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P80 form of the human tumor necrosis factor receptor is involved in DNA fragmentation

Masahiro Higuchi, Bharat B. Aggarwal*

Cytokine Research Laboratory, Department of Clinical Immunology and Biological Therapy, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

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Two different types of TNF receptors with molecular masses of 60 kDa (p60) and 80 kDa (p80) have been identified. TNF is known to cause DNA fragmentation in certain tumor cell lines but the role of p60 and p80 in this action is not understood. In the present study, we examined the role of these receptors in TNF-induced DNA fragmentation. Treatment of U-937 cells with phorbol ester caused downregulation of both types of TNF receptors and this was accompanied by disappearance of the TNF-induced DNA fragmentation. The removal of phorbol ester led to two time-dependent events: (1) the rapid regeneration of the p80 form but not the p60 form of the TNF receptor; and (2) the reappearance of TNF-induced DNA fragmentation. These results suggest that the p80 receptor could mediate the TNF-induced DNA fragmentation.

Tumor necrosis factor; Lymphotoxin; Apoptosis; TNF receptor; DNA fragmentation; Downmodulation

1. INTRODUCTION

Tumor necrosis factor (TNF) was originally characterized as a monokine that induces necrosis of certain tumors in vivo [1] and exhibits a wide variety of biological activities in vitro, including antiproliferative activity against tumor cells, proliferation of human fibroblast, B cells, and thymocytes, antiviral effects, and induction of various genes [2]. At the early stage of action, in certain cells TNF also induces DNA fragmentation, one of the characteristic events in apoptosis [3–5]. Recently the cDNAs that encode two different TNF receptors, one with an approximate molecular mass of 60 kDa (p60) and the other of 80 kDa (p80), have been isolated [6–9]. While epithelial cells express primarily p60, myeloid cells express both p60 and p80 receptors [10,11]. Receptor-specific antibodies have been used to demonstrate that most TNF signals are transduced through the p60 receptor [12,13]. Very little is known about the type of signals transduced through the p80 receptor [11,14,15]. Furthermore both receptors are independently regulated [16,17].

Previous studies from our laboratory and others have shown that PMA, a potent activator of protein kinase C, downregulates TNF receptors [18–20], and the removal of PMA results in TNF receptor regeneration [18]. However, these studies were carried out prior to the identification of two types of receptors, therefore, the type of receptor regenerated after PMA removal is unclear. In the present report, we investigated the regeneration of TNF receptors and the transduction of signal after the removal of PMA. Our results demonstrate that only p80 form of the TNF receptor is regenerated and that this receptor is involved in TNF-induced DNA fragmentation.

*Corresponding author. Fax: (1) (713) 792-3503.

Abbreviations: TNF, tumor necrosis factor; p60 also referred to as p55, TNF receptor I, or TNF receptor type B; p80, also referred to as p75, TNF receptor II, or TNF receptor type A; FCS, fetal calf serum; PMA, phorbol myristic acetate.

2. MATERIALS AND METHODS

2.1. *Materials*

Bacteria-derived recombinant human TNF-α, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech Inc., South San Francisco, CA. Polyclonal antibodies against each type of soluble receptor were raised in rabbits and purified by receptor-affinity chromatography.

2.2. *Cell lines*

The human histiocytic cell line U-937 (ATCC no. CRL 1593) was grown in RPMI medium supplemented with fetal calf serum (FCS) (10%) and gentamycin (50 μg/ml) (essential medium). The cells were seeded at a density of 1×10^5 cells/ml in T-25 flasks (Falcon 3013, Beckton Dickinson Labware, Lincoln Park, NJ) containing 10 ml of medium and grown at 37°C in an atmosphere of 95% air and 5% CO_2. Cell cultures were split every 3 or 4 days.

2.3. *Receptor-binding assay*

Recombinant human TNF was labeled with $Na^{125}I$ using the Iodogen procedure as described previously [21]. The specific activity of labeled TNF was 30 μCi/μg. Binding assays were performed using the 96-well method as described [22]. Briefly, cells (0.5×10^6 cells/well) were incubated in a binding buffer (RPMI 1640 supplemented with 10% FCS) in a flexible 96-well plate (Falcon 3911) with ^{125}I-labeled TNF (0.5×106 cpm/ml) with or without 250 nM TNF and in the presence or absence of 2 μg/ml of anti-p60 or anti-p80 antibody for 1 h at 4°C in a total volume of 0.1 ml. Thereafter, cells were washed three times with 0.15 ml of ice-cold washing buffer (PBS containing

0.1% BSA). Cell-bound radioactivity was then determined by a Packard gamma-counter (model CD 5010). The binding of TNF to p60 or p80 receptor was calculated by subtracting TNF specific binding in the absence of antibody from that in the presence of either anti-p80 or anti-p60 receptor antibodies. Unless otherwise stated, all results were determined in triplicate and expressed as mean ± standard error.

2.4. DNA fragmentation assay

The DNA fragmentation assay was carried out by the slightly modified methods of Elias and Berry [3]. Briefly, U-937 cells were labeled with tritiated-thymidine ([^{3}H]ThdR) by incubating 2×10^5 cells/ml in essential medium with 0.5 μCi/ml [^{3}H]ThdR at 37°C for 16 h. Cells were washed free of [^{3}H]ThdR and further incubated for 1 h at 37°C. Cells were then washed twice, resuspended in RPMI-1640 medium, and plated in the 96-well plate (4×10^4/well, total volume 200 μl) with or without different concentrations of TNF. After incubation for 4 h or the indicated time, cells were lysed by the addition of 100 μl of detergent buffer (10 mM Tris-HCl, pH 8.0, containing 5 mM EDTA and 0.6% Triton X-100) for 5 min at room temperature. High-speed centrifugation was performed with microcentrifuge at $12{,}000 \times g$ for 1 min. The radioactivity in the supernatant indicated fragmented DNA release from the cells. For the total count, the cells were lysed by the addition of 0.02 ml of sodium dodecyl sulfate (20%). The percent DNA release was calculated as follows:

$$\% \text{ DNA fragmentation} = \frac{\text{counts in the supernatant}}{\text{total cell associated counts}} \times 100.$$

Unless otherwise stated, all results were determined in triplicate and expressed as means ± standard error.

3. RESULTS

3.1. *Downmodulation of TNF receptor by PMA treatment*

We first examined the effect of PMA on TNF receptors. Human histiocytic U-937 cells were treated with 100 nM PMA for 2 h at 37°C and then specific TNF binding was determined. The treatment almost completely downmodulated TNF receptors (Fig. 1).

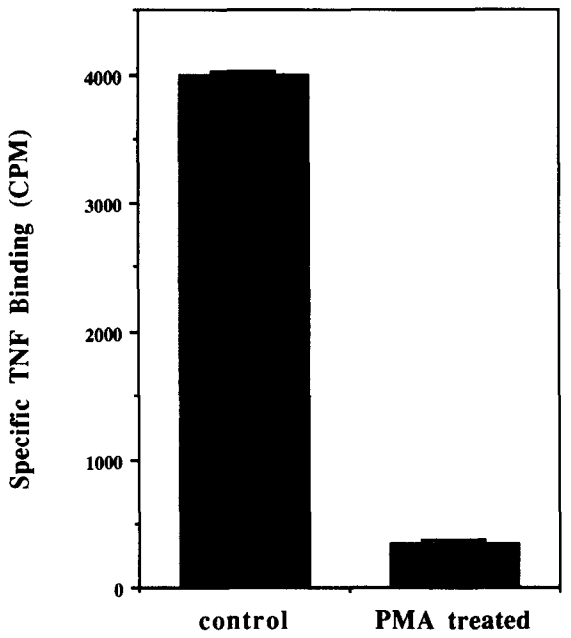

Fig. 1. Downmodulation of TNF receptor by PMA. Cells (5×10^5) were incubated with 100 nM PMA for 2 h at 37°C, washed, and tested for TNF receptors as described in section 2.

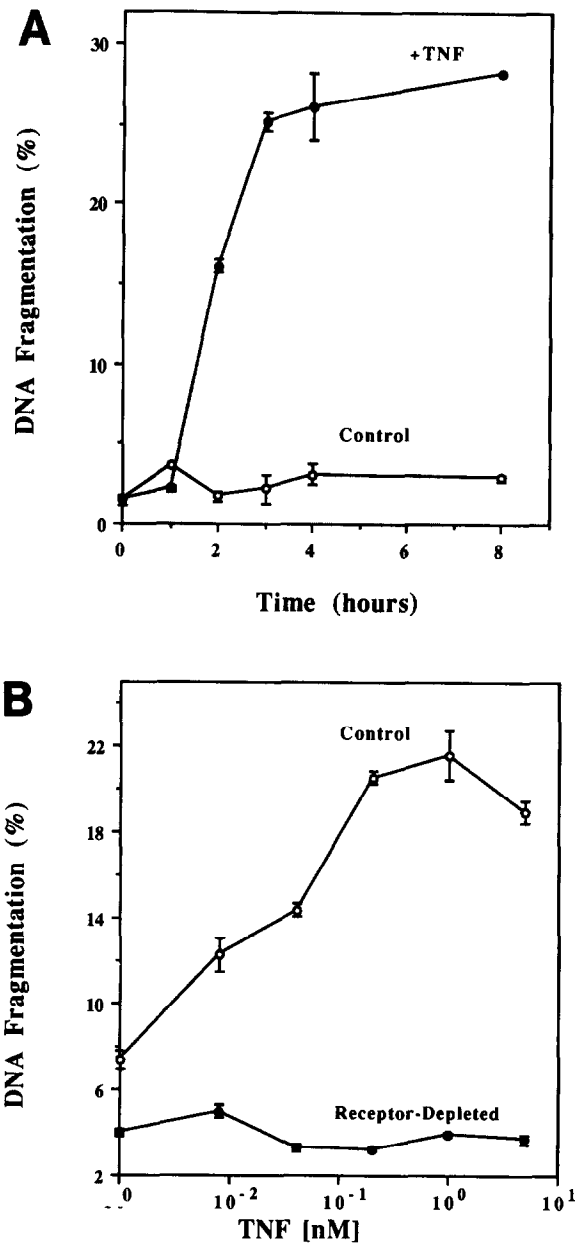

Fig. 2. (A) Time course of TNF induced DNA fragmentation. [^{3}H]ThdR pre-labeled cells (4×10^4/well) were incubated with or without 1 nM TNF for indicated times at 37°C, and then fragmented DNA was determined as described in section 2. (B) Dose–response of DNA fragmentation in control cells and cells depleted of TNF receptor. [^{3}H]ThdR pre-labeled cells (1×10^6/ml) were incubated with 100 nM PMA for 2 h at 37°C and washed. Then cells (4×10^4/well) were incubated with different concentrations of TNF for 4 h at 37°C and fragmented DNA was determined as described in section 2.

3.2. *Abrogation of TNF-induced DNA fragmentation by PMA treatment*

DNA fragmentation, one of the markers of programmed cell death, was used for the bioassay of TNF.

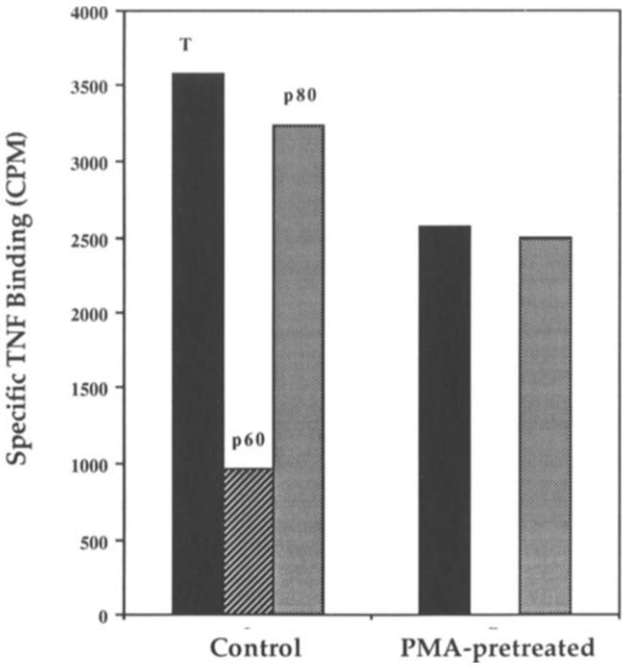

Fig. 3. Regeneration of TNF receptor after the removal of PMA. Cells (1×10^6/ml) were incubated with 100 nM PMA for 2 h at 37°C, and washed. Then the cells were resuspended in essential medium at a concentration of 5×10^5/ml and incubated for another 16 h at 37°C. Thereafter specific TNF binding to p60 and p80 was determined as described in section 2.

TNF-induced DNA fragmentation was observed 1 h after TNF addition, and plateaued after 3 h (Fig. 2A). We could thus detect TNF biological activity earlier than that with other biological assays including the cytotoxicity assay which requires 3 days of incubation. To examine the effect of PMA on TNF action, cells were prelabeled with [^{3}H]TdR, treated with or without PMA for 2 h, and incubated with different concentrations of TNF. Radioactivity released from nuclei was measured in the supernatant. In a dose-dependent manner, TNF induced DNA fragmentation in untreated U-937 cells, and the fragmentation reached its maximum at 1 nM TNF. Under these conditions, no TNF-induced DNA fragmentation in PMA-treated cells was detected (Fig. 2B).

3.3. *Regeneration of the p80 form of TNF receptor after the removal of PMA*

We previously reported that TNF receptor is regenerated after removal of PMA [18] but the type of receptor regenerated is not known. In order to investigate this, U-937 cells were treated with 100 nM PMA for 2 h, washed, incubated for an additional 16 h at 37°C, and examined for the specific TNF binding. Anti-p60 and anti-p80 antibodies were added during the binding experiment to distinguish between p60- and p80-specific binding. As we reported before [23], the p60 and p80 receptor contributed 30% and 85%, respectively, of the total binding on untreated U-937 cells (Fig. 3). However on cells pretreated with PMA, p80 receptor contributed almost entirely all of the specific TNF binding, indicating that only the p80 receptor was regenerated (Fig. 3).

3.4. *Recovery of TNF-induced DNA fragmentation after PMA removal*

We next examined the TNF-induced DNA fragmentation in cells which express only p80 receptor after regeneration. TNF caused the DNA fragmentation in both control untreated cells which express p60 and p80 receptor and in PMA pretreated cells which express only p80 receptor (Fig. 4). However, in the latter higher concentration of TNF was needed for DNA fragmentation. These results suggest that the p80 receptor can transduce the TNF signal on U-937 cells.

4. DISCUSSION

In the present report, we demonstrate that both TNF receptors are completely downregulated by PMA treatment and that after the removal of PMA, p80 but not p60 receptor is regenerated. Why only the p80 receptor and not the p60 receptor was regenerated after the removal of PMA is not clear. However, this result is con-

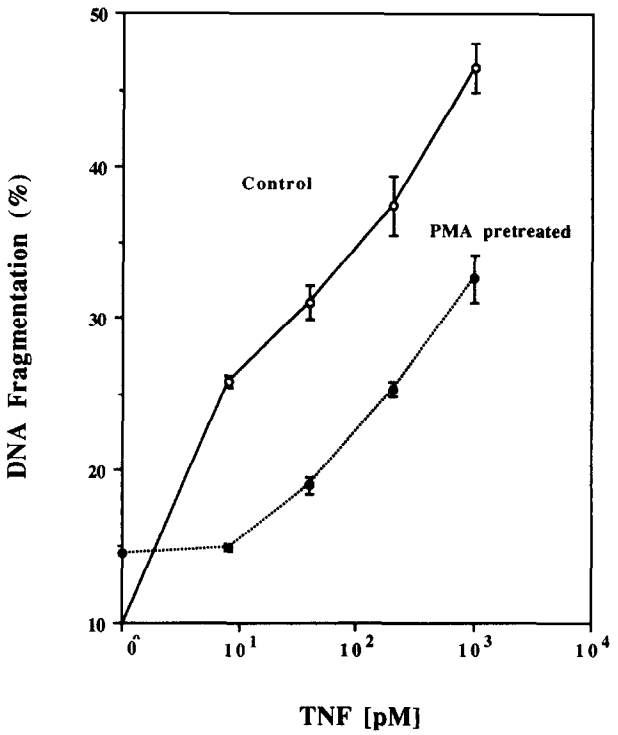

Fig. 4. Recovery of the effect of TNF-induced DNA fragmentation after the removal of PMA. [^{3}H]ThdR pre-labeled cells (1×10^6 cells/ml) were incubated with 100 nM PMA for 2 h at 37°C and washed. For receptor regeneration, the cells were resuspended in essential medium at the concentration of 5×10^5 cells/ml and incubated for another 16 h at 37°C. Cells were washed, and incubated (4×10^4/ml) with or without different concentrations of TNF for 4 h at 37°C. Fragmented DNA was then determined as described in section 2.

sistent with our previous report indicating that PMA selectively induces the mRNA for only p80 receptor [24]. Experiments with the promoter region of the murine p60 receptor gene also indicate that it does not respond to a variety of stimuli [25]. TNF-induced DNA fragmentation, used to detect the biological action of TNF, disappeared after PMA treatment in the cells which express no TNF receptor, but was recovered after the removal of PMA in the cells which express only p80 receptor, indicating that p80 receptor transduces this TNF signal. However, in the presence of PMA for a longer period of time, p80 was regenerated but no DNA fragmentation was evident (data not shown). From these observations, we conclude that PMA inhibits TNF action through two mechanisms; downregulation of the TNF receptor and inhibition of post-receptor signalling.

By using agonistic and antagonistic antibodies, it has been demonstrated that p60 receptor transduces most TNF signals including cytotoxicity, growth stimulation, differentiation and DNA fragmentation [3–5,12,13,17]. However, only very few signals have been reported to be mediated through the p80 receptor and this includes the activation of NF-kB, cytotoxicity, and stimulation of growth [11,14,15]. Since two types of TNF receptors can cross-modulate each other [23], therefore, the type of signal transduced by the individual receptor is not clear. No cell line has so far been described which expresses exclusively one type of TNF receptor. Our report clearly demonstrates that TNF can cause DNA fragmentation in the absence of any detectable p60 receptor. The concentration of TNF needed for DNA fragmentation in cells expressing only p80 receptor was found to be higher than that which express both types of TNF receptors. This difference may be either due to the affinity of TNF to p80 receptor or may also suggest the role of p60 receptor. Furthermore, we could not detect TNF-induced DNA fragmentation in epithelial cells which express primarily p60 receptor (data not shown), therefore, also suggesting the role of p80 receptor for the TNF-induced DNA fragmentation. Thus, in conclusion, our results demonstrate a selective resynthesis of p80 form of the TNF receptor in cells pretreated with phorbol ester and also document the role of this receptor in DNA fragmentation.

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