Expression of the Types A and B Tumor Necrosis Factor (TNF) Receptors Is Independently Regulated, and Both Receptors Mediate Activation of the Transcription Factor NF-κB

TNFα IS NOT NEEDED FOR INDUCTION OF A BIOLOGICAL EFFECT VIA TNF RECEPTORS*

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The expression and biological function of the types A and B tumor necrosis factor (TNF) receptors were studied using three cell types. SW480T, HEP2, and HL60 cells had, respectively, mainly the type A, only the type B, and roughly similar amounts of both receptors. Dibutyric cAMP treatment induced a 3-6-fold increase in the amount of the type A receptor in HL60 cells without affecting the amount of the type B receptor. Expression of both receptors can thus be regulated independently. HEP2 and human umbilical vein endothelial cells only showed the type B receptor, and expression of the type A receptor could not be induced in these cells. HL60 cells showed, upon Scatchard analysis, a single binding site for TNFα, and its Kd may correspond to that of the type A receptor. The approximately 7-fold lower affinity of TNFα binding to the type B receptor of HL60 cells was only detected after blocking all TNFα binding to the type A receptor. Both the types A and B receptors mediated TNFα-induced activation of the transcription factor NF-κB. The agonistic antibody htr9 to the type B receptor also activated NF-κB. Thus, signal transduction via the type B receptor may only require interaction with the receptor's extracellular domain.

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1 The abbreviations used are: TNFα, tumor necrosis factor α; cachectin; IFNγ, interferon gamma; utr1, monoclonal antibody specific for the type A TNF receptor; htr9, monoclonal antibody specific for the type B TNF receptor; HUVEC, human umbilical cord endothelial cells; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Baltimore, 1989), but the importance of NF-κB in these processes is as yet unclear.

The majority of human cells have specific high affinity binding sites for TNFα (Kull et al., 1985; Scheurich et al., 1986). We showed previously that at least two different TNFα receptor proteins exist. The type A TNFα receptor was characterized by a 100-kDa cross-linked product between TNFα and the receptor protein. The type B receptor showed a major cross-linked product of 75 kDa and a minor product of 95 kDa (Hohmann et al., 1989). The 75-kDa product may consist of one molecule of TNFα whereas the 95-kDa band contains two TNFα molecules (Smith and Baglioni, 1989). The type A receptor was the major TNFα receptor on myeloid cells, of which at least HL60 cells contained some type B receptor in addition to the type A receptor. The type B receptor was found on several epithelial cell lines such as HEp2, MCF7, and HeLa cells. These cells did not contain any detectable type A receptor (Hohmann et al., 1989). The characterization of two receptor types was later confirmed and extended by Brochhaus et al. (1990) using receptor type-specific monoclonal antibodies. The types A and B TNF receptors were shown to be proteins with apparent molecular masses of 75 and 55 kDa, respectively. Recently, two groups independently reported the cloning of the same TNF receptor gene corresponding to the type B receptor (Łotscher et al., 1990; Schall et al., 1990).

TNF receptors mediate the cellular effects of TNFα. A decrease in the number of cell surface receptors correlated with a decreased sensitivity to the cytolytic effect of TNFα in certain cells (Lehmann and Droge, 1986; Holtmann and Wallach, 1987), and half-maximal release of interleukin-1 by endothelial cells was seen with the same concentration of TNFα which showed half-maximal binding to the cells (Locksley et al., 1987). Recently, Espevik et al. (1990) reported the induction of several biological effects normally induced by TNFα by incubation of cells with an agonistic antibody to the type B TNF receptor.

In this paper, we determined the ratios of the types A and B TNF receptors on different cells and show that the expression of both receptor types can be regulated independently and that both receptors are biologically active.

MATERIALS AND METHODS

Cell Culture and Stimulation of Cells—The human cell lines HL60 and HEp2 were obtained from ATCC and maintained over many passages in RPMI 1640 medium, supplemented with 10% (v/v) fetal calf serum or 10% (v/v) horse serum. A variant of SW480 cells, SW480T, which was selected for high numbers of TNF receptors, was kindly provided by Dr. T. Espevik, University of Trondheim. SW480T cells express about 10 times more TNF binding sites on their cell surface compared with HL60 cells. Primary cell cultures of human umbilical vein endothelial cells (HUVEC) were made essentially as described (Jaffe et al., 1973), and cells derived from a single donor were analyzed in each experiment. The adherent HEp2 and SW480T cells were grown to near confluence before stimulation. The non-adherent HL60 cells were stimulated after reaching a density of 1–1.5 × 10⁶ cells/ml. Cells were incubated with either recombinant human IFNγ (Poultoukas et al., 1989) or TNFα (Hohmann et al., 1989). Both cytokines were purified from Escherichia coli cells as described. The concentrations used here refer to monomeric IFNγ and TNFα. The active forms of IFNγ and TNFα may, however, be dimers and trimers, respectively. The exact conditions of stimulation are indicated in the figure legends.

Prescission of Cells with Monoclonal Antibodies to the Types A and B TNF Receptor, Receptor Up-regulation and Binding and Cross-linking of [125I]TNFα—HL60 cells (1 × 10⁷ cells/ml) were incubated with culture medium supplemented with 1 μg/ml unlabeled TNFα or 10 μg/ml of the indicated monoclonal antibodies to TNF receptors. After reaching near confluence the adherent cell lines HEp2 and SW480T were incubated in 10-cm² dishes with 1 ml of culture medium supplemented with 1 μg/ml unlabeled TNFα or 10 μg/ml indicated monoclonal antibody/dish if not stated otherwise. After washing with PBS at 0°C, 1 nM [125I]TNFα was added, and incubation was continued for an additional 2 h. Cells were then used for cross-linking experiments using the chemical cross-linker biricoumimido-1,4-bisulfonic acid (Pierce Chemical Co.) as described previously (Hohmann et al., 1989). After cross-linking, adherent cells were released by treatment with 0.1% (v/v) Triton X-100 containing gel sample buffer. For Scatchard analysis, HL60 cells and HEp2 cells that were detached from the culture flask by treatment with 1 mM EDTA were incubated with the antibodies as described above. Instead of htr5 we used monoclonal antibody htr5, which is also specific for type B TNF receptor (Brochhaus et al., 1990). Binding of [125I]TNFα and Scatchard analysis were performed as described (Schall et al., 1990).

To test for effects of dibutyril cAMP on the number of TNFα binding sites, HL60, HEp2, and HUVEC cells were incubated for 16 h with 1 mM dibutyril cAMP as described (Scheurich et al., 1989).

Immunoprecipitation with Anti-TNF Receptor Antibodies and Ligand Binding Using Radiolabeled TNFα—HL60 cells were treated as described above and harvested by centrifugation. The medium was removed, and 10⁶ cells were resuspended in 1 ml of ice-cold phosphate-buffered saline, and 10 μg/ml purified monoclonal antibodies specific for types A and B TNF receptors (utr1 and htr9, Brochhaus et al., 1990) was added to each sample. After 1 h on ice, the cells were washed with phosphate-buffered saline and lysed with 1 ml of RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.1% SDS) supplemented with 10 mM benzamidine (Fluka) and 1 mM phenylmethylsulfonyl fluoride (Fluka). After centrifugation for 10 min at 70,000 rpm in a Beckman TL100 ultracentrifuge, immunocomplexes were collected with 50 μl of protein A coupled to Sepharose beads (Pharmacia LKB Biotechnology Inc.). The beads were washed extensively with RIPA buffer and phosphate-buffered saline and boiled in 100 μl of Laemmli sample buffer without 2-mercaptoethanol. The immunoprecipitates were subjected to SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Membranes were preincubated for at least 30 min with 1% milk powder in 50 mM Tris-HCl (7.4), 140 mM NaCl, 5 mM EDTA, and 0.02% sodium azide. They were then incubated for 2 h with [125I]TNFα (1 × 10⁶ cpm/ml of buffer) (Brochhaus et al., 1990), washed, and used for autoradiography.

Activation of NF-κB, Preparation of Nuclear Extracts, and Electrophoretic Mobility Shift Assays—For analysis of NF-κB activation, 2 × 10⁶ HL60 were incubated with 20 μl of culture medium supplemented with TNFα or antibodies as indicated in the figure legends. HEp2 or SW480T cells were stimulated after reaching near confluence in 75-cm² culture flasks. Nuclear extracts were prepared essentially as described (Dignam et al., 1983). HL60 cells were harvested by centrifugation of 5 min at 1,500 × g. Adherent HEp2 cells were released from the tissue culture flask by incubation with 1 mM EDTA, cells were then harvested by washing, and detergent extract was prepared as described (Sen and Baltimore, 1986). About 50 μl of a hypotonic lysis buffer (buffer A, Dignam et al., 1983). After 20 min, the cells were homogenized by 20 strokes with a loose fitting Dounce homogenizer. Nuclei were collected by centrifugation for 4 min at 5,500 rpm in a microcentrifuge (approximately 4,000 × g), and the proteins were extracted with 4 packed pellet volumes of high salt buffer (buffer C, Dignam et al., 1983). After 90 min, the samples were centrifuged as described above. The high salt extracts were diluted with 3 volumes of low salt buffer D (Dignam et al., 1983) containing 1% Nonidet P-40 and were used immediately for electrophoretic mobility shift assays using 4% polyacrylamide gels or kept at −20°C. Electrophoretic mobility shift assays were performed as described (Sen and Baltimore, 1986). About 5,000 cpm of 32P end-labeled DdeI/HaeIII fragment of the κ-light chain enhancer containing the NF-κB binding site was used per assay. A restriction fragment mutated in the NF-κB binding site, but otherwise identical to the wild-type fragment (Lenardo et al., 1987) was used to identify other possibly nonspecific binding sites.

RESULTS

Three Different Cell Lines Have Very Different Ratios of the Types A and B TNF Receptors—Cross-linking experiments were used to study the nature of the TNFα receptors on SW480T, HL60, and HEp2 cells. Both HL60 and HEp2 cells have about 2,300 binding sites for TNFα (see below) whereas SW480T cells have about 10 times more TNFα receptors (not
shown). A major 100-kDa cross-linked complex was seen if radioliodinated TNF was cross-linked to intact SW480T or HL60 cells (Fig. 1A, lanes 1 and 11). Cross-linked products of 95 and 75 kDa were found on HEp2 and HL60 cells (Fig. 1A, lanes 6 and 11) as reported earlier (Hohmann et al., 1989) and were also seen as minor products with SW480T cells (Fig. 1B). Binding and cross-linking of radioidinated TNFα to all cells were inhibited by more than 98% if cells were preincubated with a 300-fold molar excess of unlabeled TNFα (Fig. 1A, lanes 2, 7, and 12). Preincubation of cells with the monoclonal antibody utr1, directed against the type A TNF receptor (Brockhaus et al., 1990), inhibited the formation of the 100-kDa cross-linked products on SW480T and HL60 cells (Fig. 1A, lanes 3 and 13), but the 95- and 75-kDa products on HEp2 and HL60 (Fig. 1A, lanes 8 and 13) and SW480T cells (Fig. 1B, lane 3) were still detected. Preincubation of cells with the monoclonal antibody htr9, which is specific for the type B receptor (Hohmann et al., 1989; Brockhaus et al., 1990), completely inhibited the formation of the 95- and 75-kDa products on HEp2 (Fig. 1A, lane 9), HL60 (Fig. 1A, lane 14), and SW480T cells (Fig. 1B), had no detectable effect on the amount of the 100-kDa product on SW480T cells (Fig. 1A, lanes 1 and 4), but reduced the amount of the 100-kDa on HL60 cells (Fig. 1A, lanes 11 and 14). This reduced cross-linking to the type A receptor of untreated HL60 cells was consistently found after htr9 treatment, but the explanation for this phenomenon is at present unclear. Pretreatment of SW480T and HEp2 cells with utr1 or htr9, respectively, almost quantitatively inhibited the binding and cross-linking of TNFα to these cells (Fig. 1A, lanes 3 and 9), indicating that these cells contain mainly the type A (SW480T) or the type B (HEp2) receptor. As shown above, SW480T cells contain a small amount of the type B receptor. HL60 cells pretreated with utr1 or htr9, respectively, showed in each case about 50% of the amount of specifically bound radiolabeled TNFα when compared with untreated cells (Fig. 1A, lanes 11, 13, and 14; Fig. 2). Only incubation with a mixture of the utr1 and htr9 antibodies completely abolished the binding of TNFα to HL60 cells. HL60 cells may thus contain similar amounts of the types A and B receptors, and the amount of the type A receptor is therefore overestimated in cross-linking experiments.

**FIG. 1. Three different cell lines have drastically different ratios of the types A and B TNF receptors.**

Panel A, SW480T cells (lanes 1–5), HEp2 cells (lanes 6–10), and HL60 cells (lanes 11–15) were grown, and aliquots of each cell type were preincubated for 1 h at 0 °C either without any addition (lanes 1, 6, and 11), with TNFα (lanes 2, 7, and 12), or with the anti-TNF receptor antibodies (Brockhaus et al., 1990) utr1 (lanes 3, 8, and 13), htr9 (lanes 4, 9, and 14), or with a mixture of both antibodies (lanes 5, 10, and 15). Then saturating amounts of [125I]-TNFα were added, and incubation was continued for 2 h at 0 °C, followed by cross-linking receptor-ligand complexes. SDS extracts of cells were analyzed by SDS-PAGE and autoradiography for the indicated period of time. All samples of one cell line represent the same amount of cell equivalents. However, different cell numbers (1–2 × 10⁶ cells) were used for the different cell lines. Panel B, in a parallel experiment, SW480T cells were treated exactly as described above. A long exposure is shown to visualize the type B receptor, which represents only a minor fraction of all TNF receptors of SW480T cells. In both panels, the positions of the cross-linked products (crp) between [125I]-TNFα and the different receptor types and of monomeric and multimeric forms of TNFα are indicated on the left; the positions of molecular mass standards are on the right. In panel A, the closed arrowhead is the top of separating gel.
Expression of the Type A Receptor on HL60 Cells Can Be Regulated Independently of the Expression of the Type B Receptor—As shown above, SW480T, HL60, and HEp2 cells have very different amounts of the type A compared with the type B receptor, indicating that the expression of both TNF receptors might be regulated independently. Scheurich et al. (1989) showed that dibutyric cAMP reversibly enhanced the number of TNF receptors in HL60 cells. Treatment of HL60 cells with dibutyric cAMP resulted in a 3-fold increase in the total number of TNF receptors (Fig. 2, lanes 1 and 6) and in a drastic increase in the amount of the 100-kDa cross-linked product. This band represented the type A receptor since its formation was blocked by pretreatment with utrl (Fig. 2, compare lanes 3 and 8) but not by htr9 incubation (Fig. 2, compare lanes 6 and 9). The amount of the type B receptor was not significantly altered by the dibutyric cAMP treatment, as visualized after competition with utrl (Fig. 2, compare lanes 3 and 8). Again the formation of all cross-linked products was quantitatively inhibited by treatment with utrl plus htr9 (Fig. 2, lane 10). The increase in the total number of TNFα binding sites after treatment with dibutyric cAMP is thus the result of an increased amount of the type A receptor. Binding of TNFα to the type A receptor in dibutyric cAMP-treated cells was also not detectably decreased by incubation with htr9, in contrast to in the untreated HL60 cells (Fig. 2, lanes 4 and 9, short exposure).

To verify the result described above, TNF receptors were immunoprecipitated by binding monoclonal antireceptor antibodies to intact cells cultivated in the absence or presence of dibutyric cAMP, washing the unbound antibody away and collecting the immunocomplexes after cell lysis. This procedure allowed immunoprecipitation of only those receptors that are exposed at the cell surface. TNF receptors were then subsequently visualized in ligand blots using radiolabeled TNFα. A drastically increased amount of the type A receptor was seen in the utrl immunoprecipitates of dibutyric cAMP-treated cells (Fig. 3A, compare lanes 2 and 5, short exposure).

The amount of the type B receptor was unaffected by the dibutyric cAMP treatment (Fig. 3A, compare lanes 3 and 6, long exposure). Multiple bands were seen in the utrl immunoprecipitates. These bands are all derived from the type A receptor, as confirmed in Fig. 3B. Utrl immunoprecipitates were used for ligand blotting with radiolabeled TNFα, and incubation with the radiolabeled TNFα was done in the absence of any competitor (Fig. 3B, lane 1), in the presence of excess htr9 (Fig. 3B, lane 2), or utrl antibody (Fig. 3B, lane 3), or unlabeled TNFα (Fig. 3B, lane 4). The fastest migrating band of about 50 kDa is likely to be a proteolytic degradation product (Hohmann et al., 1989; Brockhaus et al., 1990), the band of about 75 kDa represents the intact type A receptor, and the bands with higher molecular masses represent aggregates since they disappear upon extensive reduction (not shown). Thus, the expression of both receptor types can be regulated independently.

Dibutyric cAMP Only Up-regulates Expression of the Type A Receptor on Cells Already Expressing the Type A Receptor—To test whether expression of the type A receptor could be induced in cells usually only expressing the type B TNF receptor, HEp2 cells and HUVEC were incubated without and with dibutyric cAMP, and then TNFα was cross-linked to similar aliquots of cells. As shown in Fig. 4, neither induction of the type B receptor was seen nor expression of the type A receptor. Incubation with dibutyric cAMP is thus not sufficient for induction of the type A receptor in these cells.

The Type B Receptor Mediates Activation of NF-κB by TNFα or an Agonistic Monoclonal Antibody to the Type B TNF Receptor—To determine the involvement of each TNF receptor subtype in signal transduction, activation of the transcription factor NF-κB was determined. The amount of active NF-κB was measured in nuclear extracts using electrophoretic mobility shift assays and oligonucleotides that contain either an active binding site for NF-κB or a mutated, inactive NF-κB site (data for the mutant binding site are only shown in Fig. 7). Protein-DNA complexes were separated

![Figure 2](image-url)

**Figure 2.** Expression of the type A receptor is regulated independently of the expression of the type B receptor: cross-linking experiments. Cells were cultivated in the absence (none) or presence of dibutyric cAMP as described (Scheurich et al., 1989) and were then processed exactly as described in the legend to Fig. 1. Equal cell aliquots were then applied to SDS-PAGE and analyzed by autoradiography. A shorter exposure of the part containing lanes 6–10 is shown to the right. Crp, cross-linked products. The total amount of radiolabeled TNFα bound in each case (125I-TNFα binding) was determined by γ-counting and is indicated as a percent of the binding to untreated HL60 cells.
Types A and B TNF Receptors Both Mediate NF-κB Activation

**FIG. 3.** Expression of the type A receptor is regulated independently of the expression of the type B receptor: Immunoprecipitation and ligand blotting. Panel A, untreated HL60 cells (lanes 1–5) and HL60 cells treated with dibutyric AMP (dBcAMP, lanes 4–6) were incubated on ice with the monoclonal antibodies utr1 (lanes 2 and 5) and htr9 (lanes 3 and 6) directed against type A and type B TNF receptor, respectively. Control cells were not incubated with antibodies (lanes 1 and 4). After removing unbound antibodies, detergent extracts of the cells were prepared, and antigen-antibody complexes were collected with protein G-Sepharose beads. Immunocomplexes, corresponding to 1 × 10⁶ cell equivalents, were separated on SDS-PAGE gels, transferred to an Immobilon-P membrane, and TNF receptors were then visualized with ¹²⁵I-TNFα. An autoradiogram of the membrane exposed for 4 and 20 h, respectively, is shown on the left and the right of panel A. Positions of molecular mass markers are indicated on the right. Panel B, stripes of an Immobilon-P transfer membrane containing utr1 immunoprecipitates obtained from 1 × 10⁶ HL60 cells not treated with dibutyric AMP (see panel A) were incubated with ¹²⁵I-TNFα, in the absence of antibodies (none, lane 1), in the presence of 50 μg/ml monoclonal antibodies htr9 (lane 2) or utr1 (lane 3), or in the presence of 1 μg/ml TNFα (lane 4).

**FIG. 4.** Dibutyric cAMP only affects expression of the type A TNF receptor in cells that already express the type A receptor. HEp2 cells and HUVEC were incubated in the absence or presence of dibutyric AMP (dBcAMP). Equivalent cell aliquots were then incubated with radiolabeled TNFα and cross-linker and analyzed by SDS-PAGE and autoradiography. The positions of the crosslinked products (crp) characteristic for the type B receptor (cross-linked products 75 and 95) are indicated.

from the free oligonucleotide on polyacrylamide gels. Incubation of HL60, HEp2, and SW480T cells with TNFα for 2 h at 37°C drastically increased the amount of active NF-κB (Fig. 5A). Also, incubation of cells with the monoclonal antibody htr9, which is specific for the type B receptor (see above), increased the amount of active NF-κB in these cells (Fig. 5A, lanes 3, 8, and 13). Neither incubation with IFNγ (Fig. 5A, lanes 4 and 9) nor with the monoclonal antibody 45 (Fig. 5A, lanes 5 and 10) against the extracellular domain of the IFNγ receptor (Garotta et al., 1990) had any effect although both

**FIG. 5.** NF-κB is activated via the type B receptor of HL60, HEp2, and SW480T cells by TNFα or the monoclonal antibody htr9. Panel A, activation of NF-κB by TNFα and htr9. HL60 (lanes 1–5), HEp2 (lanes 6–10), or SW480T cells (lanes 11–13) were left untreated (lanes 1, 6, and 11) or were incubated at 37°C with 10 nM TNFα (lanes 2, 7, and 12) or 10 mg/ml anti-TNF receptor antibody htr9 (lanes 3, 8, and 13). As controls, HL60 and HEp2 cells were also incubated with 1 μg/ml IFNγ (lanes 4 and 9) or 10 μg/ml monoclonal antibody 45 (Garotta et al., 1990) directed against the extracellular domain of the IFNγ receptor (lanes 5 and 10). Panel B, htr9 blocks NF-κB activation by htr9. HL60 cells were incubated for 2 h at 37°C without (none, lanes 1–3) or with 20 μg/ml monoclonal antibody htr9 (htr9, lanes 4–6). After 1 h of incubation, htr9 (4 μg/ml) (lanes 2 and 5) or TNFα (10 nm) (lanes 3 and 6) was added, and incubation was continued. Nuclear extracts were prepared and analyzed for NF-κB activity using a radiolabeled oligonucleotide containing a wild-type NF-κB binding site. The free oligonucleotide was separated from the protein-DNA complex (NF-κB) by electrophoresis through 4% native polyacrylamide gels. The position of the complex between NF-κB and DNA fragment is indicated (NF-κB).
HL60 and HEp2 cells contained high affinity receptors for IFNγ (not shown). Thus, activation of NF-κB by TNFα can occur via the type B TNF receptor, which is the TNF receptor type shared by all three cell lines (see Fig. 1). Binding of another ligand or antibody to another cell surface receptor does not lead to activation of NF-κB. The monoclonal antibody htr5, which also reacts with the extracellular domain of the type B TNF receptor (Brockhaus et al., 1990), did not activate NF-κB in HL60 cells (Fig. 5B, lanes 2 and 5) but not its activation by TNFα (Fig. 5B, lanes 2, 3, and 5). The agonistic effects of htr9 are therefore the result of binding of the antibody to the type B receptor and are not caused by a putative contaminant in the htr9 preparation, e.g., such as lipopolysaccharides. This result also suggested that either the activation of TNFα in htr5-treated HL60 cells is obtained via the type A receptor or that htr5 is not capable of blocking fully the biological effect of TNFα mediated by the type B receptor even though all binding of TNFα to the type B receptor was apparently blocked (Fig. 1). For HL60 cells, the first possibility is most likely (see also "Discussion"). Thus, the type B receptor is functional in the transmission of signals into the cell, and all three cell types contain functional type B receptors. In addition, neither TNFα nor internalization of TNFα is necessary for activation of NF-κB.

HL60 and HEp2 Cells Each Showed Only One High Affinity Binding Site for TNFα, with About 7-Fold Different Affinities for TNFα Although HL60 Cells Have Both the Types A and B Receptors—Both TNF receptors are present in roughly similar amounts on HL60 cells (see above). Untreated HL60 cells, however, showed only a single binding site for TNFα with a $K_d$ of 5.1 × 10⁻¹¹ (Fig. 6A). Preincubation of HL60 cells with htr5, which blocks all detectable binding of TNFα to the type B receptor (not shown), did not affect the affinity for TNFα binding ($K_d$ 5.2 × 10⁻¹¹ for htr5-treated cells) but reduced the number of binding sites from 2,300 to 1,050 sites (compare Fig. 6, panels A and B). The higher affinity may thus represent binding of TNFα to the type A receptor. Pretreatment of HL60 cells with utrl, which blocked all binding to the type A receptor (see Fig. 1), reduced the number of binding sites for TNFα from 2,300 to 850 sites and decreased the binding affinity for TNFα about 7-fold ($K_d$ of 3.5 × 10⁻¹⁰) compared with untreated cells (Fig. 6, panels A and C). This binding affinity was very similar to the affinity determined

![Fig. 6. HL60 and HEp2 cells each show a single binding site for TNFα with HL60 cells having slightly higher affinity for TNFα visualization of lower affinity of the type B receptor of HL60 cells only after blocking TNFα binding to the type A receptor. Panels A–D, TNFα binding (left panels) and Scatchard analysis (right panels). Triplicate samples of untreated and antibody-pretreated HL60 cells (panels A–C) and of untreated HEp2 cells (panel D) were incubated with different concentrations of [¹²⁵I]TNFα. Incubation was performed in the absence or presence of at least a 300-fold excess of unlabeled TNFα (300 nM) to determine nonspecific binding. The specific binding (difference between [¹²⁵I]TNFα binding in absence and presence of unlabeled TNFα) is shown in the left panels. Values represent the mean of triplicates ± S.E. of total binding. The nonspecific binding was less than a few percent of the total binding. For incubations with monoclonal antireceptor antibodies, HL60 cells were incubated under the same conditions as used for Figs. 1–3. htr5 and htr9 behaved identically (not shown). Scatchard analysis (right panels) was performed using the computer program LIGAND (Munson, 1983).
for the type B TNF receptor on HEp2 cells (Kd of 3.4 x 10^-10) (Fig. 6D). Cross-linking of TNFα to utrl-pretreated HL60 cells confirmed that TNFα was only bound to the type B TNF receptor at all TNFα concentrations used (not shown). At present, however, it is unclear why only one binding site is detected on untreated HL60 cells.

The Type A Receptor Mediates Activation of NF-κB by TNFα—All cell types studied by us thus far (Hohmann et al., 1989 and this paper) which express the type A receptor also express the type B receptor. Activity of the type A receptor was thus determined by specifically blocking binding of TNFα to the type A receptor using the anti-type A receptor antibody utrl. If the type A receptors were biologically active, we would expect that higher TNFα concentrations would be necessary for activation of NF-κB after utrl pretreatment whereas the concentration dependence of NF-κB activation by htr9 should be unaffected. HL60 cells were either mock incubated or were incubated with the monoclonal antibody utrl under conditions that block TNFα binding to all type A receptors and change the affinity of TNFα binding to that characteristic of the type B receptor (see above). In mock-incubated (untreated) HL60 cells, activation of NF-κB was found with TNFα concentrations below 0.5 pM and was maximal with 4-8 pM TNFα (Fig. 7, panel A). In cells incubated with utrl, higher concentrations of TNFα were needed for activation of NF-κB, at least 2 pM, whereas maximal activation was seen with 16-32 pM TNFα (Fig. 7, panel C). Quantitation of (Fig. 7, panel E) of the results described above clearly shows that NF-κB activation in utrl-pretreated cells occurs via a site with lower affinity for TNFα; the angles of both curves are different. In addition, NF-κB activation by htr9 via the type B receptor was not affected by pretreatment with utrl (Fig. 7, panels B, D, and F). Thus, the type A TNF receptor mediates NF-κB activation by TNFα.

DISCUSSION

In this paper, we studied the expression and biological activity of the types A and B TNF receptors and determined whether TNFα itself is needed for receptor-mediated activation of the transcription factor NF-κB.

Three cell lines used here have very different ratios of the types A and B receptors. SW480T cells, derived from the colon carcinoma cell line SW480 by selection for presence of a high number of TNF receptors, had about 10 times more binding sites for TNFα compared with HL60 cells and showed mainly the 100-kDa cross-linked product characteristic for the type A receptor. TNFα binding to SW480T cells was almost quantitatively blocked by the monoclonal antibody utrl. SW480T cells thus have mainly the type A TNF receptor. SW480T cells also contain a small amount of the type B TNF receptor since prolonged exposure of the autoradiograms also showed the 95- and 75-kDa cross-linked products, the formation of which was specifically inhibited by antibodies to the type B receptor. In addition, the agonistic antibody htr9 to type B receptor also activated NF-κB (see below). HEp2 showed only the 95- and 75-kDa products characteristic for the type B receptor as shown previously (Hohmann et al., 1989), and TNFα binding was only blocked by the monoclonal antibody htr9. HEp2 cells thus lack the type A receptor. HL60 cells showed both types of cross-linked products, and utrl and htr9 each blocked about 50% of the binding of TNFα to HL60 cells. HL60 cells may thus contain comparable amounts of the types A and B receptors. In primary cultures of HUVEC, we detected only the type B receptor in several experiments.

However, a small amount of the type A receptor was also detected in one experiment. At present it is unclear whether this represents contamination with other cell types or reflects individual differences caused by, e.g., stimulation of the immune system.

HL60 and HEp2 cells only showed a single binding site for TNFα and TNFβ in Scatchard analysis (Hohmann et al., 1989, 1990). The affinities of TNFα binding were about 7-fold higher for HL60 than for HEp2 cells. The higher affinity of TNFα binding seems to represent binding to the type A receptor. Preincubation of HL60 cells with the antibody htr5 blocked all detectable binding to the type B receptor and did not affect the affinity of TNFα binding. Preincubation of HL60 cells with the antibody utrl blocked all TNFα binding to the type A receptor and reduced the binding affinity for TNFα to the affinity found with HEp2 cells. Thus, the lower affinity of TNFα binding to the type B receptor can be detected in HL60 cells but only if binding to the type A receptor is blocked. In cross-linking experiments, no difference was observed between the amount of cross-linked products representing the type B receptor in untreated and utrl-treated cells. Thus, TNFα can be bound to the type B receptor in both conditions. The reason for the apparent absence of the lower affinity binding site in untreated HL60 cells is unclear. It could suggest cooperativity in TNFα binding and thus

![Fig. 7. The type A TNF receptor is biologically active.](image-url) HL60 cells were incubated for 1 h at 37°C without antibody (untreated, panels A and B) or with 10 μg of the antibody utrl against the type A TNF receptor per ml (utrl-treated, panels C and D). Under these conditions, all binding to the type A receptor was blocked (not shown and see Fig. 1). The indicated concentrations of TNFα or of the agonistic antibody htr9 against the type B receptor were then added, and incubation was continued for an additional 30 min. Nuclear extracts of identical cell aliquots were prepared and analyzed for NF-κB activity as described in the legend to Fig. 5. Autoradiograms of the gels are shown in panels A–D. Panels E and F, the results were quantified by excising the regions of the gels containing NF-κB-DNA complexes and determining the radioactivity using Cerenkov counting. Circles and squares indicate NF-κB induction in untreated and utrl-pretreated HL60 cells, respectively.

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* T. Espevik, unpublished data.
interaction between receptor types. However we did not obtain direct and conclusive evidence for receptor interaction. No co-immunoprecipitation of both receptor types was found using antibodies against each receptor type and a variety of different detergent conditions for solubilization of HL60 cell membranes.

Both the types A and B TNF receptors are functional in signal transduction. The transcription factor NF-κB was activated by TNFα in HEp2 cells, in which only the type B receptor was detected. In addition, the antibody htr9 against the type B receptor also activated the transcription factor NF-κB in SW480T, HL60, and HEP2 cells, all of which have the type B receptor. Thus, the type B TNF receptor mediates NF-κB activation. NF-κB activation by htr9 was blocked by pretreatment with the antagonistic antibody htr5, confirming that the NF-κB activation is indeed mediated by the type B receptor and is not caused by an unknown contaminant in the htr9 preparation. Pretreatment by htr5 of HL60 cells, which contain both receptor types, did not block the NF-κB activation by TNFα. The most logical explanation would be that NF-κB activation now occurs via the type A receptor. However, this result does not show directly that the type A receptor is also biologically active. NF-κB activation by TNFα (not shown), but not by htr9, was still found in htr5-treated HEp2 cells, although htr5 pretreatment blocked all detectable binding to the type B receptor in 1L60 and HEP2 cells. Inhibition of TNFα-mediated NF-κB activation is apparently more difficult than blocking (most of the) receptor binding. We showed previously that occupation of only a minor fraction of all TNF receptors of HL60 and HEp2 cells leads to maximal activation of NF-κB (Hohmann et al., 1990). The existence of other (minor) receptor types not detected by our experiments could also explain this observation and, of course, not be excluded. The type A TNF receptor also mediates activation of NF-κB by TNFα. Pretreatment of HL60 cells with utrl blocked formation of the 100-kDa product and reduced the Ke of binding of TNFα to the receptor to that specific for the type B receptor (see above). In addition, utrl pretreatment changed the concentration dependence of NF-κB activation by TNFα to that found in HEp2 cells (see also Hohmann et al., 1990) and was then characteristic for NF-κB activation by TNFα binding to a lower affinity binding site. In addition, NF-κB activation via the type B receptor by the antibody htr9 was not affected by utrl pretreatment. Of course, it remains to be established whether each of the receptors directly mediates the biological effects of TNFα or whether interaction with an as yet unknown receptor chain is necessary. At present, we also cannot exclude that signal transduction via the type A receptor requires interaction with the type B receptor, but as mentioned above, no conclusive evidence for interaction between the types A and B receptors is available.

The existence of drastically different ratios of the types A and B receptors on different cells suggested that expression of both receptor types is regulated independently and in a cell-specific manner. To test this hypothesis, we used the observation of Schrairich et al. (1989), who showed that TNF receptors on certain cells could be reversibly up-regulated by dibutyric cAMP. Five of the six cell lines for which they showed up-regulation of TNF receptors were shown previously by us to contain the type A receptor (HL60, U937, K562, SW480, and normal blood monocytes; EL4 cells were not tested by us) whereas at least one of the cells that did not show receptor up-regulation (HeLa) contained only the type B receptor (Hohmann et al., 1989; Brockhaus et al., 1990). This suggested that dibutyric cAMP may specifically up-regulate the type A receptor. Here we showed that this is indeed the case and thus that the expression of the type A and B TNF receptors can be regulated independently. In addition, we show that dibutyric cAMP does not induce expression of the type A receptor in HEp2 cells and HUVEC, both of which only showed the type B receptor in our cross-linking experiments. It seems most likely that the up-regulation of the type A receptor in HL60 cells is coupled to cell differentiation induced by overnight incubation with dibutyric cAMP (Chaplin und Niedel, 1982). Overnight incubation with forskolin did not show a similar effect (not shown). Incubation with forskolin leads to a rapid and drastic increase in the levels of intracellular cAMP (not shown), but this effect might not last long enough to induce cell differentiation.

TNFα is internalized by cells and then degraded (Tsujimoto et al., 1985). The function of this internalization process was unknown. Here we showed that the agonistic antibody htr9 activated NF-κB via the type B TNF receptor (see above). Thus, triggering of the type B TNF receptor is necessary and sufficient for activation of NF-κB. In addition, htr9 also induced cytotoxicity in U937 cells, increased the proliferation of fibroblasts, and activated human endothelial cells (Espevik et al., 1990). These data show that neither TNFα nor internalization of TNFα is required for the development of these biological effects. Internalization and degradation of TNFα by cells might only be necessary to remove receptor-ligand complexes although a possible function of TNFα internalization in other biological effects cannot be excluded. Recently, Smith et al. (1990) presented direct evidence for an intracellular role of TNFα in killing several target cells. Further work will be needed to determine the exact role of TNFα in various biological effects.

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