The Active Form of Tumor Necrosis Factor Is a Trimer

(Received for publication, December 1, 1986)

Richard A. Smith* and Corrado Baglioni
From the Department of Biological Sciences, State University of New York at Albany,
Albany, New York 12222

Natural human and recombinant human and murine tumor necrosis factors (TNF) were fractionated by gel filtration chromatography on Sephadex G-75. The active form of TNF was identified by its inhibitory activity in receptor binding assays with HeLa cells and was eluted as a protein of $M_r \approx 55,000$. Radioiodinated human and murine TNF were fractionated by gel filtration into a major peak of $M_r \approx 55,000$, corresponding to a trimer, and a minor peak of $M_r \approx 17,000$, corresponding to a monomer. Binding assays showed that the trimer was at least 8-fold more active than the monomer. The human TNF partially dissociated into monomers upon addition of the nonionic detergent Triton X-100. Isolated monomers showed low binding affinity ($K_D = 70$ nM) and reduced cytotoxicity, whereas trimers showed high binding affinity ($K_D = 90$ pm) and cytotoxicity. When $^{125}$I-TNF was bound to cells, no release of monomer was detectable, suggesting that the trimer could directly bind to cellular receptors without dissociating into subunits. Further evidence for such binding was obtained by cross-linking $^{125}$I-TNF trimers with bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone. These trimers were bound to HeLa cells, could be dissociated from cellular receptors, and elicited a cytotoxic response. These results show that trimers, whether native or cross-linked, bind to receptors and are the biologically active form of TNF.

Materials and Methods

Cytotoxicity Assay—HeLa S2 cells were grown in monolayer cultures in Dulbecco's medium supplemented with 10% heat-inactivated horse serum. For each assay, $4 \times 10^4$ cells were resuspended in 0.2 ml of culture medium containing $5 \mu$g/ml cycloheximide and the indicated concentrations of hTNF. After 18 h, the medium containing dead cells was removed and adherent cells were stained with 0.2% crystal violet in 2% ethanol (19). The dye was solubilized with 33% acetic acid, and the $A_{540}$ was measured with a Titertek Multiscan (Flow Laboratories). Cytotoxicity was expressed as a percentage of the $A_{540}$ of control cells that received cycloheximide alone.

Indication and Cross-linking—Recombinant hTNF and mTNF were radioiodinated using a solid-phase lactoperoxidase procedure (20) to a specific activity of 10-58 Ci/g. $^{125}$I-TNF was cross-linked with the bifunctional reagent BSOCOES (Pierce Chemical Co.); 200 ng of $^{125}$I-hTNF in 0.1 ml of 75 mM sodium phosphate buffer, pH 7.5, containing 0.05% BSA were reacted with 1 mM BSOCOES. After 10 min at 4°C, 0.01 ml of 1 M glycine in 0.1 M sodium phosphate buffer, pH 7.5, was added. 0.1 ml samples were applied to a Sephadex G-75 column (0.7 x 24 cm) equilibrated with 10 mM PBS, 0.1% BSA, and 0.15-0.20 ml fractions were collected.

Binding Assays—In competitive binding experiments, 0.3-0.5 ng of radioligand were incubated 5 h at 4°C with $1 \times 10^5$ cells in 0.15 ml of medium containing 5 mM MgCl$_2$ and 40 mM HEPES, pH 7.5, as previously described (20). In experiments designed to recover cell-bound radioligand, the binding assays were proportionately increased 10-fold to 1.5 ml. Following binding, the cells were centrifuged at 4°C and washed twice with 1 ml of PBS. To dissociate TNF-receptor complexes, 5 ml of 6 M GdnHCl, 0.1 M sodium phosphate buffer, pH 7.5, were added to 10$^7$ pelleted cells for 10 min at 4°C. Lysed cells were diluted with 0.1 ml of 10 mM PBS, 0.1% BSA and centrifuged for 10 min at 15,000 x g. The supernatants were chromatographed on Sephadex G-75 columns. Bindability was determined by incubating radioligands with graded amounts of excess cellular receptors, and the results were expressed as the maximum percentage of counts added that were specifically bound to HeLa S2 cells at 4°C.

Zonal Centrifugation Studies—0.3 ng of $^{35}$I-hTNF and 0.5 mg of reference protein in 0.1 ml of PBS were layered on 5-20% linear sucrose gradients. The gradients were centrifuged for 24 h at 44,000 rpm in an SW 50 rotor at 5°C, and fractions containing 1 drop were collected. Ovalbumin ($M_r = 45,000$) and BSA ($M_r = 66,000$) were used as reference proteins.

Results

Gel filtration on Sephadex G-75 was used to determine the $M_r$ value of native TNF and to establish whether iodination altered its size. Accordingly, the elution profiles of native hTNF and mTNF were compared with those of $^{125}$I-hTNF and $^{125}$I-mTNF (Fig. 1). Since small amounts of TNF were chromatographed in the presence of carrier protein, the TNF was localized by its inhibitory activity in receptor binding assays. Both recombinant TNF eluted as a single major peak (Fig. 1A). The elution profile of natural hTNF (a gift of Dr. Walter Fiers, University of Ghent) was indistinguishable from that of recombinant hTNF (data not shown). Radioiodinated hTNF and mTNF eluted as a major peak (#1) in corresponding fractions, followed by a minor peak (#2) and free $^{125}$I (Fig. 1B).
by the addition of Triton X-100 into monomers, which are binding to HeLa cells; 1,180 protein of relatively small amounts in physiological solutions, different protein of each fraction were tested for specifically bound. In subsequent experiments, the binding in a Sephadex G-75 column (0.7 cm) pre-equilibrated with PBS, 0.1% BSA for binding assays or culture medium for cytotoxicity assays. Triton X-100 was not detected (22) in the trimer or monomer peak, but was eluted at a greater column volume than hTNF monomer. In separate experiments (data not presented), it was shown by gel filtration analysis that hTNF trimers partially dissociated upon addition of low concentrations of the nonionic detergent Triton X-100. This dissociation was dependent on hTNF concentration, since the monomer/trimer ratio increased at low hTNF concentration (Fig. 2). Monomers were separated from trimers by gel filtration in a Sephadex G-75 column (0.7 cm) pre-equilibrated with either PBS, 0.1% BSA for binding assays or culture medium for cytotoxicity assays. Triton X-100 was not detected (22) in the trimer or monomer peak, but was eluted at a greater column volume than hTNF monomer. In separate experiments (data not presented), it was shown by gel filtration analysis that hTNF trimers (1 ng/ml) prepared in this manner quantitatively reassociated to trimers when the hTNF concentration was increased.

Pooled fractions of hTNF trimer and monomer were subsequently compared in competitive binding and cytotoxicity assays on an equal counts/min basis. The monomer fraction showed low binding activity and cytotoxicity compared to the trimer fraction. Binding of monomer was about 5-fold lower than that of trimer, as determined in competition binding assays (Fig. 3A). Scatchard plots of these data showed that the trimer was bound with a $K_d = 90$ pM, whereas only a small component of the monomer fraction was bound with such high affinity (Fig. 3A, inset). Most of the monomer was bound with low affinity ($K_d = 70$ nM). It seems unlikely that this binding has biological relevance, since 50% cytotoxicity of HeLa cells is observed with 2 pM hTNF (20). In parallel cytotoxicity assays, a monomer concentration 6-7-fold greater than that of trimer was needed to elicit the same biological response when tested at low concentrations (Fig. 3B). However, at the highest concentrations tested, the cytotoxicity of monomer was nearly equivalent to that of trimer. Rechromatography of the monomer fraction at the end of the incubation period showed the presence of about 10% trimer (data not shown), which could account for both the small component binding with high affinity and for the cytotoxicity at the
highest concentrations tested. These results indicated that hTNF trimer binds with higher affinity to receptors and has greater cytotoxic activity than hTNF monomer.

In the following experiment, we examined whether hTNF dissociates into monomers upon binding to receptors. A binding assay was carried out at 4 °C with low 125I-hTNF concentration, and the supernatant obtained after spinning out the cells was analyzed by gel filtration. The trimer peak was reduced in proportion to the 125I-hTNF bound to the cells, but no increase in the monomer peak could be detected (Fig. 4A). This result suggested that hTNF trimers could directly bind to receptors, but it could not be excluded that monomers or dimers were binding and that the subunits released were reassociating into trimers. Therefore, to demonstrate that trimers can bind to cells and have biological activity, the hTNF was cross-linked to prevent its dissociation.

The 125I-hTNF was reacted with the cross-linking reagent BSOCOES and compared to control 125I-hTNF by gel filtration chromatography, binding to HeLa cell receptors, and cytotoxicity assays. The cross-linked 125I-hTNF eluted with Mₜ ~ 55,000 even after treatment with 3 M GdnHCl (Fig. 4B). This demonstrated that cross-linking stabilized hTNF against dissociation. In contrast, 85% of the control 125I-hTNF treated with 3 M GdnHCl eluted with an Mₜ = 17,000 (Fig. 4C). This dissociation was in large part reversible, since after dialysis 125I-hTNF eluted as a trimer (Fig. 4D). These experiments showed that hTNF cross-linked with BSOCOES is a trimer resistant to dissociation by relatively strong denaturing reagents, such as GdnHCl. However, drastic denaturing treatment of the cross-linked trimer, such as boiling in 1% sodium dodecyl sulfate under reducing conditions, resulted in partial dissociation into dimers and monomers, as judged by gel electrophoresis (Fig. 4, inset).

A binding assay carried out with cross-linked hTNF showed that it could bind to TNF receptors of HeLa cells. These cells were treated with 3 M GdnHCl to release bound BSOCOES-125I-hTNF, and the supernatant was analyzed by gel filtration. A single peak of radioactivity was present (Fig. 4E), demonstrating that the bound hTNF could be eluted from HeLa cell receptors as a trimer. In order to determine whether cross-linking or aylation of amino groups had altered its binding to cell receptors, BSOCOES-125I-hTNF was compared with 125I-hTNF in competitive binding assays (Fig. 5). Since BSOCOES-125I-hTNF had lower bindability (39% compared to 45% for 125I-hTNF), equivalent amounts of bindable radioligands were added. The binding of both ligands was inhibited in a parallel manner by unlabeled hTNF, but only half as much unlabeled hTNF was required for 50% competition of BSOCOES-125I-hTNF. This indicated that chemical changes introduced by the cross-linking reagent resulted in partial loss of binding activity. However, binding was 90% specific for both ligands. In agreement with the loss of binding activity, the cytotoxicity of BSOCOES-125I-hTNF for HeLa cells was on average 4.5-fold less than that of control 125I-hTNF. A similar 5–10-fold decrease in cytotoxicity of BSOCOES-125I-hTNF was observed in experiments with SK-MEL-109 melanoma cells (data not shown). These results with cross-linked TNF confirmed the findings with native TNF by showing that stable TNF trimers bind to cellular receptors and elicit a biological response.

**DISCUSSION**

Natural human TNF, recombinant hTNF, and mTNF, and cross-linked 125I-hTNF coelute in gel filtration under non-denaturing conditions as a major peak with an apparent Mₜ ~ 55,000 (Figs. 1 and 4). The formation of homotrimer from recombinant TNF exist predominantly as a trimer under physiological conditions.

Gel filtration analyses show that monomers are present as
a small component of radioiodinated TNF (Fig. 1). The isolated monomers are only 12% as active as trimers in binding assays. Furthermore, 125I-hTNF can be dissociated into monomers by several treatments, such as a short incubation at pH 3.0. Several reports have indicated that the biological activity of TNF is pH-sensitive (6, 9, 11, 24). Treatment with 3 M GdnHCl also dissociates 125I-hTNF (Fig. 4C). Of particular interest is the finding that low concentrations of the nonionic detergent Triton X-100 partially dissociate hTNF into monomers (Fig. 2), suggesting that weak hydrophobic interactions may be responsible for stabilizing the trimers (25). Dissociation by Triton X-100 can be used in combination with gel filtration chromatography to obtain monomer and trimer fractions for competitive binding and cytotoxicity assays.

These 125I-hTNF monomers show low receptor binding activity when compared to trimers. Monomer binding is characterized by high and low affinity components. The high affinity component is the same as that observed for the trimer, but rechromatography of monomer fractions shows the presence of some trimers. Therefore, small amounts of contaminating trimers may account for the high affinity binding component. In contrast, the trimer exhibits a single high affinity binding and greater cytotoxicity than monomer. At low concentrations, the cytotoxicity of the monomer fraction is 6-7-fold less than that of the trimer, but at higher concentrations monomer cytotoxicity becomes equivalent to that of trimer. This finding may be explained by the reassociation of monomers into active trimers. Other reports suggest that TNF monomers and oligomers are all active (1, 4, 12, 28).

The most direct evidence that hTNF trimer is biologically active comes from experiments wherein 125I-hTNF is cross-linked with BSOCOES. The cross-linked hTNF binds to receptors (Fig. 5) and is cytotoxic. Moreover, cross-linked hTNF trimers are recovered after binding to cells (Fig. 4E). In view of the remarkably low concentrations of hTNF that are biologically active in cytotoxicity assays (27), we are lead to speculate that these trimers may interact simultaneously or sequentially with more than one receptor. A possible result of such multiple interactions may be a heightened effective concentration at the cell surface (28). Furthermore, simultaneous binding to neighboring receptors might favor the interaction of the cytoplasmic domain of receptors (29) and either trigger or amplify the as-yet unknown signaling mechanism of the TNF receptor. In addition, dissociation of TNF into monomers at low concentrations may have some physiological relevance in the action of this factor. Since the monomer appears to be less active than the trimer, this dissociation may limit some of the deleterious effects of TNF (30) at sites remote from those where it is produced in high amounts by macrophages (31).

REFERENCES

1. Aggarwal, B. B., Kohr, W. J., Hass, P. E., Moffat, B., Spencer, S. A., Henzel, W. J., Bringman, T. S., Nedwin, G. E., Goeddel, D. V., and Harkins, R. N. (1985) J. Biol. Chem. 260, 2345-2354
2. Baglioni, C., McCandless, S., Tavernier, J., and Fiers, W. (1985) J. Biol. Chem. 260, 13385-13397
3. Shirai, T., Yamaguchi, H., Ito, H., Todd, C. W., and Wallace, B. (1985) Nature 313, 803-806
4. Williamson, B. D., Carswell, E. A., Rubin, B. Y., Prendergast, Y. S., and Old, L. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5397-5401
5. Marmenout, A., Fransen, L., Tavernier, J., Van der Heyden, J., Tizard, R., Kawashima, E., Shaw, A., Johnson, M.-J., Semon, D., Muller, R., Ruyschsaert, M.-R., Van Vliet, A., and Fiers, W. (1985) Eur. J. Biochem. 152, 515-522
6. Beutler, B., Mahoney, J., Le Trang, N., Pekala, P., and Cerami, A. (1985) J. Exp. Med. 161, 984-995
7. Green, S., Dobrjansky, A., Carswell, E. A., Kassel, R. L., Old, L. J., Fiore, N., and Schwartz, M. K. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 381-385
8. Kull, F. C., Jr., and Cuatrecasas, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7932-7936
9. Takeda, Y., Shirakawa, S., Sugimoto, M., Woo, H.-J., Higuchi, M., and Osawa, T. (1985) Cell. Immunol. 96, 277-289
10. Haranaka, K., Satomi, N., Sakurai, A., and Nariuchi, H. (1985) Int. J. Cancer 36, 396-400
11. Ruff, M., and Gifford, G. E. (1980) J. Immunol. 125, 1671-1677
12. Kull, F. C., Jacobs, S., and Cuatrecasas, P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5756-5760
13. Rubin, B. Y., Anderson, S. L., Sullivan, S. A., Williams, B. D., Carswell, E. A., and Old, L. J. (1985) J. Exp. Med. 162, 1099-1104
14. Tsujimoto, M., Yip, Y. K., and Viček, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7626-7630
15. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3666-3678
16. Williamson, B. D., Carswell, E. A., Rubin, B. Y., Prendergast, J. S., and Old, L. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5397-5401
17. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A., and Shepard, H. M. (1986) Science 230, 943-945
18. Viček, J., Palombella, V. J., Henryksen-DeStefano, D., Swenson, C., Feinman, R., Hirai, M., and Tsujimoto, M. (1986) J. Exp. Med. 163, 632-643
19. Zacharchuk, C. M., Drysdale, B. E., Meyer, M. M., and Shin, H. S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6341-6345
20. Smith, R. A., Kirstein, M., Fiers, W., and Baglioni, C. (1986) J. Biol. Chem. 261, 14871-14874
21. Martin, R. G., and Ames, B. N. (1981) J. Biol. Chem. 236, 1372-1379
22. Yoshida, K., Ishihara, K., Ohara, S., Asoomo, Y., and Hotta, K. (1980) Anal. Biochem. 106, 162-165
23. Daves, G. E., and Stark, G. R. (1970) Proc. Natl. Acad. Sci. U. S. A. 66, 651-656
24. Wang, A. M., C reasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., Van Arsdell, J. N., Yamamoto, R., and Mark, D. F. (1985) Science 228, 149-154
25. Hahnen, A., McCaslin, D. R., Fries, E., and Tanford, C. (1979) Methods Enzymol. 56, 734-749
26. Fahn, T., Toker, L., Budilovsky, S., Aserkta, D., Eshhar, Z., and Wallach, D. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3814-3818
27. Ruff, M. R., and Gifford, G. E. (1981) Infect. Immun. 31, 380-385
28. Goldstein, L. (1978) Methods Enzymol. 44, 397-443
29. Heffets, D., and Zick, Y. (1986) J. Biol. Chem. 261, 889-894
30. Tracey, K. J., Beutler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Miliksk, I. W., HÄHR, R. J., Fahey, T. J., Zentella, A., Albert, J. D., Shires, G. T., and Cerami, A. (1986) Science 234, 470-474
31. Beutler, B., and Cerami, A. (1986) Nature 320, 584-588