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ARTICLES

Cell Density-Dependent Regulation of Cell Surface Expression of Two Types of Human Tumor Necrosis Factor Receptors and Its Effect on Cellular Response

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Abstract Tumor necrosis factor (TNF) is a multipotential cytokine known to regulate the growth of a wide variety of normal and tumor cells. It has been shown that the density of cells in culture can modulate the growth regulatory activities of TNF, the mechanism of which, however, is not understood. In this report, we investigated the effect of cell density on the expression of TNF receptors. The receptors were examined on epithelial cells (e.g., HeLa), which primarily express the p60 form, and on myeloid cells (e.g., HL-60) known to express mainly the p80 form. We observed that binding of TNF to both cell lines decreased with increase in cell density. Scatchard analysis of binding on HeLa and HL-60 cells revealed a 4- to 5-fold reduction in the number of TNF receptors without any significant change in receptor affinity in both cell types at high density. The decrease in TNF receptor numbers at high cell density was also observed in several other epithelial and myeloid cell lines. The downmodulation at high cell density was unique to TNF receptors, since minimum change in other cell surface proteins was observed as revealed by fluorescent activated cell sorter analysis. Neutralization of binding with antibodies specific to each type of the receptors revealed that both the p60 and p80 forms of the TNF receptor were equally downmodulated.

A decrease in leucine incorporation into proteins was observed with increase in cell density, suggesting a reduction in protein synthesis. Since inhibition of protein synthesis by cycloheximide also leads to a decrease in TNF receptors, it is possible that the density-dependent reduction in TNF receptor number is due to an overall decrease in protein synthesis. The density-dependent decrease in TNF receptors was accompanied by a decrease in intracellular reduced glutathione levels. A reduction in the number of receptors on TNF sensitive tumor cells induced by cell-density correlated with increase in resistance to the cytokine. © 1994 Wiley-Liss, Inc.

Key words: tumor necrosis factor, protein synthesis, cell density, cell proliferation, receptors, glutathione

INTRODUCTION

Human tumor necrosis factor (TNF) is a macrophage-derived cytokine with multiple biological activities, including antiproliferative effects against tumor cells, proliferative effects on normal cells, antiviral and antimicrobial effects, and immunomodulatory effects [see Aggarwal and Vilcek, 1991]. This cytokine interacts with cells through specific, high-affinity receptors [Aggarwal et al., 1985]. Recently two different forms of TNF receptors, with molecular masses of 60 kDa (p60) and 80 kDa (p80), have been identified [Hohmann et al., 1989; Brockhaus, 1990]. While the p60 form of the TNF receptor is expressed predominantly on epithelial cells, the p80 form is expressed mainly on myeloid cells [Hohmann et al., 1989; Brockhaus, 1990]. Expression of TNF receptors is controlled by multiple factors, including cytokines, protein kinases, phosphatases, and certain proteolytic enzymes [for review see Tsujimoto and Oku, 1991; Porteau et al., 1991].

Several reports suggest that the density of cells in culture can regulate the biological response of cells to growth regulatory cytokines [Patek et al., 1989; Palombella, 1989; van der Bosch, 1992; Kirstein, 1986]. Cell-mediated cyto-

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toxicity has also been shown to be regulated by the density of the target cells [Patek et al., 1989; van der Bosch, 1990; Keller, 1976]. The antiproliferative effects of TNF against certain cells have been shown to be reduced when the cell density reaches confluence [Patek et al., 1989]. In subconfluent and rapidly growing cells, TNF is growth-inhibitory, while in density-arrested cultures it stimulates DNA synthesis [Palombella and Vilcek, 1989]. Responsiveness to TNF is dependent on the cell cycle, the most sensitive phase being G2-M phase [Ruff and Gifford, 1981; Darzynkiewicz et al., 1984; Watanabe, 1987].

Whether the cell density-dependent response of target cells to TNF is due to the modulation of receptor expression or to other mechanisms is not clear. Receptors for several cytokines, namely, transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), have been reported to be modulated by cell density [Rizzino et al., 1988; Veomett et al., 1989; Holley et al., 1977]. These studies raised the question of whether cell surface expression of TNF receptors is also dependent on cell density and if so, whether the two forms of TNF receptor, are regulated similarly. In this study we report the downmodulation of both p60 and p80 forms of the TNF receptors with increase in cell density and an accompanying decrease in intracellular glutathione levels, protein synthesis and susceptibility of cells to TNF.

MATERIALS AND METHODS

Materials

Gentamicin, RPMI 1640, and DMEM medium, and fetal calf serum (FCS) were obtained from GIBCO, Grand Island, NY. Carrier-free $Na^{125}I$ was purchased from Amersham Corp., Arlington Heights, IL. Tetrachlorodiphenylglycouril, bovine serum albumin, cycloheximide, and gelatin were obtained from Sigma Chemical Co., St. Louis, MO. Propidium iodide was obtained from Calbiochem (La Jolla, CA). Monoclonal antibodies (MoABS) against CD11a (alpha chain of leukocyte function associated antigen-1, LFA-1), CD18 (beta chain of LFA-1), CD71 (transferrin receptor), and HLA-A,B,C antigens as well as FITC-conjugated rabbit anti-mouse immunoglobulin F(ab)'s were purchased from DAKO (Santa Barbara, CA) and MoAbs against CD13 (OKM13), CD33 (MY9), and CD3 (OKT3) were obtained from Coulter (Hialeah, FL) Becton Dickinson (Mountain View, CA) and Ortho (Raritan, NJ), respectively. Bacteria-derived recombinant human TNF purified to homogeneity with a specific activity of 5×10^7 units/ml was kindly provided by Genentech Inc., South San Francisco, CA. The highly purified form of the recombinant extracellular domains of TNF receptors p60 (TNF receptor I) and p80 (TNF receptor II), with molecular masses of 30 kDa (sp60) and 40 kDa (sp80), respectively, were kindly provided by Dr. Tadahiko Kohno of Synergen, Boulder, CO [Kohno et al., 1990]. Polyclonal antibodies in rabbits were raised against each type of receptor and purified by receptor-affinity chromatography.

Cells

The following cell lines obtained from American Type Cell Culture Collection (Rockville, MD) were used in these studies: HL60 (acute promyelocytic leukemia, ATCC CRL 1593), U937 (histiocytic lymphoma, ATCC CCL 240), THP-1 (acute monocytic leukemia, ATCC TIB 202), HeLa (epithelioid cervical carcinoma, ATCC CCL 2), MCF7 (breast adenocarcinoma, ATCC HTB 22), and A375 (human melanoma, ATCC CRL 1619). The HL60, U937, MCF7, A375, and THP-1 cell lines were grown in RPMI 1640 medium, whereas HeLa cells were grown in DMEM medium, supplemented with 10% FCS, and 50 μg/ml gentamicin.

The cells were seeded at 1×10^5 cells/ml (nonadherent cell lines) or 0.25×10^5 cells/ml (5.7×10^3 cells/cm^2) (adherent cell lines) in 175 cm^2 tissue culture flasks (Falcon 3028, Becton Dickinson Labware, Lincoln Park, NJ) and grown at 37°C in an atmosphere of 95% air and 5% CO_2. The density of cells one day after the subculture was considered low, and that reached in 3 days (HeLa) and 4 days (HL-60) later as high. In some experiments adherent cells were grown for 4–5 days until they reached confluence. The adherent cells were passaged routinely every third day at 60–80% confluency. For receptor-binding assay the adherent cells were detached from the flasks by a short treatment with 0.05% trypsin/0.5 mM EDTA mixture (4 min, 37°C), as used routinely for passage of these cell lines. In preliminary experiments with nonadherent cells, it was determined that this trypsin treatment had no effect on the TNF receptors.

Receptor-Binding Assay

Recombinant human TNF was labeled with $Na^{125}I$ using the IODOGEN procedure as described previously [Aggarwal et al., 1985]. The specific activity of labeled TNF was 17–22 μCi/μg. Binding assays were performed in 96-well microplates (Falcon 3911, Becton Dickinson Labware, Oxnard, CA) as described [Higuchi and Aggarwal, 1992a]. Briefly, 1×10^6 cells/well were incubated in a binding buffer (RPMI 1640-10% FCS) with ^{125}I-labeled ligand (2×10^5 cpm) and with or without 200 nm unlabeled ligand (100-fold excess) for 1 h at 4°C in a total volume of 0.1 ml. Thereafter, cells were washed three times with ice-cold PBS-0.1% BSA, and cell-bound radioactivity was determined by a Packard gamma counter (model CD 5010). To determine the dissociation constant (K_d) and receptor number on cells at low and high density cultures, Scatchard analysis was performed by adding increasing amount of labeled TNF to 3×10^5 of HeLa cells and 4×10^5 HL60 cells in the presence or absence of a 100-fold excess of unlabeled TNF. K_d and receptor numbers were calculated according to the method described by Scatchard [Scatchard, 1949].

Indirect Immunofluorescence Assay

Cells (5×10^5) were incubated with 1 μg of monoclonal antibodies in 100 ul total volume of phosphate buffered saline (PBS) containing 0.1% BSA and 2 mg/ml human immunoglobulin for 80 min at 4°C. FITC-conjugated rabbit anti-mouse immunoglobulin F(ab)'z diluted 1/20 with PBS containing 0.1% BSA was used as a second antibody (50 ul/well, 4°C, 30 min). Each sample was analyzed by flow cytometry (Ortho Cytoron Absolute) after fixation with 1% formalin. Gating was performed for the living cells in FSC and SSC parameters. The relative fluorescence intensity (mode channel number) of 5,000 cells per sample was determined.

Flow Cytometric Analysis of the DNA Content

Flow cytometric analysis of the DNA content of cells was performed with the method described by Nicoletti et al. [Nicoletti et al., 1991]. Briefly, 4–6 $\times 10^5$ cells in 100 ul of culture medium were lysed by adding 0.9 ml of a mixture of 0.1% Triton X-100 and 0.1% Na-citrate. This was followed by staining the nuclei with 30 ug/ml propidium iodide. Samples were analyzed by Ortho Cytoron Absolute flow cytometer. The percentage of cells in S phase were analyzed by means of DNA cell-cycle analysis software (Ortho Cell Program, simple methods).

Antiproliferative Assays

In nonadherent cell lines, the antiproliferative assays were done using the modified tetrazolium salt (MTT) assay as previously described [Hansen et al., 1989]. Briefly, 1×10^4 cells were incubated in the presence or absence of a serial dilution of TNF in a final volume of 0.2 ml for 72 h at 37°C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37°C, 50 μl of the *extraction* buffer (20% sodium dodecyl sulphate, 50% dimethyl formamide) was added. After an overnight incubation at 37°C, the optical densities at 570 nm were measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with the extraction buffer as a blank.

In the case of adherent cell lines, cells were plated in 96-well Costar plates (2,500 cells/0.1 ml/well). After incubation at 37°C for different time intervals serial dilutions of TNF were added for 72 h at 37°C. Thereafter, the viable cells were monitored by crystal violet staining according to the procedure as described [Ali-Osman et al., 1990]. Percent relative cell viability was calculated as optical density in the presence of TNF divided by optical density in the absence of the cytokine (media) multiplied by 100.

Determination of ^3H-Leucine Incorporation Into Proteins

Cells (0.01×10^6/0.1 ml) were cultured in 96-well plates for different days (1–4 days) to a density of 0.16×10^6/0.1 ml at 37°C. During the last 6 h of incubation, cells were pulsed with 1 uCi of tritiated leucine, washed with the medium, and then solubilized with 0.2% sodium dodecyl sulfate. To determine the amount of leucine incorporated into the protein, trichloroacetic acetic acid (TCA) precipitation was performed with 10% TCA at 4°C for 30 min. The TCA-insoluble fraction was harvested on a glass fiber filter, washed, and counted on a Packard scintillation counter.

Determination of Intracellular Glutathione Levels

The intracellular glutathione levels were determined by an ion-exchange high-performance liquid chromatography method as described previously [Aggarwal, 1985]. Briefly, 3 × 10^6 cells cultured at high and low density were homogenized in 10% $HCLO_4$ and centrifuged. The pellet was analyzed for protein by the method of Lowry and the supernatant was analyzed for glutathione content. The latter was carried out by the addition of 1,10-phenanthroline (2.5 mM) and iodoacetic acid (10 mM). After neutralization of the mixture with $KHCO_3$, the reaction mixture was stored for 2 h in the dark and then 1-fluoro-2,4-dinitrobenzene was added to achieve a 40 mM final concentration. After derivatization overnight, the samples were centrifuged, and the supernatants diluted with 80% methanol and chromatographed on a 3-aminopropyl-spherosorb ion-exchange column as described [Ali-Osman et al., 1990]. All determinations were made in triplicate and the results were expressed as nanomoles of glutathione per mg of protein.

RESULTS

Effect of Cell Density on TNF Binding to Epithelial and Myeloid Cells

We investigated the effect of cell density on HeLa, an epithelial cell line that mainly expresses the p60 form of the TNF receptor, and on HL-60, a myeloid cell line that displays primarily the p80 form of the receptor. These two cell types also differ in that HeLa cells adhere to plastic plates and HL60 cells do not. HeLa and HL-60 cells were cultured for 5 days starting at low cell density. Specific TNF binding was examined on different days as the cells approached high density. As indicated in Figure 1, TNF binding decreased with an increase in cell density in both cell lines. An almost 75% decrease in TNF binding to HL60 (upper panel) was observed after five day culture, during which cell density increased from 0.2 × 10^6 cells/ml (low density) to 1 × 10^6 cells/ml (high density). The decrease in TNF binding in HeLa cells was more rapid (lower panel). An approximately 85% decrease in binding occurred when the cell density increased from 75 cells/mm^2 (day 1) to 200 cells/mm^2 (day 3). At that time, cells appeared to be ~80% confluent.

To determine whether the decrease in TNF binding was due to a decrease in the number of TNF receptors or to a change in receptor affinity, we performed Scatchard analysis on HeLa and HL-60 at low and high cell densities (Fig. 2). The specific binding of TNF to both HL 60 (upper panel) and HeLa (lower panel) cells at

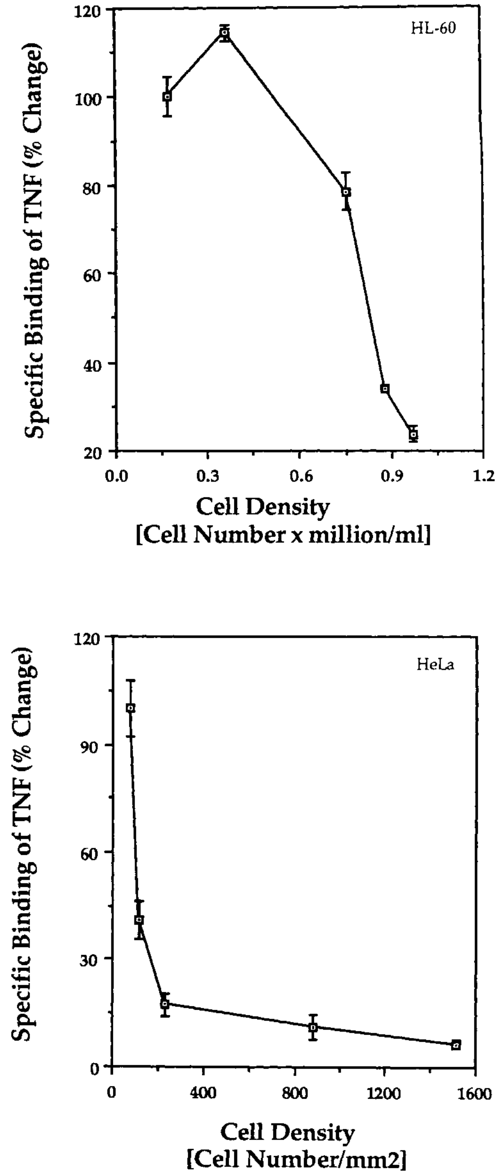

Fig. 1. Effect of cell density on TNF binding to HL-60 (upper panel) and HeLa (lower panel) cells. HeLa and HL-60 cells were cultured starting at low density of 57 cells/mm^2 and 0.1 × 10^6 cells/ml, respectively, for 5 days. On day 1 when TNF binding was initiated, the cell density was 75 cells/mm^2 (HeLa) and 0.2 × 10^6 cells/ml (HL-60). The cell viability was above 98% throughout the experiment as indicated by trypan blue staining. TNF binding assay was performed every day using 1 × 10^6 cells/well (see Materials and Methods), and the values of specific binding at each day were compared to the specific binding obtained on day 1 (100%). Each value represents average and standard error of three determinations of a representative experiment.

saturating concentration was almost sixfold lower in low-density cultures than in high-density cultures. Scatchard analysis of the data at high and low density for each cell line is

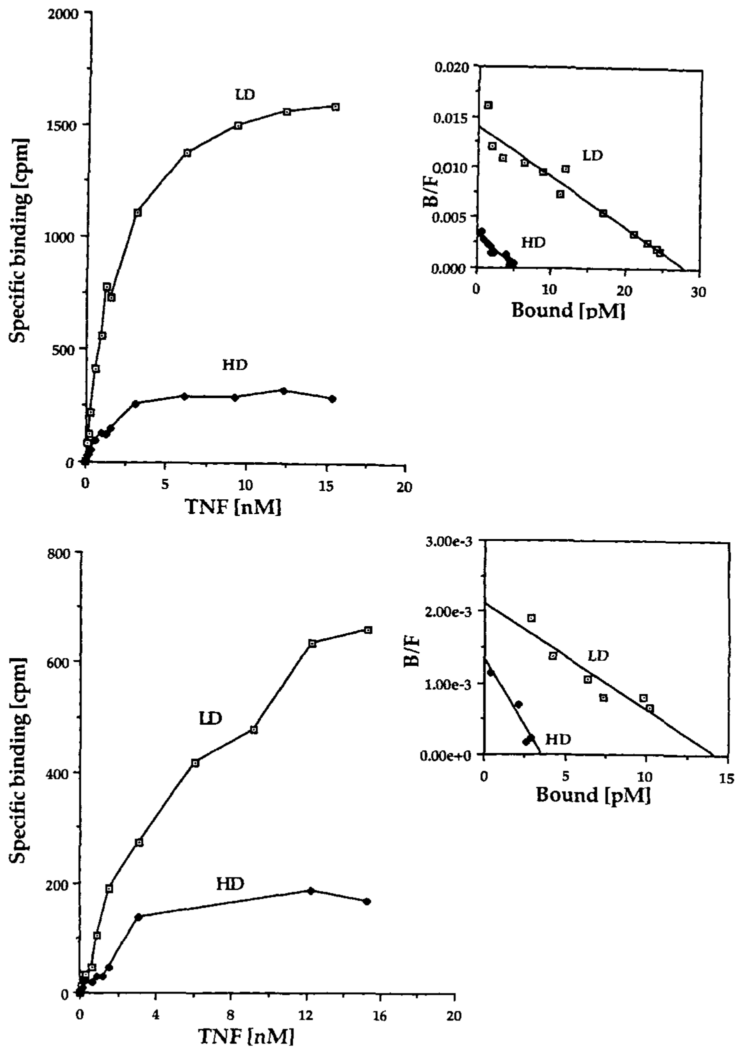

Fig. 2. Specific binding of TNF and Scatchard analysis (inset) on HL-60 (upper panel) and HeLa (lower panel) cells at low and high density. Cells were cultured as described in legend to Figure 1. For HL-60, the values for low density (LD) and high density (HD) are 0.2×10^6 cells/ml (day 1) and 1×10^6 cells/ml (day 4), respectively. For HeLa, the values are 103 cells/mm^2 (day 1) and 1,029 cells/mm^2 (day 3), respectively. TNF binding assay was performed as described in Materials and Methods by adding increasing amount ^{125}I-TNF to the cells in the presence or absence of a 100-fold excess of unlabeled ligand. All measurements were carried out in triplicate. Each point in the figure represents the average of three determinations.

shown in insets of Figure 2. When K_d and receptor numbers were calculated, we found a five-fold reduction in the number of TNF receptors at high cell densities in HL-60 cells and no change in the K_d values (Table I). HeLa cells also showed a four-fold decrease in total receptor number at high cell density, but the receptor affinity increased by 2- to 3-fold. The decrease in TNF receptors at high cell density was found to be not due to its effect on the cell viability (Table I).

The decrease in TNF receptors at high cell density could occur because more cells may accumulate in one phase of the cell cycle as compared to the other. Therefore we analyzed the cells for G1, S, and G2 M phases of the cycle by FACS analyzer. These results (Table II) show approximately 10% difference in the proportion of cells in G_1 phase as well as $S+G_2+M$ phases between low and high cell density. Thus changes in cell cycle distribution cannot be responsible for the decrease of TNF receptor numbers at high cell density.

To investigate whether our findings are unique to TNF receptors or they are general phenomena with other cell surface antigens, expressions of other characteristic cell surface markers like

TABLE I. TNF Receptors and Their Affinity on HeLa and HL60 Cells at Low and High Cell Density*

Cell line	Density	Viability	TNF receptors/cell	Kd (nM)
HL60	Low	100.0	4,209	1.99
	High	88.1	802	1.61
HeLa	Low	100.0	2,841	6.72
	High	97.0	714	2.84

*Scatchard analysis was performed with cells derived from low and high density cultures as described in legend to *Figure 2 and in* Materials and Methods. Cell viability was determined by trypan blue exclusion on 200 cells.

TABLE II. Cell Cycle Distribution of HL60 and HeLa Cells at Low and High Density

Cell lines	Time (days)	Cell density[a]	G_1	% Cells in S	G_2 + M
HL60	1	0.11	48.1	35.0	18.9
	2	0.19	50.4	30.0	19.6
	3	0.37	52.7	31.7	15.5
	4	0.68	57.5	28.7	13.8
HeLa	1	69	45.3	32.5	22.2
	2	141	44.0	35.9	20.1
	3	475	46.6	31.8	21.6
	4	731	55.7	26.7	17.6

[a]HL-60 and HeLa cells were seeded at 0.1×10^6 cells/ml and 57 cells/mm^2 (1×10^6 cells/20 ml medium), respectively, for 1–4 days as described in the legend to Figure 1. Determination of DNA content of cells derived from cultures on different days was performed by propidium iodide staining as described in Materials and Methods. Cell density for HL-60 is expressed as X million cells/ml and that for HeLa as X cells/mm^2.

myeloid differentiation antigens (CD13, CD-33), class I HLA molecules, cell adhesion molecules (leukocyte function-associated antigen-1, CD11a/CD16), and proliferation markers (transferrin receptor, CD71) were tested on HL60 cells. The fluorescence intensity of monoclonal antibody binding was compared in cells derived from low and high density. Our results show (Table III) a 10–15% reduction in fluorescent intensity at high cell density with antibodies against myeloid differentiation antigens and transferrin receptors (CD 71) but no differences were detected in the expression of the other cell surface markers studied. The extent of reduction in the expression of cell surface molecules differs among the different markers, and it seems to be much less than that observed with TNF receptors. These observations indicate that the phenomenon observed at high density is unique to the TNF receptor.

TABLE III. Effect of Cell Density of the Expression of Different Cell Surface Antigens on HL60 Cells*

	Mode channel number		
Antigens	Low cell density	High cell density	Reduction in mode channel number
HLA-A,B,C	223.6	207.4	16.2
CD13	163.7	130.1	33.6
CD33	143.6	121.0	22.6
CD11a	189.4	180.0	9.4
CD18	141.3	131.5	9.8
CD71	176.4	145.8	30.6
CD3 (negative control)	77.9	58.2	19.7

*Cells were cultured for 4 days as described in the legend to Figure 1 starting low cell density. The relative fluorescence intensity of staining of cells derived from low and high cell density cultures was determined by indirect immunofluorescence as described in Materials and Methods. At both cell densities per cent of positive cells with all the MoAbs was higher than 90% as compared to a negative control MoAb of the same isotype (anti-CDS, IgG1).

To examine whether the effect of cell density on TNF receptors was characteristic only of HeLa and HL-60 cell lines, we tested other cell lines. Among epithelial cell lines, we examined the human breast adenocarcinoma line, MCF7 and the melanoma cell line A-375; among myeloid cell lines, the human histiocytic lymphoma cell line U-937 and monocytic leukemia cell lines THP-1 were examined. Our results (Table IV) indicate that in both epithelial and myeloid tumor cell lines TNF binding was reduced at high cell density. The decrease in TNF binding was more pronounced with epithelial cell lines than with myeloid cell lines (60–80% and 30–70%, respectively). Changes in TNF binding to MCF7 cells were very similar to that in HeLa cells, starting at subconfluence, while the decreases in TNF binding to A-375 cells occurred only when the cells had reached confluence (data not shown). Changes in TNF binding to U-937 and THP-1 cells were similar to that observed in HL-60 cells.

High Cell Density Downregulates Both the p60 and p80 Forms of the TNF Receptor

In contrast to HeLa cells, which express only the p60 form of the TNF receptor, HL-60 cells express both form [Hohmann et al., 1989; Brockhaus, 1990]. Therefore we examined whether the reduction of TNF binding observed in HL-60

TABLE IV. Effect of Cell Density on Specific Binding of TNF to Various Human Cell Lines*

Cell line	LD	SB (cpm)	HD	SB (cpm)	Change (%)
Epithelial (adherent) cells/mm^2					
Cervical carcinoma (HeLa)	35	1,487 ± 54	989	257 ± 92	82.7
Breast adenocarcinoma (MCF-7)	37	2,866 ± 159	248	1,198 ± 72	58.2
Melanoma (A-375)	41	1,201 ± 75	1,464	287 ± 12	76.1
Myeloid (nonadherent) 10^6 cells/ml					
Acute promyelocytic (HL-60)	0.2	3,997 ± 176	0.9	1,351 ± 27	66.2
Histiocytic lymphoma (U-937)	0.2	7,511 ± 309	1.2	3,949 ± 145	47.4
Acute monocytic (THP-1)	0.1	821 ± 31	1.2	571 ± 41	30.5

*Cells were cultured for 4–5 days as described in the legend to Figure 1 starting at low cell density. The specific binding (SB) of TNF was determined on day 1 (LD; low density) and day 4 or 5 (HD; high density) after seeding the cells as described in Materials and Methods. Each value represents the average of three determinations.

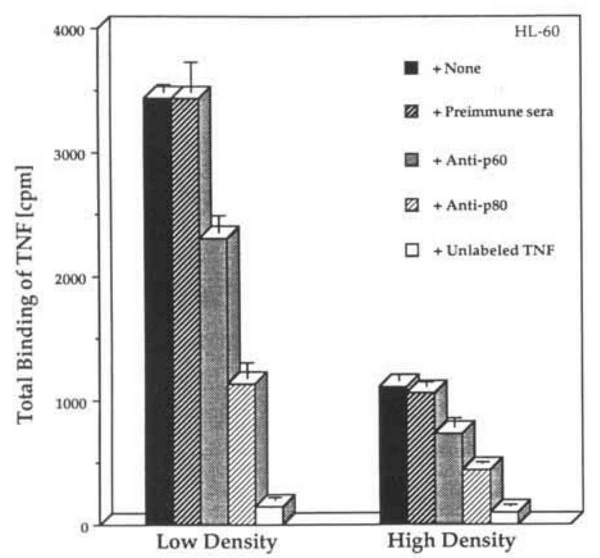

Fig. 3. Effect of cell density on the expression of p60 and p80 forms of TNF receptors on HL-60 cells. HL-60 cells (1 × 10^5 cells/ml) were cultured for 4 days as described in legend to Figure 1. At low (0.2 × 10^6 cells/ml on day 1) and high density (0.9 × 10^6 cells/ml on day 4) standard TNF binding assay was performed as described in Materials and Methods in the presence or absence (black bars) of 2.4 μg/ml polyclonal, affinity-purified anti-p60 (shaded bars), or anti-p80 (cross-hatched bars) antibody as well as preimmune IgG (dark shaded bars). The nonspecific binding was measured in the presence of 200 nM unlabeled TNF (empty bars). Each value represents the average and standard error of three determinations.

cells at high density was due to a decrease in the expression of only one or both kind of receptors. Polyclonal antibodies raised against the extracellular portion of p60 and p80 receptors inhibited the total binding of TNF by 34% and 66%, respectively, at low cell density (Fig. 3). At high density, 32% and 59% inhibition of p60 and p80 was found, respectively. These results indicate that both kinds of receptors were responsible for the overall decrease in the number of TNF receptors on HL-60 cells at high density.

Effect of Inhibition of Protein Synthesis on TNF Binding

Because the decrease in TNF binding to myeloid cells was observed at a cell density that also decreases the cell growth rate, it is possible that decreased cell metabolism led to overall decrease in the rate of protein synthesis and thus the synthesis of TNF receptors. Figure 4 clearly indicates that in both U937 (upper panel) and HL60 cells (lower panel), leucine incorporation into the proteins decreased with increases in cell density. These results suggest that higher cell density slows the metabolic processes.

In order to determine whether the inhibition of protein synthesis actually led to the decrease in TNF receptors, we examined the effect of protein synthesis inhibitor cycloheximide (CHX) on TNF binding to HL-60 cells at low density. Our results show (Fig. 5) a 30% decrease in the specific binding of TNF after exposure of cells to CHX (10 ug/ml) for 2 h, and a further decrease, of up to 65%, by the end of a 6 h incubation period. Thus it is clear that inhibition of protein synthesis can decrease the binding of TNF to cells.

Effect of Cell Density on the Biological Response to TNF

We examined the association of decrease in the number of TNF receptors with the biological response of cells to TNF. As U-937 and MCF 7, unlike HeLa and HL60, have been shown to be highly sensitive to the antiproliferative effects of TNF, we used these cells in this study. The U-937 cells were cultured either at low (0.2 × 10^6 cell/ml) or high (0.9 × 10^6 cells/ml) density, as were MCF7 cells (37 vs. 248 cells/mm^2). Both cell lines were cultured in 96-well microplates. As shown in Figure 6, TNF (1,000 units/ml)

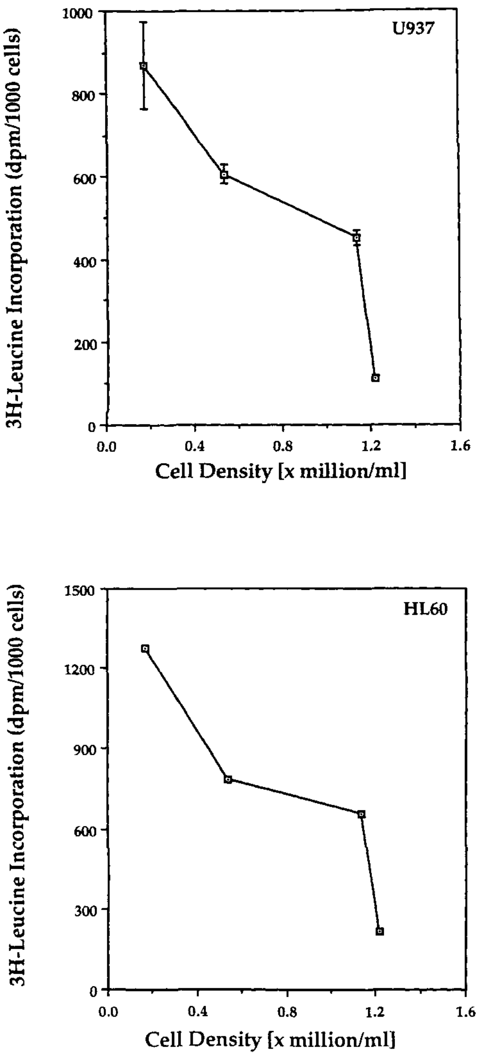

Fig. 4. Effect of cell density on protein synthesis in U-937 (*upper panel*) and HL-60 (lower panel) cells. Cells (0.01 × 10^6/0.1 ml) were cultured in 96-well plates for different days (1–4 days) to a density of 1.6 × 10^6/ml at 37°C. During last 6 h of incubation, cells were pulsed with 1 uCi of tritiated leucine, then washed with the media and solubilized with 0.2% sodium dodecyl sulfate. In order to determine the incorporation of leucine into the protein, trichloroacetic acetic acid (TCA) precipitation was performed with 10% TCA at 4°C for 30 min. The TCA insoluble fraction was harvested on a glass fiber filter and counted on a Packard Scintillation Counter.

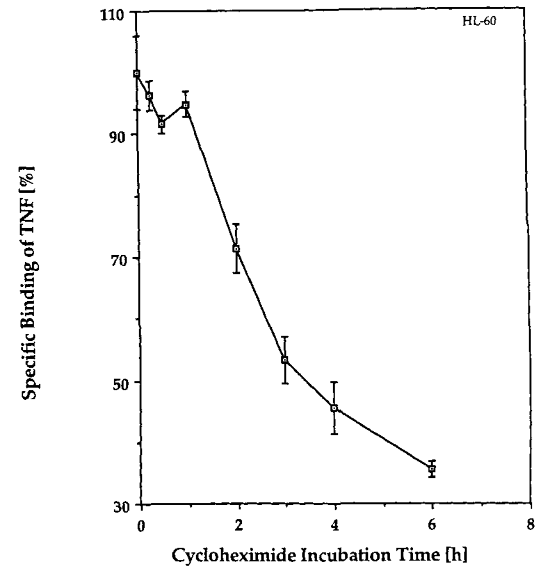

Fig. 5. Effect of cycloheximide on specific binding of TNF to HL-60 cells; 1 × 10^6 HL-60 cells obtained from low-density cultures (0.2 × 10^6/ml) were incubated for different times at 37°C in 0.2 ml volumes in 96-well microplates in the presence of 10 μg/ml cycloheximide. Thereafter, cells were washed three times with ice-cold binding medium, and TNF binding assay was performed in the presence or absence of 100-fold excess of unlabeled ligand as described in Materials and Methods. The values of specific binding in the absence of cycloheximide were expressed as 100%. Each value represents the average of three determinations.

induced approximately 40% cytotoxicity in U-937 cells when plated at low density but no cytotoxicity when these cells were allowed to grow to high density before the cytotoxicity assay. The cytotoxicity against MCF7 cells was also reduced by 66% after preculturing the cells to high density before TNF cytotoxicity assay. These results indicate that the cell density-dependent decrease in TNF receptors is accompanied by a decrease in the biological response to this cytokine.

Effect of Cell Density on Intracellular Glutathione Levels

One of the mechanisms reported to modulate cellular resistance to TNF is change in intracellular glutathione [Zimmerman et al., 1989a,b; Ishii et al., 1992]. Therefore, we examined the effect of cell density on the cellular reduced glutathione levels. The results of these experiments are shown in Table V. An approximately 40–50% decrease in cellular glutathione level was observed in several cell lines at high cell density. Thus it is possible that glutathione plays an important role in decreasing TNF receptors, which in turn may decrease the biological response of the cytokine.

DISCUSSION

In this report, we demonstrate that the density at which cells are grown affects the number of TNF receptors in both adherent (epithelial) and nonadherent (myeloid) tumor cell lines, which differentially express the p60 and p80 forms of the TNF receptors respectively. A decrease in receptor number with an increase in cell density was observed. Both the p60 and p80 forms were equally affected. We also found that the decrease in receptors with increased density was accompanied by a decrease in protein synthesis, an increase in resistance of the biological response to the cytokine, and a decrease in intracellular glutathione levels.

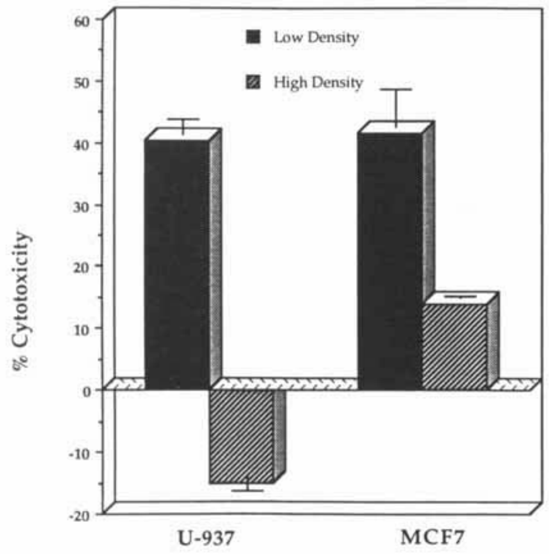

Fig. 6. Effect of cell density on the biological response of U-937 and MCF7 cells to TNF; 1 × 10^4 cells of U-937 and 2 × 10^3 cells of MCF7 cell lines were plated into the wells of 96-well microplates in 0.1 ml volumes. Low-density cultures (0.2 × 10^6/ml for U-937 and 37 cells/mm^2 for MCF-7) and high density cultures (0.9 × 10^6 cells/ml for U-937 and 248 cells/mm^2 for MCF-7) were incubated with 1,000 U/ml TNF in a total final volume of 0.2 ml for 3 days at 37°C. Thereafter, the viable cells was determined by either crystal violet staining (for MCF7 cells) or MTT assay (for U-937 cells) and the results were calculated for percent cytotoxicity as described in Materials and Methods. All determinations were made in triplicate.

Our finding that TNF-binding to various tumor cell lines decreases as the cell density increases is in agreement with results reported for other cytokines. The decreases in cellular binding of TGF-β, PDGF, EGF, and FGF to non-transformed mouse fibroblast-derived and to human carcinoma cell lines has been described when the culture density increased [Rizzino et al., 1988; Veomett et al., 1989; Holley, 1977]. Our results with the HL60 cell line show that the decrease in TNF binding at high cell density is due to the reduction in TNF receptor density rather than receptor affinity. These results are similar to those previously reported for TGF-β and EGF [Rizzino et al., 1988, 1990] and for FGF [Veomett et al., 1989; Neufeld and Gospodarowicz, 1985]. In the case of HeLa cells, however, we observed not only a decrease in the number of TNF receptors, but also a 2–3 fold increase in receptor affinity. Our finding is not unique to the TNF receptors as the expression of other cell surface molecules like differentiation antigens and transferrin receptors also decreased at high density in HL60 cells, although not to the same extent. TNF receptors seem to be more sensitive to downregulation at high cell density than the other markers investigated.

Recently two different form of TNF receptors have been isolated [Hohmann et al., 1989; Brockhaus, 1990]. It has been shown that the p60 form of the receptor is involved in the cytotoxic effects of TNF whereas the p80 form is responsible for the proliferation of certain cells [Tartaglia et al., 1991]. We found that the expression of both forms was diminished at high density. Previously, we have shown that there is a trans-downmodulation of TNF receptors in U-937 cells, i.e., the downmodulation of p60 will cross-

TABLE V. Effect of Cell Density on the Levels of Glutathione in Different Cell Lines*

	GSH levels (nmoles/mg protein)							
	Low cell density			Mean ± SD	High cell density			Mean ± SD
Myeloid cell lines								
U-937	32.2	28.4	30.3	30.3 ± 1.55	11.3	17.9	18.5	15.9 ± 3.3
HL-60	48.2	51.2	57.3	52.2 ± 3.8	27.8	26.8	29.6	21.9 ± 6.6
THP-1	30.1	31.1	34.6	31.9 ± 1.9	23.1	23.0	24.2	22.5 ± 5.4
Epithelial cell lines								
HeLa	59.0	60.5	51.3	56.9 ± 4.0	30.8	30.2	29.9	24.4 ± 5.8
A-375	86.1	82.1	82.1	83.4 ± 1.9	27.1	37.0	36.9	26.3 ± 6.7
MCF-7	90.1	89.4	—	89.8 ± 0.35	54.9	52.4	51.5	52.9 ± 1.4

*Cells were cultured at low (0.2–0.3 × 10^6 cells/ml for nonadherent and 50–80 cells/mm^2 for adherent) and high (0.9–1.2 × 10^6 cells/ml for nonadherent and 900–2,000 cells/mm^2 for adherent) density; 3 × 10^6 cells of each type were analyzed at each cell density for reduced glutathione contents as described in Materials and Methods. Each determination was made in triplicate.

downmodulate p80 and vice versa [Higuchi and Aggarwal, 1992b]. It is possible that the co-downmodulation of p60 and p80 receptor at high HL-60 cell density occurs by a similar mechanism. Perhaps the presence of the p80 form of the receptor is responsible for the slower kinetics observed with HL-60 cells than with HeLa cells, which do not express p80 form.

Most likely, different mechanisms are responsible for the downregulation of the TNF receptors in the adherent epithelial cells as opposed to the nonadherent myeloid cells. For instance, we observed that HeLa and MCF7 cells grow in smaller or bigger islets even at subconfluent densities, while A-375 cells do not. Decrease in binding of TNF to A-375 cells was observed at much higher density (at confluence) than in the case of HeLa or MCF7 cells. It is possible that signals triggered by close proximity or cell-to-cell contact can decrease the number of TNF receptors. An association has been observed between resistance of target cells to TNF-induced lysis in mouse L cells, U-937 cells, and Chinese hamster ovary cells and the presence of gap junctions and tumor cell morphology [Fletcher et al., 1987; Matthews and Neale, 1988], which supports the hypothesis that increasing the proximity of cells either by changing the microenvironment or by increasing cell-to-cell contact may induce different biological responses to TNF.

In the case of the myeloid (nonadherent) cell lines investigated in our study, nonpermissive conditions for growth at high density, e.g., depletion of growth factors, nutrients, etc., may result in G1/G0 arrest. It has already been found that TNF-binding is dependent on cell cycle: it is highest at G2-M phase in mouse L-M fibroblast-derived cells [Watanabe et al., 1987]. Therefore it is possible that the decrease in the number of TNF receptors on myeloid cells reflects a cell cycle-dependent downregulation of the receptors. However, there was only a slight change in the cell cycle distribution in high density cultures as compared to low density. Cell viability was also at least 97% in both low and high density cultures. These observations rule out the role of these possibilities in the decrease of the expression of TNF receptors. The overall decrease in protein synthesis we found at high density may result in inhibition of TNF receptor biosynthesis in particular. Our results also show that inhibition of protein synthesis in HL-60 cells could indeed induce the downregulation of TNF receptors. These results are consistent with previous reports with the human ME-180 cervical carcinoma cell line [Aggarwal and Eessalu, 1987; Ruggiero et al., 1987].

Our results also show that the biological response of target cells to TNF is dependent on the density of target cells. We found that the sensitivity of MCF7 and U-937 cell lines to TNF-induced lysis was significantly reduced as the cell density increased. This can be explained, at least partially, by reduction of the number of TNF receptors on cells at high density. Our data may support the finding that the highest sensitivity to TNF cytotoxicity in mouse L fibroblast cell line occurs in the late stage of mitosis [Darzynkiewicz, 1984]. In a synchronized culture of mouse L-M cells, peak TNF cytotoxicity coincide with peak TNF binding in G2-M phase [Rizzino, 1988]. In other studies the highest sensitivity of mouse L cells to TNF-induced lysis was also detected in late mitosis [Darzynkiewicz et al., 1984]. Our results are also in agreement with the finding that the cells of other mouse fibroblast derived-cell lines (L929, B/C-N) cultured at high density became resistant to lysis by TNF [Patek et al., 1989].

Previously it has been reported that increase in cell density lead to depression of intracellular glutathione levels [Waxman, 1990]. We too found a significant decrease in cellular glutathione contents at high density, when cells develop resistance to TNF. These results are in agreement with previous reports which demonstrated that tumor cells resistant to TNF express reduced levels of glutathione [Zimmerman et al., 1989a,b; Ishii et al., 1992]. In contrast tumor cells that develop resistance to various chemotherapeutic agents are known to express elevated levels of glutathione [Ali-Osman et al., 1990; Meijer et al., 1992; Waxman, 1990; Kuchan and Milner, 1992]. The relationship of glutathione to the cell surface expression of TNF receptors or to the alteration of biological response is not clear. TNF is known to induce superoxide dismutase (SOD) in various cell types and the latter is involved in protection of cells from TNF [Wong et al., 1989; Visner et al., 1990]. But it is unlikely that the reduced glutathione (GSH) serves a similar function to SOD in the protection of cells from TNF by quenching superoxide radical; it would require elevation of GSH in TNF resistant cells rather than the decrease we found.

Overall, our results can partially explain cell density-dependent changes in susceptibility of tumor cells to lysis by TNF, and they also support the possibility that certain cells sensitive to TNF, when growing as tumors, can develop resistance to this cytokine by expressing a decreased number of TNF receptors. Our results may also provide an explanation for a great deal of variation in TNF receptor number reported by different laboratories [Tsujimoto and Oku, 1991].

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