKF, we demonstrated the presence of receptor sites on various cells for which TKF competed and found that on sensitive cells such as L929 only receptor-bound TKF could induce cytolysis.

Normal embryonic fibroblasts, lymphoma EL4 cells, and melanoma B16 cells were insensitive to TKF, although they were found to have receptors. Thus, the presence of receptors may not be directly related to the sensitivity of the cells to TKF. Possibly the receptors of sensitive and insensitive cells differ qualitatively, and only the former are effective for triggering cytolysis.

Recently, TNF was found to be a multifunctional protein (16). Recombinant human TNF-$\alpha$ was shown to augment the growth of normal diploid fibroblasts (17). It was also shown to stimulate osteoclastic bone resorption and inhibit bone collagen synthesis (18). Furthermore, TNF-$\alpha$ was suggested to be the same protein as cachectin, a lipoprotein lipase inhibitor (19, 20). All these observations suggest multifunctions of TNF and the presence of qualitatively different receptors. The situation may be the same with TKF. Therefore, receptors on TKF-$\alpha$-like cytotoxic protein produced by J774.1 cells in response to Sarcophaga lectin (5). From studies on the binding of $^{125}$I-labeled TKF to various cells, we concluded that there are specific receptors for TKF on the surface of cells, as found for TNF (8, 12−15). This conclusion was drawn because $^{125}$I-labeled TKF retained sufficient biological activity to assess specific binding and cytotoxicity. Using this TKF, we demonstrated the presence of receptor sites on various cells for which TKF competed and found that on sensitive cells such as L929 only receptor-bound TKF could induce cytolysis.

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