Retinoids Downregulate Both p60 and p80 Forms of Tumor Necrosis Factor Receptors in Human Histiocytic Lymphoma U-937 Cells

By Klara Totpal, Madan M. Chaturvedi, Ruth LaPushin, and Bharat B. Aggarwal

Because retinoids are known to modulate the growth and differentiation effects of tumor necrosis factor (TNF), we investigated the effect of all-trans-retinoic acid (RA) on the cell surface expression of TNF receptors in human histiocytic lymphoma U-937 cells. RA decreased the specific binding of 125I-labeled TNF to these cells in a dose- and time-dependent manner. The maximal decrease occurred when cells were treated with 1 μmol/L RA for 24 hours at 37°C. Scatchard analysis of the binding indicated that the decrease by RA was caused by a decrease in receptor number and not by a decrease in affinity. The downmodulation of TNF receptors was also confirmed by covalent receptor-ligand cross-linking studies. Receptor-mediated internalization of the ligand was also found to be decreased on treatment of cells with RA. Northern blot analysis also indicated a decrease in the transcript of the receptor. By using antibodies specific to either the p60 or p80 form of the TNF receptor, we found that both receptors were downregulated by RA. RA treatment also decreased TNF receptors on acute monocytic leukemia cell line THP-1. Other analogues of RA, specifically 9-cis-RA, (E)-4-[2-(5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB), and 3-methyl-TTNPB, which differ in their specificity towards different RA receptors, were also active in downregulating TNF receptors. 3-Methyl-TTNPB, which is more specific for the RXR form of the RA receptor, was found to be most potent. The downregulation of TNF receptors by RA correlated with the downmodulation of the antiproliferative effects of TNF against U-937 cells. Overall, our results indicate that RA downmodulates both the p60 and p80 form of the TNF receptor on cells of myeloid origin, which correlates with the cellular response.

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MATERIALS AND METHODS

Materials. RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Whittaker MA Bioproducts (Walkersville, MD). Fetal bovine serum (FBS) and gentamicin were from Gibco (Grand Island, NY). Bacteria-derived recombinant human TNF (specific activity, 5 x 10^7 U/mg) was kindly supplied by Genentech, Inc (South San Francisco, CA). Carrier-free Na^125I was purchased from Amersham (Arlington Heights, IL); PD-10 (prepacked Sephadex G-25 medium) columns were from Pharmacia Fine Chemicals (Piscataway, NJ); iodogen and gelatin were from Sigma Chemical (St Louis, MO). The highly purified form of the recombinant extracellular domains of TNF receptors p60 and p80 were kindly provided by Dr T. Kohno (Synergen, Boulder, CO). Polyclonal antibodies were raised in rabbits against each type of receptor and purified by receptor-affinity chromatography. RA was purchased from Kodak Fine Chemicals (Rochester, NY). Retinol, 9-cis retinoic acid, (E)-4-[2-(5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB), and 3-methyl-TTNPB were kindly provided by...
Dr Peter Davis (University of Texas Health Science Center, Houston, TX), (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was from Promega (Madison, WI).

Cell culture. U-937 (histiocytic lymphoma, CRL 1593) and THP-1 (acute monocytic leukemia, TIB 202) cell lines used in this study were obtained from American Type Cell Culture Collection (Rockville, MD). These cells were grown in RPMI-1640 supplemented with 10% FBS and 50 µg/mL gentamicin.

RA treatment. Solutions of RA (1 µmol/L), retinol (1 µmol/L), 9-cis RA (5 µmol/L), TTNPB (5 µmol/L), and 3-methyl-TTNPB (5 µmol/L) were prepared in dimethyl sulfoxide (DMSO) and then diluted in the appropriate medium. Cells (0.2 to 0.5 x 10^6/mL) were incubated with RA in a serum-free RPMI-1640 medium supplemented with 50 µg/mL gentamicin at 37°C for 0 to 24 hours in 12-well plates or in T25 tissue culture flasks. The medium was then removed, and the cells were washed, counted for viability, and then examined for TNF receptors. An appropriate DMSO control was run wherever necessary.

Receptor binding assays. Receptor binding assays were performed as described previously.4,5 TNF was labeled with Na^251 using the Iodogen method. Briefly, 10 µg of TNF in a 20-µL volume was placed onto a film of 50 µg of Iodogen and incubated for 10 minutes at 4°C in the presence of 1 µCi of carrier-free Na^251. Free iodine was removed by gel filtration on a PD-10 (Sephadex G-25) column equilibrated with phosphate-buffered saline (PBS) containing 0.1% gelatin. More than 96% of the iodine in the final product was incorporated in the protein as determined by trichloroacetic acid (TCA) precipitation. The specific activity of the labeled TNF ranged from 20 to 30 mCi/mg.

Binding assays were performed in flexible 96-well plates precoated with 0.2 mL of FBS for 24 hours at 4°C. The binding medium (RPMI-1640) contained 10% FBS. Cells (0.4 to 0.5 x 10^6/mL) were incubated with 101251-TNF in the absence (total binding) or in the presence of 100 nmol/L unlabeled ligand (nonspecific binding) for 1 hour at 4°C. The cells were washed three times with ice-cold medium (PBS containing 0.1% bovine serum albumin [BSA]) at 4°C, and the cell-bound radioactivity was determined in a γ-counter (Cobra-Auto-Gamma; Packard Instrument Co, Meriden, CT). All determinations were performed in triplicate. Specific binding of the 1251-labeled TNF was calculated by subtraction of nonspecific binding from the total binding. Inhibition of specific binding by RA was calculated from the specific binding obtained from the untreated cells (100%). The dissociation constant (kd) and the number of receptors were calculated by Scatchard analysis as described.46

To examine the expression of p60 and p80 form of the TNF receptors, affinity-purified antibodies specific against each receptor were used. Cells (0.5 x 10^6/mL) were preincubated with the antibody (2 µg/mL) for 1 hour at 4°C and then examined for specific binding of labeled TNF as described above. Specific binding of TNF observed on cells pretreated with anti-p60 antibody is due to p80 receptor and that observed with anti-p80 antibody-pretreated cells is due to the p60 receptor.

Covalent cross-linking of TNF to cell surface receptors. The cross-linking procedure was performed according to the previously described method.47 Briefly, cells (2 x 10^6) were incubated with 2.5 nmol/L 125I-labeled TNF in 1 mL RPMI-1640 for 1 hour at 4°C, washed three times with ice-cold PBS to remove unbound ligand, and then treated with 0.1 µmol/L (final concentration) ethylene glycol-bis (succinimidyI) carbonate (EGS), the cross-linking reagent. After 45 minutes of incubation at room temperature, EGS was quenched by 10 minutes of incubation with 20 µmol/L ammonium chloride (final concentration). The cells were washed with ice-cold PBS and solubilized with solubilization buffer (50 mMol/L Tris, pH 7.5) supplemented with aprotinin (2 µg/mL), phenylmethylsulfonyl fluoride (1 µmol/L), leupeptin (2 µg/mL), N-P(40 (0.5%), sodium chloride (200 mMol/L), and β-mercaptoethanol (0.1%). After 10 minutes of incubation at 4°C, the samples were centrifuged and the supernatants were analyzed on 7% sodium dodecyI sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and exposed to a Phosphorimagre screen (Molecular Dynamics, Sunnyvale, CA), and the image was recorded and quantitated using “Image Quant” software.

Receptor-mediated internalization of TNF. The ligand internalization experiments were performed according to the procedure as described previously. Briefly, U937 cells (0.5 x 10^6 cells/mL) were incubated either with or without RA (1 µmol/L) for 24 hours at 37°C in serum-free medium. The cells were then washed and incubated in a fresh medium for 2 hours at 4°C with 0.5 x 10^6 cpm/mL of 125I-labeled TNF, and then unbound 125I-TNF was removed by three washes with cold PBS containing 0.1% BSA. The kinetics of internalization of TNF was examined by incubating the cells with fresh medium at 37°C. At indicated times, medium was removed and counted for radioactivity in the gamma counter. The cell-surface bound TNF was eluted from cells by acid washing twice (0.2 mol/L acetic acid (TCA) precipitation. The specific activity of the labeled TNF ranged from 20 to 30 mCi/mg.

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To examine the expression of p60 and p80 form of the TNF receptors, affinity-purified antibodies specific against each receptor were used. Cells (0.5 x 10^6/mL) were preincubated with the antibody (2 µg/mL) for 1 hour at 4°C and then examined for specific binding of labeled TNF as described above. Specific binding of TNF observed on cells pretreated with anti-p60 antibody is due to p80 receptor and that observed with anti-p80 antibody-pretreated cells is due to the p60 receptor.

Northern blot analysis of the expression of p60 and p80 mRNAs. U-937 cells (0.5 x 10^6/mL) were treated with 1 µmol/L RA for different times. Total cellular RNA was extracted from cells as described previously. For Northern blot analysis, RNA samples (20 µg) were denatured with formamide and formaldehyde and electrophoresed in 0.8% agarose gel containing 0.67 mol/L formaldehyde at 75 V for approximately 3 hours. RNA was alkali-transferred to Hybond N+ nylon membrane (Amersham Corp). After alkali transfer, the membrane was rinsed with 2 X SSC (1 X SSC: 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate, pH 7.0). It was prehybridized at 65°C for 1 hour in hybridization buffer containing 7% SDS and 1 mol/L EDTA in 0.5 mol/L sodium phosphate buffer, pH 7.2. The membrane was then hybridized for 16 to 20 hours with 32P-labeled cDNA probes for p60 and p80 (approximate specific activity, 5 x 10^6 cpm/µg DNA) in the hybridization buffer, plus denatured salmon sperm DNA (0.2 mg/mL). After hy-
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bridization, the membrane was washed twice with 2X SSPE (1X SSPE: 0.18 mol/L NaCl, 0.01 mol/L sodium phosphate, and 1 mmol/1 EDTA) containing 0.1% SDS at room temperature, followed by another wash with 1X SSPE containing 0.1% SDS at 65°C. The blots were then exposed to phosphorimagery screen (Molecular Dynamics, Sunnyvale, CA), and the images were recorded and quantitated using "Image Quant" software. To show the equal loading of lanes, the probes were stripped off the membrane by washing the filters twice with 0.5% SDS at 95°C for 30 minutes. The membrane was then hybridized with a cDNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Antiproliferative assays. To examine the antiproliferative effects of TNF, U937 cells (0.5 x 10^6 cells/mL) were treated for 16 hours with RA (1 μmol/L) or DMSO (0.02%) as a control in serum-free RPMI-1640 medium. Subsequently, cells (5 x 10^5 cells/0.2 mL) were washed and plated in a 96-well flat-bottom plate (Falcon cat. no. 3072) in a complete medium containing different concentrations of hTNF. After 72 hours at 37°C, cells were assayed for viability using the Promega CellTiter 96 AQueous Non-Radioactive Proliferation Assay kit (cat no. G5421). Cell viability was expressed by plotting the absorbance at 490 nm. Antiproliferative effects of TNF were calculated by using following formula:

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\text{Antiproliferative Effects (% of control)} = 100 - \left[ \frac{\text{Absorbance With TNF}}{\text{Absorbance Without TNF}} \right] \times 100
\]

RESULTS

RA downregulates cell surface expression of TNF receptors. To examine the effect of retinoic acid on TNF receptors, U-937 cells were treated with different concentrations of RA at 37°C for 24 hours, washed, and then analyzed for cell surface expression of TNF receptors (Fig 1, upper panel). RA decreased the specific binding of 125I-labeled TNF on U-937 cells in a dose-dependent manner. The maximum decrease (approximately 50%) in binding was observed when cells were treated with 1 μmol/L RA. This concentration had no effect on the viability of U-937 cells, as determined by cell counting or by [3H] thymidine incorporation (data not shown). The decrease of TNF receptors by RA was also not due to an effect on protein synthesis, as evaluated by [3H] leucine incorporation (data not shown). Next, we studied the time-course of downregulation of TNF receptors on U-937 cells by RA. The cells were exposed to 1 μmol/L of RA at 37°C for different times and then assayed for specific binding of 125I-TNF. Binding decreased in a time-dependent manner throughout the RA treatment (Fig 1, lower panel). A 58% decrease in binding was observed after 24 hours. When we examined the effect of RA (1 μmol/L for 24 hours) on TNF receptors in acute monocytic leukemia cell line, THP-1, a 56% decrease in specific binding of TNF (1,000 cpm ν 342 cpm) was observed.

We used Scatchard analysis to determine the effects of RA treatment on the number and/or binding affinity of TNF receptors. The cells were treated with 1 μmol/L RA for 24 hours at 37°C, washed, and then analyzed for receptor binding characteristics by incubating cells with variable amounts of 125I-TNF in the absence or presence of a 100-fold excess of unlabeled TNF (Fig 2, upper panel). For untreated cells, the receptor numbers/cell and dissociation constant (kd) were 4,554 sites/cell and 1.0 nmol/L, respectively, but for cells treated with 1 μmol/L RA, the values were 3,044 receptor numbers/cell and 105 nmol/L, respectively. These results indicated that RA decreased the number of TNF receptors, whereas the affinity of the receptors did not change significantly (Fig 2, lower panel).

To determine whether the downmodulation of TNF receptors by RA affects ligand internalization, we examined the rate of internalization and the dissociation of the ligand from
To further confirm the RA-mediated downmodulation of TNF receptors, we performed receptor-ligand cross-linking studies on U-937 cells. $^{125}$I-labeled TNF was cross-linked to the cell surface receptors on control and RA-treated cells using EGS, and the ligand-receptor complexes were visualized after SDS-PAGE (Fig 4). We observed a single band with an approximate molecular mass of 97 kD in untreated U-937 cells. A band with the same molecular mass was also observed in RA-treated cells, but computer-assisted densi-

the cell surface. The results of these experiments, shown in Fig 3, clearly indicate that RA decreased both the internalization of the ligand into the cells (upper panel) and dissociation of the ligand from the cell surface (lower panel). These results suggest that RA downmodulates the TNF uptake by the cells.

To further confirm the RA-mediated downmodulation of TNF receptors, we performed receptor-ligand cross-linking studies on U-937 cells. $^{125}$I-labeled TNF was cross-linked to the cell surface receptors on control and RA-treated cells using EGS, and the ligand-receptor complexes were visualized after SDS-PAGE (Fig 4). We observed a single band with an approximate molecular mass of 97 kD in untreated U-937 cells. A band with the same molecular mass was also observed in RA-treated cells, but computer-assisted densi-

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tometry showed that the amount of cross-linked TNF-receptor complex was 50% of the control value (Fig 4). These studies show the downregulation of p80 receptor by RA, but it was not possible to visualize the p60 receptor in either control or RA-treated cells.

**RA decreases the mRNAs of TNF receptors.** We investigated whether downregulation of the cell surface expression of TNF receptors is accompanied by a decrease in receptor mRNA levels. Northern blot analysis (Fig 5) of control and RA-treated U-937 cells showed a decrease in the mRNAs coding for the p60 and p80 forms of the TNF receptor. Therefore, the downregulation of the cell surface expression of TNF receptors induced by RA was a consequence of a decrease in mRNA levels.

**RA downregulates both the p60 and p80 forms of TNF receptors.** It has been previously reported from our laboratory that 60% to 80% of the total TNF-binding sites on U-937 cells can be attributed to the p80 form of the TNF receptor, with p60 receptors constituting the other 20% to 40% of the binding. To determine which receptor was influenced by RA, we used receptor-specific antibodies. The results of these experiments, shown in Fig 6, indicate that RA downmodulated both forms of the TNF receptors to a similar extent.

**Downmodulation of TNF receptors by RA is not due to its shedding.** We also investigated the possibility that RA decreases the number of cell surface TNF receptors by causing their release (shedding) into the medium. For these experiments, U-937 cells were treated with 1 μmol/L of RA at 37°C for 6 hours and 24 hours. The shed form of the TNF receptor in the culture medium was analyzed by an ELISA kit. RA treatment had no effect on the shedding of the p60 and p80 forms of the receptor (data not shown).

**RA analogues also downregulate TNF receptors on U-937 cells.** The intracellular effects of RA are mediated by two families of retinoid receptors, RA receptors (RARs) and retinoic X (RXRs) receptors. Whereas all-trans-RA binds to RAR, 9-cis-RA binds with high affinity to the RXR family. Similarly, TTNPB and 3-methyl-TTNPB have higher affinities for RAR and RXR, respectively. To investigate the role of different retinoid receptors in TNF binding, we treated U-937 cells with different retinoid analogues (1 μmol/L) for 24 hours and then examined them for TNF receptors. The results indicate that, under identical conditions, 3-methyl-TTNPB was maximally effective (80%) and retinol was least effective (10%) in decreasing TNF receptors (Fig 7). No significant difference was observed between cis and all-trans forms of RA (45% to 50%), and TTNPB was as effective as all-trans-RA. These observations suggest that both classes of retinoid receptors are involved in the modulation of TNF receptors on U-937 cells.

**RA also downregulated the cellular response of TNF in U-937 cells.** TNF has been shown to exhibit antiproliferative effects against U-937 cells. To correlate the decrease of TNF receptors by RA with the TNF-dependent cellular responses, we examined the antiproliferative effects of TNF on treated and untreated U-937 cells. The results shown in Fig 8 indicate that TNF decreases the rate of proliferation of these cells and this decrease is significantly abolished when cells are pretreated with RA. These results thus suggest that decrease of TNF receptors by RA could protect the cells from the antiproliferative effect of TNF.
DISCUSSION

In the present report, we show that all-trans-RA decreases the cell surface expression of TNF receptors on U-937 cells. The decrease in TNF receptors by RA was not unique to U-937 cells but was also observed with another myeloid cell line, THP-1. Scatchard analysis showed that RA downregulated TNF receptor number without significantly changing the receptor affinity. Furthermore, both forms of the TNF receptor were downmodulated by RA, which was accompanied with the decrease in the cellular response to TNF. Studies with analogues of RA indicated that the downregulation of TNF receptor was mediated through both RAR and RXR forms of the RA receptor.

Our results indicate that both types of TNF receptor in U-937 cells are downmodulated by RA when treated for 24 hours. In contrast, Winzen et al. showed that RA treatment of HL-60 cells increased TNF binding. The reason for this discrepancy is not clear. The treatment of HL-60 cells with RA under our experimental conditions had no effect on TNF receptors (data not shown). This difference may represent a difference in the experimental conditions. For instance, during our experiment, we treated the cells with RA in serum-free medium for 24 hours, whereas they used serum-containing medium and long-term treatment with RA. Despite an increase in binding of TNF by RA on HL-60, no change in the mRNA of the TNF receptor was reported. These results are also inconsistent with ours on U-937 cells, in which we showed changes in TNF receptor levels by binding, by receptor-ligand cross-linking and receptor-specific blocking antibodies, and by mRNA analysis. Besides myeloid cell lines, we found the modulation of TNF receptors by RA also in nonmyeloid cell lines such as T-47D (breast carcinoma) and H-596 (lung carcinoma).

As we showed for the modulation of TNF receptors by RA, the receptors for IL-6, AMF, and EGF have also been shown to be downmodulated by RA. In contrast, the receptors for IL-2, TGF-β, and NGF are increased by RA. How RA induces the receptors for some cytokines while downmodulating that of others is not clear. Because RA modulates the expression of several different cytokines, it is possible that the expression of cytokines by RA could in turn lead to the modulation of TNF receptors. The cell surface expression of TNF receptors can be downregulated by a number of different cytokines including IL-1, granulocyte monocyte colony-stimulating factor, and TNF. However, it is unlikely that RA-mediated downmodulation of TNF receptor is through the induction of TNF, because the latter has recently been shown to be also downmodulated by RA.

There are several reports that show that activators of protein kinase C (PKC) downmodulate TNF receptors, and RA has been shown to induce PKC in certain cells; therefore,
in the present studies, we found that the downregulation of TNF receptors by RA accompanied with a decrease in the antiproliferative effects of TNF. Overall, our results indicate that RA can downmodulate TNF receptors in myeloid cells and this may have a role in well-described modulation of the cytokine responses by RA.

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