Brief Definitive Report

Binding and Regulation of Cellular Functions by Monoclonal Antibodies against Human Tumor Necrosis Factor Receptors

By M. R. Shalaby, A. Sundan, H. Loetscher,* M. Brockhaus,* W. Lesslauer,* and T. Espevik

*From The Institute of Cancer Research, University of Trondheim, N-7006 Trondheim, Norway; and *Central Research Units, F. Hoffman-La Roche, CH-4002 Basel, Switzerland.*

Summary

The present study was undertaken to further characterize the interaction of monoclonal antibodies (mAbs) against tumor necrosis factor (TNF) receptors with different targets, and to assess their ability to influence TNF effects on U937 and human endothelial cell (HEC) functions. Actions of recombinant TNF-α on U937 and HEC were effectively inhibited by Htr-5 and Utr-1, and to a greater extent by a combination of both mAbs. These observations indicate that TNF interaction with antigenically different components of membrane receptors (p55 and p75) represents a crucial step in transduction of signals for TNF toxicity against U937 and TNF activation of HEC functions.

TNF-α, also called cachectin, is a protein produced mainly by macrophages (1). Besides its antitumor activities, TNF-α has a multitude of biologic and immunologic functions (2–5). Recent studies showed the existence of at least two distinct cell surface receptors for human TNF-α (6). The same investigators raised mAbs against TNF-binding proteins from HL60 and U937 cells, designated Htr and Utr mAbs, respectively (7). Studies of these mAbs showed that Htr-5 and Htr-9 recognize a TNF-binding protein of ~55 kD, whereas Utr-1 specifically interacts with a protein of ~75 kD molecular mass (7). More recently, both human TNF receptors have been cloned and expressed, and were shown to have binding properties similar to their native counterparts (8–10). The existence of two antigenically distinct TNF-binding proteins points to possible differences in signal transduction after TNF interaction with each of these receptors. Accordingly, we extend our previous studies (11) in order to examine the ability of Htr-5 and Utr-1 to influence TNF-α interaction and the ensuing biologic effects on U937 and endothelial cells.

Materials and Methods

Cytokines and mAbs. The rTNF-α (1) used in this study (Genentech, Inc., San Francisco, CA) was >98% pure and had a specific activity of 4.8×10^7 U/mg rTNF-α, as determined in a standard cytotoxicity bioassay (12). The generation of mAbs Htr-5, Htr-9, and Utr-1 against human TNF receptors from HL60 and U937 cells has been reported (6, 7). Biotinylation of mAb and rTNF-α was performed as described (13).

Cells. U937 and HL60 cells were obtained from American Type Culture Collection, Rockville, MD. Human endothelial cells (HEC) were obtained by collagenase treatment of human umbilical cord veins and seeded at uniform density (1.5×10^5/well) in 24-well plates as detailed previously (14, 15).

Flow Cytometric Analysis of TNF Receptor mAbs Binding. Aliquots of 10^6 cells were mixed with biotinylated (B) mAb (10 μg/ml) and streptavidin PE, and analyzed by FACS essentially as described (11). Experiments involving the blocking of BmAb binding were performed by pretreating the cells with rTNF-α (50 μg/ml) for 45 min before addition of the biotinylated reagents.

Cytotoxicity Assay. 100 μl of ^{51}Cr-labeled U937 cells were seeded in each well of FB microtiter plates (Costar, Cambridge, MA) together with 100 μl of complete medium containing various concentrations of rTNF-α with or without mAbs to TNF receptors (10 μg/ml). After 20 h of incubation, supernatants were collected, and their radioactivity was determined. Percent cytotoxicity expressed as specific lysis was calculated as follows: percent specific lysis = $100\times (A - B)/(C - B)$; where A represents the mean cpm in test supernatants, B represents the mean cpm in supernatants of targets alone (spontaneous ^{51}Cr release), and C represents the mean cpm in supernatants of targets lysed with 0.25% SDS (maximum ^{51}Cr release).

HEC Assays. TNF receptor mAbs or rTNF-α were used for activation of confluent HEC monolayers for IL-6 production and for adhesion of ^{51}Cr-HL60 cells essentially as described previously (11, 15). Brief descriptions of these procedures are given below (see Tables 1 and 2).

Results

Binding of TNF Receptor mAbs to U937 Cells. The binding of BUtr-1 and BHtr-9 to cells was examined in a series of experiments. Representative results in Fig. 1 show that the two mAbs bound to U937, indicating that these cells express both Htr and Utr types of TNF receptors, though to

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/11/1517/04 $2.00
Volume 172 November 1990 1517–1520

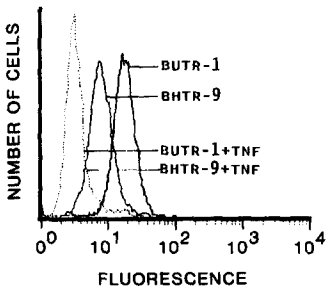

Figure 1. Binding of Utr-1 and Htr-9 to U937. Flow cytometric analysis of U937 stained with BUtr-1 or BHtr-9 (10 μg/ml). The dotted curves represent histograms obtained by analysis of cells pretreated with rTNF-α (50 μg/ml) before staining with respective antibody.

different extents. Compared with BHtr-9, more pronounced binding of BUtr-1 to U937 was evident, as reflected by greater fluorescence intensity (Fig. 1). The data also show that U937 pretreated with rTNF for 45 min completely lacked the ability to bind to BUtr-1 or BHtr-9. The results of additional binding experiments (not shown) demonstrated that pretreatment of U937 cells with Htr-9 resulted in a slight reduction of BrTNF binding, whereas Utr-1 caused an almost complete inhibition of BrTNF binding, indicating that Utr-1 can occupy (and block) a predominant structure that is involved in the interaction of TNF with U937. These data are consistent with results published by Brockhaus et al. (7), and indicate the heterogenous nature of membranal structures involved in TNF interaction with U937.

Inhibition of TNF-α Cytotoxicity on U937 by TNF Receptor mAbs. Based on the results presented in Fig. 1, it was of interest to examine whether mAb Utr-1 could influence the cytotoxicity induced by TNF on U937. The results (Fig. 2) show that Utr-1 is just as effective as Htr-5 in causing

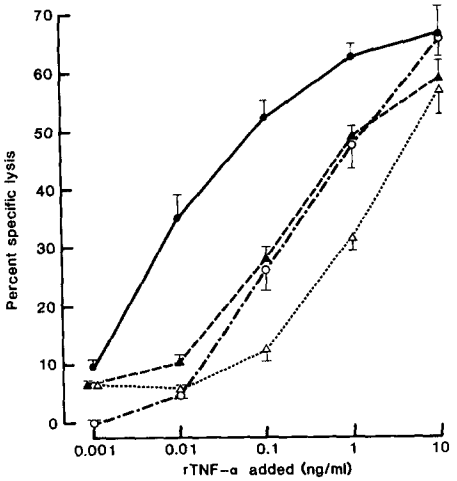

Figure 2. Inhibition of rTNF-α cytotoxicity by Utr-1 and Htr-5. ^{51}Cr-U937 were incubated in the absence or presence of various concentrations of rTNF-α (●) with or without the addition of Utr-1 (○), Htr-5 (▲), or both (△). After 20 h, specific cell lysis was determined based on the amount of radioactivity released in the supernatant, as detailed in Materials and Methods.

Table 1. *The influence of TNF Receptors mAbs on rTNF-α Stimulation of IL-6 Production from HEC*

Agent(s) added to HEC culture	IL-6	Stimulation index
	pg/ml	
None	275	1.0
Htr-5 (2.5 μg/ml)	425	1.54
Utr-1 (2.5 μg/ml)	333	1.21
Both mAbs	333	1.21
rTNF-α (10 U/ml)	1,162	4.22
rTNF-α + Htr-5	691	2.50
rTNF-α + Utr-1	675	2.45
rTNF-α + both mAbs	408	1.48

Cultures of confluent HEC monolayers were incubated in duplicate with and without the addition of various agents as indicated. IL-6 activity in 24-h supernatants was measured, and the data are presented as the mean of triplicate IL-6 determinations; SD ≤ 10%. Stimulation index is calculated by dividing IL-6 values from test cultures by IL-6 value in control cultures.

Table 2. *Regulation of Tumor Cell Adherence to Endothelial Cells by TNF Receptors mAbs*

Treatment of HEC cultures	Percent adherence of HL60
None	16
rTNF-α (1 U/ml)	45
Htr-9 (1 μg/ml)	36
Htr-5 (2 μg/ml)	18
Utr-1 (2 μg/ml)	17
rTNF-α (1 U/ml) + Htr-5 (2 μg/ml)	38
rTNF-α (1 U/ml) + Utr-1 (2 μg/ml)	24
rTNF-α (1 U/ml) + both mAbs	16
Htr-9 + Htr-5	14
Htr-9 + Utr-1	37
Htr-9 + both mAbs	16

Cultures of confluent HEC monolayers were incubated in the absence or presence of various agents as indicated. After 4 h, the cultures were washed twice with HBSS, and each culture received 0.5 × 10^5 ^{51}Cr-HL60 in 0.25 ml medium and was further incubated for 1 h. Nonadherent cells were aspirated, the cultures were washed, and adherent ^{51}Cr-HL60 were lysed for determination of retained radioactivity. Each treatment was performed in duplicate, and the mean cpm from four lysate aliquots counted is expressed as a percentage of the mean maximum cpm.

significant inhibition of TNF cytotoxicity, and that greater inhibition can be obtained by a combination of both mAbs.

Regulation of TNF-α Actions on Endothelial Cells by TNF Receptor mAbs. A series of experiments was performed to examine whether Utr-1 alone and in combination with Htr-5 can regulate the effects of TNF-α on HEC functions. Consistent with data reported recently (15), the results in Table 1 show that rTNF-α caused a significant stimulation of IL-6 production after 24 h of incubation. rTNF-α stimulation of IL-6 production was reduced substantially in the presence of Htr-5 or Utr-1, and to a greater extent when both mAbs were added to the cultures. Furthermore, the ability of Htr-5 and Utr-1 to affect rTNF-α-induced adhesiveness of HEC was tested in an adhesion assay using ^{51}Cr-HL60, and the results are presented in Table 2. Monolayers of HEC treated with 1 U/ml of rTNF-α or 1 μg/ml of Htr-9 (which is known to mimic TNF-α effect in this system [11]) showed an increased adhesiveness amounting to two- to threefold greater than control value, as reflected by the adherence of ^{51}Cr-HL60. Htr-5 or Utr-1 by themselves did not increase HEC adhesiveness. However, the presence of either Htr-5 or Utr-1 partially reduced rTNF-α effects, and the presence of both mAbs inhibited completely the effects of rTNF-α to control level. These results indicate that HEC express both Htr and Utr types of TNF receptors. Further, while Htr-5 completely blocked Htr-9 effects (Table 2), the presence of Utr-1 did not interfere with Htr-9-induced adhesiveness, indicating the lack of crossreactivity between Utr-1 and Htr-9.

Discussion

In the present study, we examined further the nature of TNF interaction with U937 and HEC, and the ensuing biologic response in the presence of mAbs, particularly Utr-1, which has been shown to recognize a TNF-binding protein of ~75 kD (p75) in myeloid cells (7). Previously, we showed that mAb Htr-9 bound to U937 (11), and here we confirm this result and further demonstrate that biotinylated Utr-1 bound efficiently to U937. Flow cytometric analysis of U937 cells stained with BmAbs with and without pretreatment with TNF indicate that the predominant molecular component of TNF receptors in U937 cells is a molecular structure with specificity for Utr-1.

Previously, we demonstrated that the p55 TNF receptor is involved in mediating TNF actions on U937, HEC, and fibroblasts (11). The present study shows that mAbs Htr-5 and Utr-1 can protect U937 cells to an equal extent against the cytotoxic effects of TNF, and that greater protection can be obtained by a combination of both mAbs. These results indicate that both p55 and p75 molecular components of the TNF receptors are involved in the transduction of signals leading to the cytotoxic action of TNF. This explanation is supported by the fact that Htr-5 and Utr-1 protected U937 against TNF cytotoxicity. It is interesting to note that at 0.01 ng/ml TNF (Fig. 2), either Htr-5 or Utr-1 tested alone completely blocked the cytotoxic action of TNF, while at relatively higher concentrations of TNF (0.1 ng/ml), the combination of both mAbs was required. Of relevance to these results is the observation that the blocking by Utr-1 of the p75 receptor decreases the affinity of TNF binding to the p55 receptor on U937 cell surface (Sundan, A., unpublished results). Similar data of decreased TNF affinities to the p55 receptor were also obtained in experiments of TNF binding to HL60 in the presence of Utr-1 (Hohmann, P., manuscript in preparation). In the present study, a decrease in the TNF affinity to one type of receptors, due to the blocking of another receptor by mAb, may explain the complete reversal of the cytotoxic action noted when low concentrations of TNF are used (Fig. 2). Under otherwise similar experimental conditions, excess TNF (i.e., higher concentrations) may compensate for the decrease in the affinity of TNF binding to its target leading to partial, rather than complete, inhibition of TNF action. The observation that TNF can exert an effect on U937 at a low concentration of 0.01 ng/ml (Fig. 2), which is equivalent to ~0.58 pM, is of interest. This result suggests that an extremely low occupancy of both types of TNF receptors may be sufficient to trigger intracellular events. Furthermore, both Htr-5 and Utr-1 antibodies inhibited TNF stimulation of IL-6 production by HEC, and together caused a complete inhibition of TNF-induced adhesiveness of HEC monolayers. It is tempting to suggest that the activation of endothelial cells by TNF is also mediated by TNF interaction with both the Htr (p55) and the Utr (p75) types of TNF receptors. The importance of this observation is further emphasized in light of the fact that the inflammatory nature of TNF is in part due to its ability to influence endothelial cell functions (4, 5). In this regard, mAbs Htr-5 and Utr-1 may have a potential use as antiinflammatory agents by virtue of their abilities to suppress TNF-associated endothelial activation.

Collectively, the data in this report demonstrate that TNF interaction with both Htr (p55) and Utr (p75) types of receptors can trigger cytotoxic- (U937) as well as cell function–enhancing effects (HEC). Signals responsible for induction of different TNF actions may therefore be conveyed via distinct post-receptor pathways.

Address correspondence to M. R. Shalaby, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080.

Received for publication 3 July 1990 and in revised form 13 August 1990.

References

1. Pennica, D., G.D. Nedwin, J.S. Hayflick, P.H. Seeburg, R. Derynck, M.A. Palladino, W.J. Kohr, B.B. Aggarwal, and D.V. Goeddel. 1984. Human tumor necrosis factor: precursor structure expression and homology to lymphotoxin. *Nature (Lond.).* 312:724.
2. Cerami, A., and B. Beutler. 1988. The role of cachectin/TNF in endotoxic shock and cachexia. *Immunol. Lett.* 9:28.
3. Wong, G.H.W., and D.V. Goeddel. 1986. Tumor necrosis factor α and β inhibit virus replication and synergize with interferons. *Nature (Lond.).* 323:819.
4. Gamble, J.R., J.M. Harlan, S.J. Klebanoff, and M.A. Vadas. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA.* 82:8667.
5. Shalaby, M.R., M.A. Palladino, Jr., S.E. Hirabayashi, T.E. Eessalu, G.D. Lewis, H.M. Shepard, and B.B. Aggarwal. 1987. Receptor binding and activation of polymorphonuclear neutrophils by tumor necrosis factor-alpha. *J. Leukocyte Biol.* 41:196.
6. Hohmann, H.P., R. Remy, M. Brockhaus, and A.P.G.M. van Loon. 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNF-α). *J. Biol. Chem.* 264:14927.
7. Brockhaus, M., and H. Loetscher. 1990. Identification of two types of TNF receptors on human cell line by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 87:3127.
8. Loetscher, H., Y.E. Pan, H. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer. 1990. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell.* 61:351.
9. Schall, T.J., M. Lewis, K.J. Koller, A. Lee, G.C. Rice, G.H.W. Wong, T. Gatanaga, G.A. Granger, R. Lentz, H. Raab, W.J. Kohr, and D.V. Goeddel. 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell.* 61:361.
10. Smith, C.A., T. Davis, T. Anderson, L. Solam, M.P. Beckmann, R. Jerzy, S.K. Dower, D. Cosman, and R.G. Goodwin. 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science (Wash. DC).* 248:1019.
11. Espevik, T., M. Brockhaus, H. Loetscher, U. Nonstad, and R. Shalaby. 1990. Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. *J. Exp. Med.* 171:415.
12. Kramer, S.M., and M.E. Craver. 1986. Serum-free in vitro bioassay for the detection of tumor necrosis factor. *J. Immunol. Methods.* 93:210.
13. Updyke, T.V., and G.L. Nicoloson. 1984. Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices. *J. Immunol. Methods.* 73:83.
14. Wall, R.T., L.A. Harker, L.J. Quadruci, and G.E. Striker. 1978. Factors influencing endothelial cell proliferation in vitro. *J. Cell. Physiol.* 96:203.
15. Shalaby, M.R., A. Waage, and T. Espevik. 1989. Cytokine regulation of interleukin-6 production by human endothelial cells. *Cell. Immunol.* 121:372.