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Cell Biology

A high molecular weight component of the human tumor necrosis factor receptor is associated with cytotoxicity

(cell lysis/stimulation of fibroblast proliferation/receptor structure)

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ABSTRACT We compared the molecular structure of the receptor to human recombinant tumor necrosis factor (HurTNF) on cells of different tissue origin that differ in their response to one of the known activities of TNF. We studied *(i)* tumor cell lines that respond to the cytotoxic action of TNF and resistant variants that bind TNF, *(ii)* normal cell lines that are stimulated to proliferate by TNF and those that are not affected by TNF, and *(iii)* peripheral blood granulocytes whose activation is also augmented by TNF. Using ^{125}I-labeled HurTNF, we found that it bound mainly to four cellular polypeptides (138, 90, 75, and 54 kDa), three of which were found in every cell type examined and one (138 kDa) that was observed only in a human breast carcinoma cell line (MCF-7) that is highly responsive to the cytotoxic action of TNF. The 138-kDa polypeptide was not found in resistant variants of MCF-7 that bind TNF. In contrast to the other polypeptides, the 138-kDa protein was detected 30 min after incubation at 4°C, as compared to 5 min. Scatchard analysis and cross-linking data suggest a model for the TNF receptor structure whereby the receptor is composed of noncovalently linked membrane-bound polypeptides that bind TNF with high affinity (K_d, 0.05–0.8 × 10^{-9} M) with the 138-kDa protein being the least abundant and/or even absent in most cells.

Tumor necrosis factor (TNF) was discovered in the sera of animals primed with bacillus Calmette–Guérin and challenged with endotoxin (1–3). This substance was found to cause hemorrhagic necrosis of some transplantable mouse and human tumors with no apparent effect on the host (1, 4, 5), and to exhibit primarily cytotoxic activities against tumor but not normal cells *in vitro* (6, 7). Studies with recombinant TNF (8–10) confirmed the selective cytotoxicity of TNF against tumor cells and have delineated a growth stimulatory role for TNF on some tumor cells and normal diploid fibroblasts (11, 12). In addition, TNF was found to augment the phagocytic and cytotoxic activities of polymorphonuclear neutrophils (13) and to stimulate their adherence to umbilical vein endothelium (14).

These varied effects of TNF suggest specificity in the interaction of the molecule with each cellular type. A likely site for this specificity is at the TNF receptor. Several investigators have thus far used native and recombinant TNF to characterize the TNF receptor mainly on tumor and some normal cell lines (15–22). It was found that nearly all cells possess receptors for TNF. However, no direct comparison was made of the TNF receptor structure on cells that respond to the different actions of TNF. We therefore compared the biochemical characteristics and molecular structure of the TNF receptor on cells of different tissue origin that differ in their response to one of the known actions of TNF. We have found that *(i)* TNF binding to all the cell types is specific, saturable, and time, temperature, and dose dependent; *(ii)* consistent with physiologic concentrations necessary for response, the binding reached maximal level at 1.5 nM on the granulocytes and saturated at 15 nM on the normal and tumor cell lines, suggesting that there may be diversity in TNF receptor occupancy and biological response for the different cell types; *(iii)* the ^{125}I-labeled human recombinant TNF (^{125}I-HurTNF) bound to predominantly four cellular proteins (138, 90, 75, and 54 kDa), of which three were found in every cell type and the fourth (138 kDa) was observed only in a human cell line that is sensitive to the cytotoxic action of TNF. These results suggest that the TNF receptor(s) may act independently or as multiple subunits, and that one of these subunits may trigger TNF cytotoxic action *in vitro*.

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MATERIALS AND METHODS

TNF. Highly purified ≥90% HurTNF expressed in *Escherichia coli* was used in this study. The specific activity of the material was 1.2 × 10^7 units per mg of protein (8). TNF was radioiodinated by the use of chloramine T-derivatized polystyrene beads (23). Briefly, 2 mCi (1 Ci = 37 GBq) of carrier-free Na^{125}I (New England Nuclear) was added to a glass tube containing four Iodobeads (Pierce) in 200 μl of Dulbecco's phosphate-buffered saline (pH 7.4) (DPBS) for 5 min at 27°C. Twenty micrograms of purified HurTNF was then added and incubated for an additional 7.5 min. The sample was loaded on a Sephadex G-25 column (Pharmacia) that had been equilibrated with DPBS/2% fetal bovine serum. Fractions were collected and assayed for both radioactivity and cytotoxicity.

Cell Lines. The human cell lines breast carcinoma MCF-7, BT-20, and SKBR-3; fibrosarcoma HT-1080; colon carcinoma HT-29; normal fibroblasts Hs27F; and fetal intestinal epithelial cell line FHs74Int were obtained from the Naval Biosciences Laboratory (Oakland, CA). A TNF-resistant variant of the MCF-7 cell line was developed at Cetus by cloning the cell line in the presence of HurTNF (250 units/ml) and then subculturing the survivors in increasing quantities of TNF for ≈3 months.

The following human cell lines were gifts from the indicated individuals: ZR-75-1, breast carcinoma (A. Frankel, Cetus); ACHN, renal carcinoma (E. Borden, University of Wisconsin); and JW5, B lymphoblastoid (J. Larrick, Cetus). The human cervical carcinoma ME180 was obtained from the American Type Culture Collection. The human foreskin fibroblasts trisomic for chromosome 21, GM-2504, were obtained from the Human Cell Repository (Camden, NJ). Normal human granulocytes were obtained by separating whole blood on Ficoll/Hypaque columns.

The mouse cell line L929S (TNF sensitive) was a gift from Lloyd Old (Sloan–Kettering Institute, New York). The

Abbreviations: HurTNF, human recombinant tumor necrosis factor; ^{125}I-HurTNF, ^{125}I-labeled HurTNF.

BALB/3T3 normal fibroblasts were obtained from the American Type Culture Collection.

All cell lines were cultured in standard medium formulations with the addition of 10% fetal bovine serum as described (24).

Affinity Cross-Linking. The homobifunctional agent 1,5-difluoro-2,4-dinitrobenzene (Sigma) was freshly prepared as a 1 nM solution in 5% dimethyl sulfoxide/95% DPBS. Monolayers of cells were incubated with 1.5 nM ^{125}I-HurTNF in growth medium with 10% fetal calf serum for 8 hr at 4°C on a rocking platform. Excess ^{125}I-HurTNF was decanted, cells were rinsed three times with cold DPBS, and 1 nM cross-linking agent and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride/5 μM pepstatin A/5 μM leupeptin/0.5 mM *o*-phenanthroline; Sigma) were added for an additional 16 hr at 4°C. Cell monolayers were then rinsed three times with cold DPBS, and cells were scraped, centrifuged, and lysed in 2% Triton X-100 for 30 min at 0°C. Cell lysates were centrifuged. Supernatants were harvested and analyzed by 7.5% $NaDodSO_4$/polyacrylamide gel electrophoresis (24).

RESULTS

HurTNF was iodinated to a specific radioactivity of 0.05–0.2 mCi/μg, which was equivalent to an average of 1 atom of ^{125}I per molecule. Iodinated TNF was fully biologically active, as determined on the mouse L929 TNF-sensitive cell line. Analysis of the radiolabeled HurTNF by $NaDodSO_4$/PAGE followed by autoradiography (Fig. 1 *Inset*) revealed two radioactive bands: a major band corrresponding to the 17-kDa monomer and a minor band apparently corresponding to a nonreducible 34-kDa dimer.

Time Course of HurTNF Binding. ^{125}I-HurTNF bound to specific receptor sites on the breast carcinoma, MCF-7, in a time- and temperature-dependent manner (Fig. 2). At 4°C, the steady state was reached in 30–60 min, whereas at 37°C, there was a higher level of cell-associated TNF and a plateau was reached in 2 hr. This is probably due to binding and internalization of TNF. Internalization of TNF has been noted to occur in other cell types (18, 19). The level of nonspecific binding at both temperatures was <20% of the total bound radioactivity (data not shown).

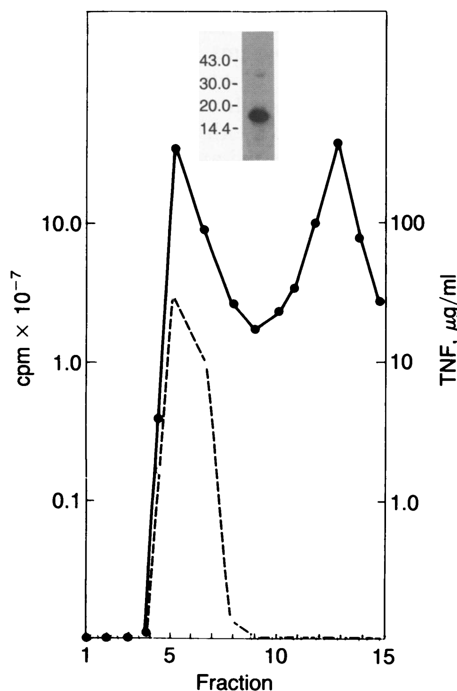

FIG. 1. Elution profile of ^{125}I-labeled TNF and analysis by $NaDodSO_4$/15% polyacrylamide gels. HurTNF was labeled with Iodobeads and was fractionated on a Sephadex G-25 column as described. ●, Radioactivity; ○, TNF cytotoxic activity. A 25-pg aliquot of ^{125}I-HurTNF (fraction 5, 2500 cpm) was autoradiographed (*Inset*); molecular mass markers (kDa) are indicated.

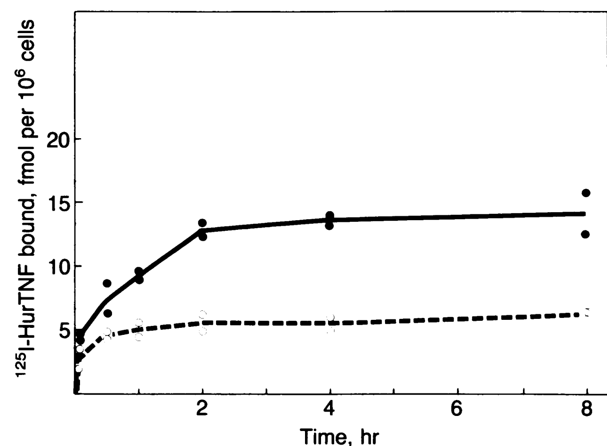

FIG. 2. Time course of binding of ^{125}I-HurTNF to specific receptors. Monolayers of MCF-7 (1 × 10^6 cells per well) were incubated in the presence of 1 nM ^{125}I-HurTNF for the indicated times at either 4°C (○) or 37°C (●). The amount of ^{125}I-HurTNF specifically bound was then determined as described.

The steady-state level of specific binding at 37°C was 2.4-fold higher than that attained at 4°C and did not change even after 8 hr of incubation. This is consistent with the finding by Rubin *et al.* (16) with a subclone of the murine L929 fibrosarcoma cell line (LM cells), human HeLa cells, and native LukII TNF.

Specificity of ^{125}I-HurTNF Binding. Fig. 3 depicts dose-dependent inhibition of binding of ^{125}I-HurTNF to MCF-7 cells by increasing concentrations of unlabeled HurTNF. When incubated for 2 hr at 4°C in the presence of 1 nM ^{125}I-HurTNF, the MCF-7 breast carcinoma cells bound 0.1–4% of the input radioactivity, >90% of which could be competed for by unlabeled TNF. β-interferon, interleukin 2, tumor growth factor α, interleukins 1α and -β, and epidermal growth factor did not compete for radiolabeled TNF (<20%; data not shown). These results indicate that ^{125}I-HurTNF binds to a specific receptor site on these cells. The binding of HurTNF to these sites is inhibited by antibodies that neutralize the cytotoxic activity of HurTNF (Fig. 4*A*).

Affinity and Number of TNF Receptors. Composite binding

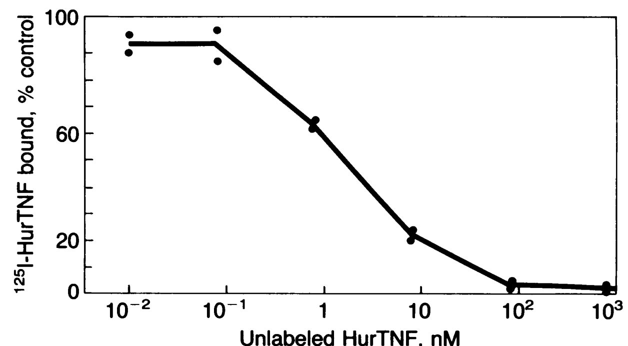

FIG. 3. Specificity of ^{125}I-HurTNF binding to cellular receptors. MCF-7 (1 × 10^6 cells per well) were incubated for 2 hr at 4°C in the presence of 1.0 nM ^{125}I-HurTNF and the indicated concentrations of unlabeled HurTNF. Total radioactivity bound to cells in each well was then determined. Duplicate determinations are expressed as percentage of ^{125}I-HurTNF bound to control cells (4000 cpm).

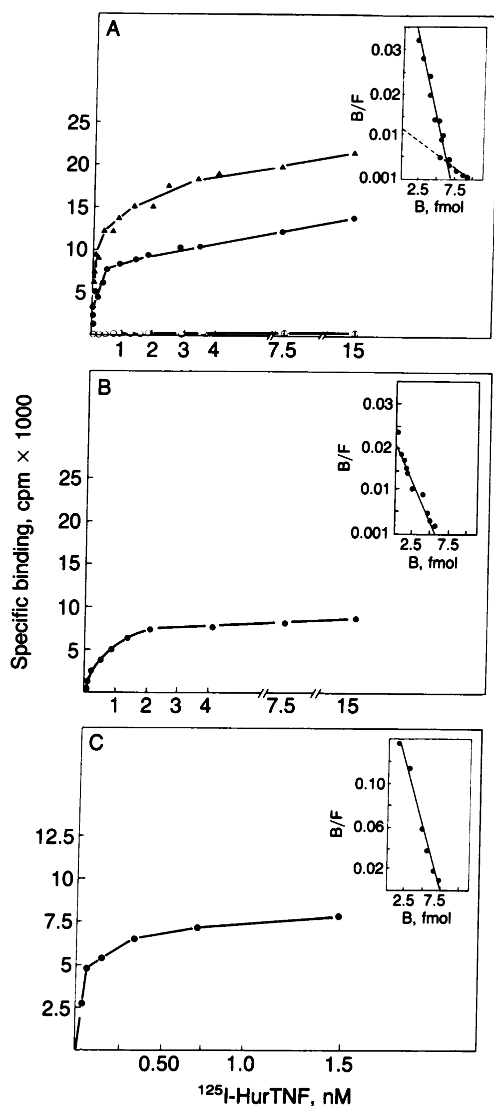

FIG. 4. Equilibrium saturation binding of ^{125}I-HurTNF to MCF-7 (breast carcinoma) (*A*), Hs27F (normal fibroblasts) (*B*), and peripheral blood granulocytes (*C*). Monolayers of MCF-7 cells and Hs27F (*A* and *B*) (10^6 cells per well in 35-mm tissue culture dishes) were incubated for 2 hr at 4°C (●) or 37°C (▲) (MCF-7 only) with various concentrations of ^{125}I-HurTNF with (nonspecific) and without (total) 1000-fold excess of unlabeled HurTNF. Rabbit polyclonal antibodies to TNF (diluted 1:100) were incubated with 1.5 nM TNF for 2 hr at 37°C and then bound to MCF-7 cells (○), as described above. The granulocytes (*C*) were suspended at 3×10^6 cells per ml in snap-top tubes and incubated for 8 hr at 4°C with various concentrations of labeled and unlabeled TNF as described above. Results represents specific binding (total nonspecific: B, bound; F, free).

isotherms obtained with various concentrations of ^{125}I-HurTNF and unlabeled HurTNF indicated that saturation of HurTNF receptors at 4°C and 37°C (shown for MCF-7 only) is achieved at 15 nM HurTNF in both MCF-7 cells (Fig. 4*A*) and Hs27F cells (Fig. 4*B*), but at 1.5 nM HurTNF in fresh peripheral blood granulocytes (Fig. 4*C*). Scatchard analysis of ^{125}I-HurTNF binding to MCF-7 cells at 4°C (Fig. 4*A Inset*) results in a curvilinear plot; the K_d values for the two components are 0.13 and 0.8×10^{-9} M. In contrast, Scatchard analysis of ^{125}I-HurTNF binding to Hs27F cells (Fig. 4*B Inset*) and fresh granulocytes (Fig. 4*C Inset*) showed a first-order interaction between HurTNF and its receptors in these cells. The K_d values for Hs27F and the granulocytes are 0.3 nM and 0.05 nM, respectively.

The number of receptors in the MCF-7 cells were 2500 high-affinity sites and 4000 lower-affinity sites per cell, while in Hs27F and the granulocytes there were 3000 and 300 high affinity sites per cell, respectively.

^{125}I-HurTNF Binding to Other Cell Lines. In addition to the three cell lines described above, we evaluated TNF receptor numbers on several human and rodent cell lines that vary in their response to the cytotoxic action of TNF (Table 1). Eight human tumor cell lines displayed an ability to specifically bind ^{125}I-HurTNF. We found that there was no correlation between receptor number and response. A decrease (by a factor of ≈10) in TNF receptor number was found in the MCF-7 TNF-resistant variant as compared to the parent line. Interestingly, the cell line did not respond to TNF even upon actinomycin D treatment. In contrast, the HT-1080 fibrosarcoma cell line did not respond to TNF cytotoxic action, except when treated with actinomycin D. Under these conditions, the cells bound 3 times less ^{125}I-labeled TNF. A decrease in TNF receptor number in actinomycin D-treated cells is not surprising since actinomycin D inhibits transcription. These findings confirm that a TNF receptor is necessary for response and that receptor number may not necessarily correlate with response.

We studied the TNF receptor on four normal human, lymphoblastoid, fibroblastic, and epithelial cell lines, and normal peripheral blood granulocytes (Table 1). We found that all sources of normal cells display a cellular receptor for TNF. There was no correlation between response—i.e., growth stimulation or cellular activation—and receptor number. None of the normal cell types was sensitive to the cytotoxic action of TNF.

Similar to human cells, rodent cell lines bound human ^{125}I-HurTNF and displayed low receptor number. Although

Table 1. Bioresponse and estimated HurTNF receptor number of human and rodent cells

	Cell line	Response	Estimated no. of receptors
	Human tumor cell lines		
MCF-7-S	Breast carcinoma	Cytotoxic	2500
MCF-7-R	Breast carcinoma	No response	200
	+ actinomycin D	No response	200
BT-20	Breast carcinoma	Cytotoxic	1700
ZR75-1	Breast carcinoma	Cytotoxic	1200
SKBR3	Breast carcinoma	Cytostatic	500
HT-1080	Fibrosarcoma	No response	3900
	+ actinomycin D	Cytotoxic	1300
HT-29	Colon carcinoma	Cytostatic	400
ME-180	Cervical carcinoma	Cytotoxic	2000
ACHN	Renal carcinoma	Cytostatic	2800
	Human normal cells and cell lines		
JW5	B lymphoblastoid	No response	200
Hs27F	Foreskin fibroblast	Growth stimulated	2200
Hs74Int	Fetal intestinal epithelial	Growth stimulated	7000
GM-2504	Trisomic foreskin fibroblast	No response	3800
Granulocytes		Activated	300
	Rodent cell lines		
L929	Fibrosarcoma	Cytotoxic	1400
3T3	Fibroblasts	No response	1700

Bioresponse to HurTNF was as described (8, 10), in which cytotoxic response is defined as cell lysis in 5 days, cytostatic response is a decrease in cell density, activated data were obtained from ref. 25, and growth stimulation is a dose-dependent increase in uptake of [^{3}H]thymidine following exposure to TNF.

we did not test many rodent cell lines, there was no correlation between receptor number and response to TNF.

Kinetics of Affinity Labeling of MCF-7 Cells with ^{125}I-HurTNF. We attempted to characterize the structural characteristics of the cellular receptors for TNF by cross-linking intact cells with ^{125}I-HurTNF using the homobifunctional agent 1,5-difluoro-2,4-dinitrobenzene (26). Fig. 5 shows an autoradiogram from a 7.5% $NaDodSO_4$/polyacrylamide gel of 1.5 nM ^{125}I-HurTNF bound to MCF-7 cells for 5, 30, 120, and 480 min, and then cross-linked for 16 hr at 4°C because cross-linking is inefficient. Three major labeled species were visible after 5 min of binding: 90 kDa, 75 kDa, and 54 kDa (Fig. 5*A*, lane 1); a fourth species (≈138 kDa) was more easily detectable at 30 min (lane 2). The intensity of the 138-kDa band increased with time and plateaued after 120 min (lanes 3 and 4). The kinetics of appearance and plateau of all four TNF-binding molecular species is consistent with the kinetics of binding and saturation of ^{125}I-HurTNF to cells at 4°C as shown in Fig. 2. Affinity cross-linking of ^{125}I-HurTNF to the breast carcinoma cells (MCF-7) in the presence of 0.75 μM unlabeled TNF inhibited all labeled species (lane 5).

Radiolabeled cross-linked TNF in the species described for the receptor(s) in this report were not detected (Fig. 5*B*). Our findings on cross-linking TNF to itself are consistent with those published by Kull *et al.* (15).

Affinity Labeling of Human Cell Lines with ^{125}I-HurTNF. We examined the molecular structure of the TNF receptor on cell lines that possess the TNF receptor, yet vary in their response to TNF. Specifically, we selected three tumor cell lines that respond to the cytotoxic (MCF-7), cytostatic (SKBR-3), and show no effect (MCF-7 R variant). Similarly, we selected a normal fibroblastic line that is growth stimulated (Hs27F) and another that is not affected by TNF (GM-2504). Fig. 6 shows an autoradiogram of cross-linked ^{125}I-HurTNF to the cell lines described above. Similar to the molecular structure of the TNF receptor on MCF-7 cells, three species (90 kDa, 75 kDa, and 54 kDa) were observed on all normal and tumor cell lines. However, the 138-kDa band was observed only on the MCF-7 TNF-sensitive cell line and was not apparent in any of the affinity-labeled $NaDodSO_4$/polyacrylamide gels of the other tumor and normal cell lines. This finding suggests a possible role for the 138-kDa band in the sensitivity of the MCF-7 tumor cell line to the cytotoxic action of TNF.

DISCUSSION

In this study, we show that human cell types derived from a breast tumor, foreskin fibroblasts, and peripheral blood granulocytes bind HurTNF through similar receptors, as judged by the affinity for TNF, which varied maximally ≈30-fold. However, we found differences in the concentrations necessary to achieve saturation (receptor occupancy) and biological response to TNF in the different cell types. It was interesting that lower concentrations of TNF were required to elicit a response from the granulocytes, as described (13), than from the MCF-7 cells. The concentration dependence of the biological response of the MCF-7 cells may not be surprising in light of the relatively higher number of TNF receptors (2500–4000 per cell), as compared to the granulocytes (300 per cell), compounded with the high affinity of HurTNF binding. In the case of the granulocytes, a biological response seems to be elicited at very low levels of cell-bound TNF. Diversity in hormone receptor systems and disparity of receptor occupancy and biological response has been discussed by Cuatrecases and Hollenberg (27). They described the effects of insulin in two cell systems: stimulation of glucose oxidation by rat epididymal fat cells (half maximal response at 50 pM) and stimulation of fibroblasts (half maximal response at 1 nM). Such disparity in receptor occupancy, saturation, and bioresponse was also described recently for interleukin 1 (28). Specifically, the half-maximal response for a T-lymphoma cell line to respond to interleukin 1 by producing interleukin 2 was at 5×10^{-14} M, and that for growth stimulation of a fibroblast cell line was 5×10^{-11} M.

Various investigators (15, 20, 21, 29, 30) have used the homobifunctional agent disuccinimidyl suberate to affinity label the TNF receptor. The results of these studies have been variable. Kull *et al.* (15) described 90-kDa and 75-kDa polypeptides in the TNF-sensitive mouse L929 cells, and Israel *et al.* (20) have confirmed that result in human cells (U937, KG-1, and FSII). In contrast, Tsujimoto *et al.* (29, 30) have described a 100-kDa polypeptide in human U937, HeLa, and HT-29 cells, while Scheurich *et al.* (21) have found a 93-kDa polypeptide in U937 and K562 cells. Similar to Kull and Israel (15, 20), we have also detected the 90-kDa and 75-kDa polypeptides in human cells when we used disuccinimidyl suberate (data not shown). However, when we used 1,5-difluoro-2,4-dinitrobenzene to affinity label the TNF receptor, under conditions of specific binding, we detected two additional polypeptides: a 138-kDa and a 54-kDa peptide. Specifically, we observed the 90-kDa, 75-kDa, and 54-kDa polypeptides on all TNF-binding human cell types studied, and we detected the additional 138-kDa polypeptide on the MCF-7 breast carcinoma that is highly susceptible to TNF cytotoxicity. A difference in the labeling pattern of the atrial natriuretic factor receptor by disuccinimidyl suberate and

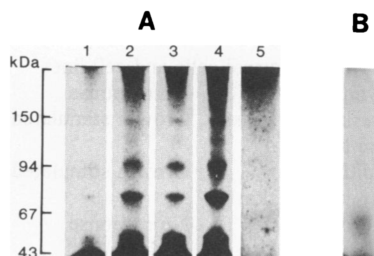

FIG. 5. Kinetics of binding and chemical cross-linking of ^{125}I-HurTNF to MCF-7 cells. ^{125}I-HurTNF (1.5 nM) was added to monolayers of MCF-7 cells (10^6 cells per well in 35-mm tissue culture dishes) for 5, 30, 120, and 480 min, and then cross-linked for 16 hr at 4°C. Samples were processed as described in the text and legend of Fig. 4. (*A*) Lanes 1–4, 5, 30, 120, and 480 min; lane 5, ^{125}I-labeled TNF cross-linked to MCF-7 cells in the presence of 0.75 μM unlabeled TNF. (*B*) ^{125}I-labeled TNF (15 pM) cross-linked to itself in solution for 16 hr at 4°C.

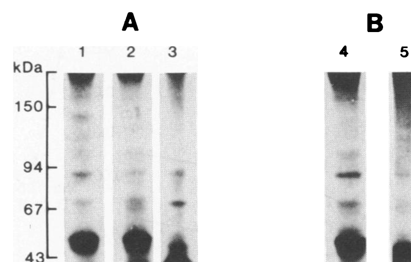

FIG. 6. ^{125}I-HurTNF cross-linking to intact tumor and normal human cell lines. Cells (10^6 cells per well in 35-mm tissue culture dishes) were incubated with 1.5 nM ^{125}I-HurTNF for 8 hr at 4°C, washed, and incubated for an additional 6 hr at 4°C in the presence of 1 nM 1,5-difluoro-2,4-dinitrobenzene and protease inhibitors. Cells were harvested, lysed, pelleted, and the supernatants were processed for $NaDodSO_4$/polyacrylamide gel electrophoresis as described in the text. (*A*) Tumor cell lines. Lanes 1–3, MCF-7 TNF sensitive, MCF-7 TNF resistant, and SKBR-3 sensitive to cytostatic action of TNF at high concentrations. (*B*) Normal cell lines. Lanes 4 and 5, Hs27F, foreskin fibroblasts that are stimulated to proliferate by TNF, and GM-2504, normal fibroblasts unresponsive to TNF.

1,5-difluoro-2,4-dinitrobenzene cross-linking agents has been reported (26). It was thought that the difference may be related to the spanning lengths and reactivities of the cross-linking agents.

We compared the molecular structure of the TNF receptor on cells that bind TNF specifically yet vary in their biological response to TNF. We purposely subcloned the TNF-sensitive cell line MCF-7 and selected for TNF-resistant variants that bind TNF. Using the homobifunctional reagent 1,5-difluoro-2,4-dinitrobenzene, we found that ^{125}I-HurTNF is preferentially cross-linked to four cellular polypeptides (138, 90, 75, and 54 kDa) in the TNF-sensitive MCF-7 cell line. In contrast, ^{125}I-HurTNF was preferentially cross-linked to only three cellular polypeptides (90, 75, and 54 kDa) on the MCF-7 TNF-resistant variant. Hence, the major difference in the TNF receptor structure between the two cell lines was detection of the 138-kDa polypeptide. This finding suggested that the response to the cytotoxic action of TNF resides in, or may be associated with, the 138-kDa protein. This prompted us to investigate further the molecular structure of the TNF receptor on other TNF-resistant and -responsive cell lines.

Interestingly, ^{125}I-HurTNF was found to preferentially cross-link to three cellular polypeptides (90, 75, and 54 kDa) in cell lines that do not respond to the cytotoxic action of TNF. These polypeptides were detected in the human tumor cell line SKBR-3, which responds to the cytostatic action of TNF at high doses (>5000 units/ml; see ref. 10), in the normal diploid fibroblasts (Hs27F), which are stimulated to proliferate in the presence of TNF, and in the trisomic 21 fibroblastic cell line (GM-2504), which does not appear to respond to TNF. The 138-kDa polypeptide was not detected in any of the cell lines described above. These findings provide further evidence for a connection between the 138-kDa polypeptide and cellular response to the cytotoxic activity of TNF.

If the 138-kDa band is the critical polypeptide for the TNF cytotoxic response, what role do the other TNF binding species play? What is the relationship of these polypeptides to each other? We feel that it is unlikely that the lower molecular weight polypeptides are cleavage products of the higher molecular weight polypeptides because all cross-linking experiments were performed in the presence of a cocktail of protease inhibitors. Although our studies do not fully delineate the TNF receptor structure, we feel we have suggestive evidence to propose a tentative model for the TNF receptor structure. We propose that the TNF receptor(s) is composed of noncovalently linked membrane-bound polypeptides that bind TNF with an apparent K_d of $0.05–0.8 \times 10^{-9}$ M. Each cell type may display different ratios of these polypeptides in the membrane, with the 138-kDa protein being the least abundant and/or even absent in most cells. Our Scatchard and cross-linking data support our contention that these polypeptides occur in different numbers on the membrane since the amount of the bound ^{125}I-HurTNF varied severalfold among the cell lines. The migration of these bands in $NaDodSO_4$ gels did not change appreciably under reducing and nonreducing conditions (data not shown). We hypothesize that the 138-kDa polypeptide has a similar high-affinity binding to TNF as the other polypeptides, but that it occurs at quantities less than the others since it was detected 30 min after binding.

To delineate the exact association of the four polypeptides with each other may be difficult at this time. However, the idea of the TNF receptor being composed of multiple high-affinity subunits is consistent with receptor structures for other peptides such as interleukin 2 (31), transforming growth factor β (32), and insulin (33). Similar to our studies, detection of these subunits was possible through cross-linking experiments.

Although our data are in agreement with the finding that there is a single class of high-affinity receptors for TNF on most cells (15, 22), we have routinely observed a curvilinear Scatchard plot of TNF binding on some TNF-sensitive cell lines—namely, BT-20 and ZR75-1—including the breast carcinoma MCF-7. There are two possible interpretations of this observation: (*i*) the likely existence of low-affinity binding sites for TNF and/or (*ii*) the curvilinear plot may have been an artifact that was generated by the use of large quantities of the ligand. Thus far, our cross-linking studies on the molecular characterization of the TNF receptor used only low quantities (1.5 nM) of TNF and hence favored the detection of only high-affinity sites for TNF.

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