The Type B Receptor for Tumor Necrosis Factor-α Mediates DNA Fragmentation in HL-60 and U937 Cells and Differentiation in HL-60 Cells

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Tumor necrosis factor-α (TNF) binds to two specific cell surface receptors, types A and B, which are both present on HL-60 and U937 cells, and induces monocytoid differentiation in HL-60 cells and early DNA fragmentation in HL-60 and U937 cells. To further define the receptors’ roles, we studied how monoclonal antibodies (MoAbs) against each receptor affected TNF-induced cellular responses. HTR-9, an MoAb against the type B (low affinity, 55 Kd) receptor, reproduced all of these effects in a dose-dependent manner. UTR-1, an MoAb against the type A (high affinity, 75 Kd) receptor, had no effect in saturating doses, but supersaturating doses enhanced DNA fragmentation threefold. TNF and interferon gamma (IFN-γ) synergistically induced morphologic differentiation and monocytic antigen expression, while the anti-type B receptor MoAb was synergistic for morphologic response, but not antigen expression. Our results indicate that (1) the type B receptor mediates some responses to TNF in HL-60 and U937 cells, (2) the type A receptor does not stimulate these responses, (3) the TNF molecule is not necessary for some of these actions, and (4) TNF-induced morphologic changes and surface antigen expression in HL-60 cells may be regulated by separate postreceptor pathways.

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MATERIALS AND METHODS

Cell lines. HL-60 and U937 cells were grown in RPMI 1640 (Cellogel, Mediatech, Washington, DC) supplemented with 20% Controlled Process Serum Replacement-3 (CPSR-3; Sigma, St Louis, MO), glutamine, penicillin, streptomycin, and sodium pyruvate. Assays were performed in assay medium that contained 5% CPSR-3, but was otherwise the same as the growth medium. Cells were passed at 1 to 2 x 10^7/mL and grown at 37°C in the presence of 4% to 6% CO2. Studies were performed on passages 23 to 50 for HL-60 and 50 to 95 for U937.

Cytokines and antibodies. Recombinant (r)-TNF-α, specific activity 5.02 x 10^7 U/mg, and r-IFN-γ, specific activity 3 to 5 x 10^7 U/mg, were supplied by Genentech, South San Francisco, CA. The IgG1-monoclonal antibodies HTR-9 and UTR-1 were generously provided by Genentech. These antibodies are described in detail elsewhere.

We have previously investigated the phenomenon of DNA fragmentation, which occurs rapidly following TNF treatment of HL-60 cells. DNA fragmentation has been described in several systems as an important event in apoptosis, or programmed cell death. This process differs from toxic cell death, and is important in such regulatory systems as embryologic development and lymphocyte killing by steroids. However, in HL-60 cells, TNF-induced DNA fragmentation precedes cell death, but differentiation into monocytoid cells. Thus, TNF-induced DNA fragmentation may contribute to the regulation of gene expression and differentiation.

We used MoAbs against the TNF receptors to study the roles of the types A and B TNF receptors in mediating DNA fragmentation in U937 and HL-60 cells, and monocytic differentiation in HL-60 cells. The rationale of this study was both to contribute to the emerging understanding of the roles of these receptors in TNF effects in general, and also to determine if DNA fragmentation and differentiation of HL-60 are triggered by the same or by distinctive receptor-mediated pathways. In this report, we demonstrate that these responses are mediated mainly by the type B, lower-affinity receptor.

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Iodination of TNF. TFN was labeled with 125I using the Iodogen method. Briefly, 1 μg of Iodogen reagent (Pierce, Rockford, IL) was plated onto Eppendorf microcentrifuge tubes. TFN, 0.75 to 1.0 μg, was added to 50 μL of phosphate-buffered saline (PBS; 10 mmol/L NaPO4, 100 mmol/L NaCl, pH 7.4) plus 0.02% Tween 80 (Sigma) was reacted for 7 to 8 minutes. The reaction was terminated by adding 450 μL PBS plus 5 mg/ml KI, 0.02% Tween 80, and 0.1% bovine serum albumin. Gel filtration was performed on a NAP-1 column (Pharmacia, Piscataway, NJ) eluted with 25 mmol/L Tris; the labeled TNF was recovered in the first 1-mL fraction. Specific activities of 40 to 60 mCi/mg TNF were obtained. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography demonstrated that the majority of the radioactivity was found in a band of 17 Kd, representing TNF monomer, with smaller bands at 34 and 51 Kd (dimer and trimer). TNF radiolabeled in this manner had the same biological activity as native TNF.

Binding experiments. Cells, 1.0 x 10⁶, were preincubated in 0.5 mL assay medium only and with medium plus 10 μg unlabeled TNF, 25 μg HTR-9, and/or 25 μg UTR-1 for 4 to 10 hours at 4°C. 125I-TNF was then added, and all cells were incubated an additional 3 hours at 4°C. Cells were washed with PBS at 4°C. Radioactivity of the cell pellet was measured in a Beckman 5500 Gamma Counter (Fullerton, CA). Specific binding was determined by subtracting the radioactivity of cells preincubated with unlabeled TNF (nonspecific binding) from the radioactivity of the other samples.

DNA fragmentation assay. U937 or HL-60 cells were labeled with ³H-deoxythymidine (³H-dThd; Dupont NEN) by incubating 2.0 to 3.0 x 10⁶ cells, at 2.0 x 10⁶ cells/mL in assay medium with 1 μCi/ml ³H-dThd at 37°C for 2 hours. Cells were washed free of ³H-dThd, and 2.0 x 10⁶ cells were resuspended, in duplicate, in flat-welled Falcon 3047 plates (Becton-Dickinson, Mountain View, CA), in 1.0 mL assay medium alone and with different concentrations of TFN and/or the MoAbs. Cells, 2.0 x 10⁶, were saved for assessment of total cell labeling with ³H-dThd. Cells were incubated for 3 hours at 37°C, then pelleted and lysed with a hypotonic detergent buffer (1.0 mmol/L Tris, 1.0 mmol/L EDTA, 0.2% Triton X-100) for 30 minutes at 37°C. High-speed centrifugation was performed (12,000g for 20 minutes) on a Beckman J2-21M centrifuge. The pellet contains all cell organelles; radioactivity in the supernatant represents DNA released into the cell due to DNA fragmentation. The cells saved to measure total cell ³H labeling were lysed by freezing and thawing followed by overnight incubation with 1.0% Triton X-100 at 37°C. They were not centrifuged after disruption, and were counted in the same manner. Intracellularly released radioactivity was expressed as a percentage of whole cell radioactivity; for the control cells was counted, and were expressed in the same manner. Intracellularly released radioactivity was expressed as a percentage of whole cell radioactivity; for the control cells was typically 0.5% to 2.0%.

Differentiation assay. HL-60 cells were seeded in 5 mL complete medium at 2.0 x 10⁶/mL for 3 to 4 days at 37°C in 4% to 6% CO₂, with 1,000 U/mL TFN, 10 μg/mL HTR-9, 10 μg/mL UTR-1, and 100 U/mL IFN-γ, alone and in combinations. Cells were counted daily. When they reached a count of 1.5 x 10⁶/mL, cells were evaluated for monocytoid differentiation. Cytosins of 1 x 10⁶ cells were prepared in a Shandon Southern Cytocentrifuge (Shandon Southern Products Limited, Cheshire, England), 800 rpm for 10 minutes. Cytosin slides were stained with Wright's stain and morphology was evaluated by light microscopy. Flow cytometric studies were performed according to standard techniques, using MoAbs directed against the monocyte antigens CDw14 (Coulter, Hialeah, FL) and CD11b (Becton-Dickinson). Antibody binding was detected with a fluoresceinated F(ab')₂ goat anti-mouse antibody (Caltag, San Francisco, CA). Controls for immunofluorescence included the second-step antibody alone to control for nonspecific binding. Flow cytometry was performed on a Becton-Dickinson FACScan.

RESULTS

Effects of MoAbs on 125I-TNF binding to HL-60, U937 cells. Specific binding of 125I-TNF to HL-60 and U937 cells was determined after preincubation of the cells with assay medium alone (control), or with medium plus unlabeled TNF, HTR-9, UTR-1, or both MoAbs. Specific binding was reduced to 30.5% ± 14.5% of control in the presence of HTR-9 and 33.2% ± 12.9% in the presence of UTR-1. Preincubation with both MoAbs reduced binding almost to the level of nonspecific binding found after preincubation with cold TNF.

Effects of MoAbs and TNF on induction of DNA fragmentation in U937, HL-60 cells. Cells were labeled with ³H-dThd, incubated with medium alone (control), or with TNF and/or varying concentrations of the MoAbs. Radioactivity present in the postnuclear supernatant was measured. Results from treatment of U937 cells with MoAbs or 1,000 U/mL TNF alone are shown in Fig 1, and results of combination treatment in Fig 2.

TNF, 1,000 U/mL, induced DNA fragmentation of about eight times that of control cells. HTR-9 stimulated DNA fragmentation at low doses (1 μg/mL), with a dose-response curve that reached a plateau at 10 μg/mL. The maximum amount of HTR-9-induced DNA fragmentation was three times control levels, but only about 35% of the amount induced by 1,000 U of TNF. Addition of HTR-9 to TNF did not change DNA fragmentation.

UTR-1, alone in doses up to 10 μg/mL, did not induce DNA fragmentation and did not change DNA fragmentation when added to TNF. However, with doses of UTR-1 of 50 μg/mL, DNA fragmentation was increased threefold,
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...changed by anti-type B receptor MoAb HTR-9, but is increased by anti-type A receptor MoAb UTR-1 in high doses. Cells were treated with TNF alone were assigned a value of 1. Each bar represents the mean ± SE of three or more experiments. 

Effects of TNF and MoAbs on growth and differentiation of HL-60 cells. HL-60 cells were incubated with TNF, IFN-γ, and MoAbs, alone and in combinations, and were counted and evaluated for evidence of monocytic differentiation. No changes in the growth curve were noted with any treatment compared with control cells. Cellular morphology was evaluated by light microscopy after Wright’s staining of cytospin slides, and flow cytometry (FCM) was performed to evaluate expression of monocye cell surface antigens CDw14 and CD11b. Morphologic changes are shown in Fig 3, and histograms of fluorescence of FCM experiments are shown in Figs 4 and 5.

Control cells incubated without cytokines and MoAbs had fine chromatin with one or more prominent nucleoli, a small amount of cytoplasm, prominent azurophilic granules, and occasional vesicles (Fig 3A). TNF (500 U/mL) induced a characteristic pattern of differentiation with some monocytoid and osteoclast-like features, as previously reported. Approximately 20% of cells became binuclear or multinuclear with denser chromatin and less prominent nucleoli, and most cells had a reduced nuclear/cytoplasmic ratio and pale blue cytoplasm without granules (Fig 3B). FCM demonstrated increased expression of the monocye antigens CDw14 and CD11b compared with control cells (Fig 4A). HTR-9 (10 μg/mL) induced identical changes as TNF, both morphologically and in antigen expression (Fig 3C). The fluorescence histograms of the population of HTR-9–treated cells superimposes exactly with the curve of TNF-treated cells (Fig 4A). The combination of TNF and HTR-9 also produced a response identical to TNF alone. UTR-1 (10 μg/mL) did not induce any changes in cell morphology or antigen expression compared with control cells. The fluorescence curves of antigen expression in these cells superimpose exactly on the control cells (Fig 4B). The combination of TNF and UTR-1 resulted in morphological and antigenic response identical to the response to TNF alone (Fig 4C), i.e., UTR-1 did not inhibit the TNF-induced changes.

TNF (500 U/mL) and IFN-γ (100 U/mL) produced a synergistic effect on morphology (as previously reported) and antigen expression. Approximately 40% of cells became binuclear or multinuclear, with some giant multinuclear cells. IFN-γ alone induced no morphologic changes compared with control cells, indicating a synergistic and not additive effect. The FCM fluorescence histograms demonstrate that both TNF and IFN-γ induce expression of CDw14 and CD11b, but the combination of cytokines increases this expression (Fig 5A). On a log scale, the peak channel shift increases approximately 15 to 20 U, and both curves are shifted approximately 40 U to the right. When added to HTR-9, IFN-γ also enhanced the morphologic changes; more than 50% of cells became binuclear or multinuclear. However, a synergistic effect on antigen expression is not apparent on the fluorescence histograms (Fig 5B). There is no increase in channel shift in the cells treated with HTR-9 plus IFNγ compared with cells treated with each agent alone.

DISCUSSION

In this report, we studied the activities of MoAbs against TNF receptors and demonstrated that the type B TNF receptor mediates DNA fragmentation in U937 and HL-60 cells, and monocytoid differentiation in HL-60 cells. The type A receptor appears not to stimulate these events, and may be either inhibitory or have no effect.

Previous work with the MoAbs HTR-9 and UTR-1 has demonstrated high-affinity binding and influence on TNF’s biologic effects in several systems. They each compete with TNF for cell binding and reduce the specific binding of 125I-TNF in HL-60 and U937 cells to 30% to 50% of total TNF-specific binding. Competition with both of the antibodies together completely blocks 125I-TNF binding. HTR-9 acts as an agonist for TNF actions in that it induces lysis of U937 cells, growth of fibroblasts, production of interleukin-6 by endothelial cells, and NF-κB activity in HL-60 and HEP-2 cells. UTR-1 inhibits TNF-induced activation of NF-κB activity in HL60 cells. These results imply that these MoAbs recognize an area at or near the TNF binding domain and active site of the receptor, that type B mediates all of the actions reported above, and that both receptor types can mediate NF-κB activity.

We first confirmed the finding that U937 and HL-60 cells have similar numbers of types A and B, and can thus be used to investigate the roles of both receptors in TNF’s cellular effects. Treatment with the MoAb against one receptor in doses of 10 μg/mL or greater is sufficient to saturate the receptors, and any biologic effect produced by simple interaction of MoAb and receptor should be appar-
ent. Additionally, saturating one type of TNF receptor with MoAb ensures that any TNF added must bind to the other, unbound, receptor type.

Our results indicate that type B, but not type A, is responsible for the induction by TNF of DNA fragmentation in U937 and HL-60 cells, and monocytoid differentiation in HL-60s. The direct stimulatory effect of HTR-9 to produce these changes indicates that the TNF molecule is not necessary to cause some TNF-associated effects. Since no MoAb exists that acts as an agonist for type A, evidence that it does not stimulate DNA fragmentation is indirect, but can be inferred from our findings. When the high-affinity type A receptor is bound by UTR-1, TNF must bind only to the lower-affinity type B receptor. Hohmann et al noted that type A blockade by UTR-1 resulted in a change in the dose-response curve to TNF, with decreased NF-κB activation.5

No such change was seen in our studies; amounts of DNA fragmentation and differentiation were the same as those found with TNF alone at all doses. Our results confirm previous findings demonstrating that the responses to the types A and B receptors can be dissociated. The specific biologic effects associated with each receptor appear to vary greatly among distinctive cell types. Although antiproliferative or cytotoxic effects are frequently associated with the type B receptor,5-7,16-18 inhibition of erythropoiesis has been recently associated with the type A receptor.19 Similarly, growth stimulatory effects have been attributed to either the type A,18 type B,17,20 or both types of receptors21 in particular cell types. Growth factor release induced by TNF has recently been associated with type A receptors.20

The finding that a 50-µg/mL dose of UTR-1 causes a threefold increase in DNA fragmentation is interesting,
and has several possible explanations. Although we cannot completely exclude that this property of UTR-1 is mediated by its ability to block the type A receptor, this appears unlikely since a dose of 10 μg/mL of UTR-1 saturates the receptor. A more likely explanation is that UTR-1 may recognize, with lower affinity, another cell surface epitope that also stimulates DNA fragmentation. One such example is Fas, a 200-Kd antigen that appears not to be one of the described TNF receptors. Anti-Fas acts on TNF-sensitive cells to produce cytolysis identical to TNF, but does not stimulate other TNF responses. Fas is co-downregulated with the TNF receptor, and may represent a receptor multimer or receptor-associated protein. Perhaps UTR-1 can similarly induce DNA fragmentation by a non-TNF receptor mechanism.

Alternatively, very high doses of UTR-1 may stimulate or remove an inhibition to DNA fragmentation by changing the characteristics of TNF receptor aggregation, altering signal transduction. Evidence of receptor clustering as a mechanism of controlling cell response exists for TNF and other cytokines. The native structure of TNF is a trimer, so it is likely to aggregate its receptors on the cell surface. Another MoAb, HTR-1, is an IgM molecule that recognizes the TNF type B receptor and is a more potent inducer of
Fig 4. Flow cytometric analysis shows that monocyte antigens CDw14 and CD11b are expressed in HL-60 cells treated with TNF and anti-type B receptor MoAb HTR-9, but not with anti-type A receptor MoAb UTR-1. Cells were incubated and prepared as described in the Methods. Dashed curves represent fluorescence of cells incubated in medium alone. These curves of control cells superimpose on the fluorescence curve of treated cells incubated with second step antibody alone. (A) TNF and HTR-9 stimulate expression of CDw14 and CD11b. (B) UTR-1 does not stimulate CDw14 and CD11b expression. (C) UTR-1 does not inhibit TNF-induced antigen expression.

TNF effects than any IgG MoAb tested. Its multimeric structure is more likely to facilitate receptor aggregation compared with the divalent IgG molecules, and this aggregation may account for its more potent TNF-like activity. Another cytokine system in which receptor multimerization is important is platelet-derived growth factor (PDGF). Two types of PDGF receptor occur (α and β), and receptor dimerization is vital to signal transduction. Homodimer and heterodimer receptor clusters are formed depending on which dimer of PDGF is present, and the different receptor dimers appear to produce different biologic effects. An analogous situation would imply the generation of TNF receptor trimers. In such a system, the dose, size, and number of receptor binding sites of TNF or other agonists, and the ratio of receptor types, could affect the cellular responses.

Another poorly understood aspect of TNF signal transduction involves the internalization of the TNF-receptor complex, which has been demonstrated but is of unknown significance. Evidence from microinjection and lysosomal inhibition studies suggests that internalization is
important for some but not all TNF actions. We confirmed earlier findings that the presence of the TNF molecule, and therefore the TNF-receptor complex, is not necessary for TNF effects.\textsuperscript{7,17,18} It is unknown whether the MoAb-receptor complex is internalized.

The experiments of combined treatment of TNF or HTR-9 with IFN-\(\gamma\), addressing the question of which receptor is responsible for TNF's interaction with other cytokines, were inconclusive but suggested that the mechanisms involved in stimulating morphologic changes may be distinct from those producing synergy in antigen expression. A dose of HTR-9 sufficient to mimic TNF's effect on morphology and antigen expression produced additive morphologic changes with IFN-\(\gamma\) but, unlike TNF, antigen expression was not enhanced. Thus, mere stimulation of the active site of the type B receptor was insufficient to induce synergy with IFN-\(\gamma\) for some cellular responses. This differs from results using anti-Fas, in which cytotoxicity of the MoAb was enhanced following pretreatment with IFN-\(\gamma\).\textsuperscript{22} Possible explanations include the need for participation of the type A receptor or the TNF molecule itself in the synergy pathway.

It is unclear whether TNF induces multinuclearity via nuclear division without cell division or by cell fusion. Weinberg and Larrick considered the latter, since it is the mechanism by which multinuclearity occurs after IFN treatment of nondividing monocytes, and TNF treatment of osteoclasts.\textsuperscript{13} However, conclusive data are lacking.

The occurrence in a system of DNA fragmentation that does not result in cell death remains intriguing.\textsuperscript{5,10} DNA fragmentation may be a mechanism of control of gene expression, in this case leading to differentiation. The linkage of both DNA fragmentation and differentiation with the type B receptor described here is consistent with our hypothesis that DNA fragmentation may contribute to differentiation.

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