0026-895X/03/6405-1251–1258$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 64:1251–1258, 2003

Vol. 64, No. 5
2646/1106809
Printed in U.S.A.

Expression of the Angiogenic Factor Thymidine Phosphorylase in THP-1 Monocytes: Induction by Autocrine Tumor Necrosis Factor-α and Inhibition by Aspirin

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Received May 8, 2003; accepted August 18, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The angiogenic factor thymidine phosphorylase (TP) is highly expressed in human monocytes and macrophages, and its expression has been linked to the pathology and progression of solid tumors, rheumatoid arthritis, and gastric ulcers. In this study, TP mRNA and enzyme activity were found to be up-regulated upon the induction of differentiation of the human monocyte cell line THP-1 by phorbol 12-myristate 13-acetate (PMA). TP expression in THP-1 cells was similarly increased by tumor necrosis factor-α (TNFα). Because monocytes and macrophages are a predominant source of TNFα, the up-regulation of TP upon THP-1 differentiation could have been caused by the autocrine production of TNFα. In support of this hypothesis, PMA increased TNFα mRNA levels; furthermore, the increase in TP expression with PMA treatment was partially blocked by a neutralizing antibody to TNFα, particularly at the earlier time points. This data also suggested there may be additional mechanisms regulating TP expression upon PMA treatment of the cells. The induction of TP by TNFα was mimicked by an antibody to the TNFα receptor R2 (TNF-R2; p75), but not by an antibody to TNF-R1 (p55), suggesting that the TNF-R2 plays a role in the regulation of TP expression. The PMA-induced increase in TP expression was blocked by aspirin but not by the related agent indomethacin, suggesting that aspirin's effect was not caused by the inhibition of cellular cyclooxygenases. An alternative mechanism by which aspirin inhibits gene expression is the modulation of the transcription factor NFκB, and the TNFα-induced increase in TP mRNA was blocked by a cell-permeable NFκB inhibitory peptide. Furthermore, TNFα increased and aspirin (but not indomethacin) decreased NFκB DNA-binding activity in THP-1 cells. In conclusion, the modulation of TP expression in monocytes by pro- and anti-inflammatory agents suggests that its angiogenic-related actions could contribute to the inflammatory response associated with a number of pathophysiological conditions.

Thymidine phosphorylase (TP; also known as platelet-derived endothelial cell growth factor) is an angiogenic factor that has been found to be chemotactic for endothelial cells and to induce neovascularization in vivo (Miyazono et al., 1987; Ishikawa et al., 1989; Finnis et al., 1993; Sumizawa et al., 1993). These actions are not mediated by TP directly but rather by 2-deoxyribose, a metabolite of the 2-deoxyribose-1-phoshophate formed from thymidine via the catalytic activities of TP and cellular phosphatases (Haraguchi et al., 1994; Hotchkiss et al., 2003a,b). In normal human tissues, TP is strongly expressed in macrophages, including Kupffer cells and alveolar macrophages, other stromal cells, glial cells, and more weakly in some epithelia (Fox et al., 1995). TP was found to be frequently overexpressed in human solid tumors compared with adjacent uninvolved tissue, and its expression has been correlated with higher tumor microvessel density, increased tumor invasion and metastasis, and shorter patient survival time (Takebayashi et al., 1996). In human colon and other gastrointestinal tumors, TP overexpression occurred more often in tumor-associated macrophages and other stromal cells compared with expression in the colon cancer epithelial cells. High levels of expression of TP in tumor-associated macrophages have also been observed in human breast, prostate, lung, and brain tumors (Engels et al., 1997; Koukourakis et al., 1998; Lee et al., 1999; Toi et al., 1999; Okada et al., 2001; Yao et al., 2001; Sivridis et al., 2002). These findings suggested that TP in tumor-associated macrophages may play a more direct role in tumor angiogenesis, and it has been hypothesized that tumor cells can amplify their own angiogenic activity by recruiting or activating macrophages, which then express high level of angiogenic factors (Polverini and Leibovich, 1984).

This work was supported by grants R01-CA54422, RO1-CA89352, and P01-CA13330 from the National Cancer Institute.

ABBREVIATIONS: TP, thymidine phosphorylase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; PMA, phorbol 12-myristate 13-acetate; NFκB, nuclear factor κB; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Elevated levels of TP expression have also been associated with the pathophysiology of other inflammatory diseases, including: 1) rheumatoid arthritis, where TP was found to be highly elevated in synovial fluid and where there was an increase in TP mRNA in cultured rheumatoid arthritis fibroblast-like synoviocytes; 2) psoriasis, where there was an increase in TP expression in psoriatic lesions, including increased TP mRNA in lesional epidermis and increased TP expression in basal keratinocytes and suprabasal layers; and 3) gastric ulcers, in which TP was elevated near gastric ulcer margins compared with uninvolved fundic and pyloric stomach (Takeuchi et al., 1994; Creamer et al., 1997; Kusugai et al., 1997; Muro et al., 1999). Plasma TP was found to be higher in intractable gastric ulcer patients compared with either healthy persons, patients with duodenal ulcers, or patients with gastric ulcer with significant resolution (Kusugai et al., 1997). An increase in TP expression was also noted in interstitial mononuclear infiltrates in scarred kidneys occurring secondary to urinary tract diseases, suggesting that TP plays a role in the inflammatory and/or neovascularization response to renal interstitial fibrosis (Konda et al., 1999).

Tumor necrosis factor α (TNFα) is an important mediator of inflammatory responses, and it regulates multifunctional cytokines and several cellular functions in a wide variety of cells. A number of genes that can mediate angiogenesis have also been shown to be induced by TNFα, including basic fibroblast growth factor, plasminogen activator, platelet-activating factor, angiopoietin, ephrin A1, and VEGF and its receptors (Bussolino et al., 1988; Okamura et al., 1991; Ryuto et al., 1996; Giraudo et al., 1998; Kim et al., 2000; Cheng and Chen, 2001). In our previous study, we found that TNFα induced TP expression in human colon cancer WiDr cells by transactivation of the TP gene (Zhu et al., 2002). Activated macrophages are a major source of TNFα, and it has been proposed that the angiogenic activity of macrophages was mediated primarily by TNFα (Beutler and Cerami, 1986; Leibovich et al., 1987). It was reasonable to hypothesize, therefore, that the angiogenic activity associated with monocyte/macrophages was caused, in part, by the autocrine regulation of angiogenic factor expression by TNFα.

The THP-1 cell line is often used as a model of human monocytes, and it represents a relatively immature stage of monocyte differentiation. Treatment of THP-1 cells with agents such as lipopolysaccharide or phorbol-12-myristate 13-acetate (PMA) has been shown to both induce further monocytic differentiation of the cells (characterized by increased adhesion, loss of proliferation, and higher CD14 and CD54 expression) and increase the expression and release of TNFα by the cells (Sugimoto et al., 1984; Hmama et al., 1999; Rutault et al., 2001). In the present study, we used PMA-differentiated THP-1 cells as a model to test the hypothesis that an autocrine circuit mediated by TNFα regulates TP gene expression in macrophages.

TNFα mediates its biological effects by interacting with two distinct receptors, p55 (TNF-R1) and p75 (TNF-R2) (reviewed in Vandenabeele et al., 1995). Although these receptors have been shown to share partially overlapping signaling pathways, they can also mediate distinct cellular functions. The role of TNF-R1, independent of TNF-R2, in the TNFα-mediated induction of apoptosis and other cellular actions has been well described; recent studies have suggested that TNF-R2 can also mediate cell proliferation and/or apoptosis, both independent of and in conjunction with TNF-R1 (Tartaglia et al., 1991; Vandenabeele et al., 1995; Murray et al., 1997; Grell et al., 1998; Baxter et al., 1999; Chan and Lenardo, 2000; Amrani et al., 2001). A second objective of these studies, therefore, was to begin to discern the signal transduction pathways responsible for the regulation of TP by TNFα. The capacity of TNFα to induce its pleiotropic effects is attributable partly to its ability to activate the NFκB family of transcription factors. Thus, we also investigated whether NFκB activation was involved in the regulation of TP expression. These studies included an examination of the effect on TP expression of aspirin, which at higher therapeutic concentrations has been shown to inhibit the activation of NFκB through the stabilization of its inhibitory protein, IκB (Kopp and Ghosh, 1994; Yin et al., 1998).

Materials and Methods

Cell Lines and Reagents. Human monocyte THP-1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium with 10% fetal bovine serum and gentamicin in a humidified CO_2 incubator at 37°C. Anti-TP antibody was from Oncogene Research Products (San Diego, CA); anti-TNFα, anti-TNF-R1, and anti-TNF-R2 antibodies, and recombinant hTNFα were purchased from R&D Systems (Minneapolis, MN); anti-p65 NFκB (Rel A) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). PMA, aspirin, and indomethacin were from Sigma (St. Louis, MO); the NFκB cell-permeable inhibitor peptide SN50 and the inactive control peptide SN50M were from Calbiochem (San Diego, CA).

Measurement of TP and TNFα mRNA Levels. RNA was isolated from THP-1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). TP, TNFα, and GAPDH mRNA levels were determined by RT-PCR, as described previously (Zhu et al., 2002). Briefly, 2 μg of total RNA was reverse transcribed into cDNA with 200 units of Maloney leukemia transcriptase (Invitrogen) in 20 μl of reaction buffer containing 10 units of RNasin, 0.2 μg of random primers, and 0.8 mM dNTPs at 42°C for 1 h. Reactions were terminated by heating at 95°C for 10 min. The mixture was diluted 2.5 times with RNase-free water. An aliquot (2.5 μl) was used for PCR amplification with primer for TP: sense, 5′-GCTTCGTGGCCGCTGTGGTG-3′; antisense, 5′-TCTGCTCTGGGCTCTGGATGA-3′; TNFα: sense, 5′-GTCTACTTTGGGATCATTG-3′; antisense, 5′-TCAGGGATCAAAGCTGTA-3′ GAPDH: sense, 5′-CATCTCTGCCCTCTGCTG-3′; antisense, 5′-CCCTCCGACGCCTGCTTCAC-3′. The TP primer corresponds to sequences in exons 2 and 4 of the human genomic TP sequence. Reactions contained 25 μl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, and 1.25 units of Taq DNA polymerase. The TP reaction proceeded for 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. Amplified cDNAs were electrophoresed on 2% agarose gels containing ethidium bromide, gels were photographed, and bands were scanned and quantitated by densitometry using ImageQuant (Amersham Biosciences, Piscataway, NJ).

Protein Preparation and Western Blot Analysis. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.5 mM phenylmethysulfonyl fluoride (PMSF). Protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA). Protein (20 μg) was loaded onto 10% polyacrylamide gels and electrophoresed, and transferred to polyvinylidene difluoride membrane (Amersham Biosciences). The membranes were blocked by incubation in 5% nonfat dry milk in Tris-buffered saline/Tween 20 (10 mM

Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). Membranes were incubated with primary antibody at the dilution of 1:500 for 1 h at room temperature. Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:3000) for another 1 h. After washes in Tris-buffered saline/Tween 20, proteins were visualized by chemiluminescence using the enhanced chemiluminescence reagent (Amersham Biosciences) as substrate.

Preparation of Nuclear Extract. Nuclear extracts were prepared as described previously (Schreiber et al., 1989), with some modifications. Cells were washed twice with PBS, harvested by scraping into 4 ml of PBS, and centrifuged (500*g*, 5 min). The pellet was dispersed in 1 packed cell volume of hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2 μg/ml each of aprotinin, pepstatin, and leupeptin. After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% (v/v), and nuclei were pelleted by centrifugation (5000*g*, 5 min). The pelleted nuclei were dispersed in a high-salt buffer (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 1 mM PMSF, aprotinin, pepstatin, and leupeptin) to solubilize DNA-binding proteins. The suspended nuclei were gently shaken for 30 min at 4°C and centrifuged (12,000*g*, 20 min). The cleared supernatants, containing nuclear proteins, were stored in small aliquots at −70°C. Protein concentrations were determined using a Bradford assay kit.

Electrophoretic Gel Mobility Shift Assay. Nuclear proteins (6 μg) were incubated with 1 μg each of poly(dI-dC)-poly(dI-dC) and poly(dG-dC)-poly(dG-dC) in the presence of 10 fmol of [γ-^{32}P]ATP end-labeled double-stranded NFκB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCT-GAAAGGGTCCG-5′; Promega, Madison, WI) for 20 min at room temperature in a total volume of 20 μl. Oligonucleotide competition experiments were performed in the presence of 50-fold excess of nonradioactive NFκB oligonucleotide. For supershift analysis, nuclear extracts were preincubated with 2 μl of polyclonal anti-NFκB antibody (Santa Cruz Biotechnology) for 1 h at 4°C before the addition of labeled DNA probe. Samples were analyzed by polyacrylamide gel electrophoresis; dried gels were exposed to X-ray film at −70°C.

Results

PMA Increased TP and TNFα Levels in THP-1 Human Monocytes. Treatment of THP-1 cells with PMA has been previously reported to induce the differentiation of the cells to a more mature monocyte/macrophage phenotype (Hoff et al., 1992; Schwende et al., 1996; Rutault et al., 2001). When we measured TP mRNA levels in THP-1 cells undergoing differentiation, we found that PMA (20 nM) induced an increase of ~2-fold in TP mRNA levels, first detected at 4 h, reaching a maximal increase at 12 h, and sustained up to 24 h (Fig. 1A, lanes 1–6). Consistent with the increased TP mRNA levels, PMA also induced an increase in TP protein (Fig. 1A) and enzyme activity, including a statistically significant 70% increase at 48 h (Table 1). Although TNFα is highly expressed in fully differentiated macrophages, THP-1 cells express low levels of the cytokine (Rutault et al., 2001). When induced to differentiate with 20 nM PMA, however, a 2-fold increase in TNFα mRNA was observed in the THP-1 cells and, in contrast to the effect of PMA on TP mRNA, the increase was first observed at 2 h, was maximal at 4 h, and had decreased to below basal levels at 8 h (Fig. 1B). This effect of PMA on TNFα levels was consistent with previous studies in monocyte cell lines (Lopez et al., 2000; Rutault et al., 2001).

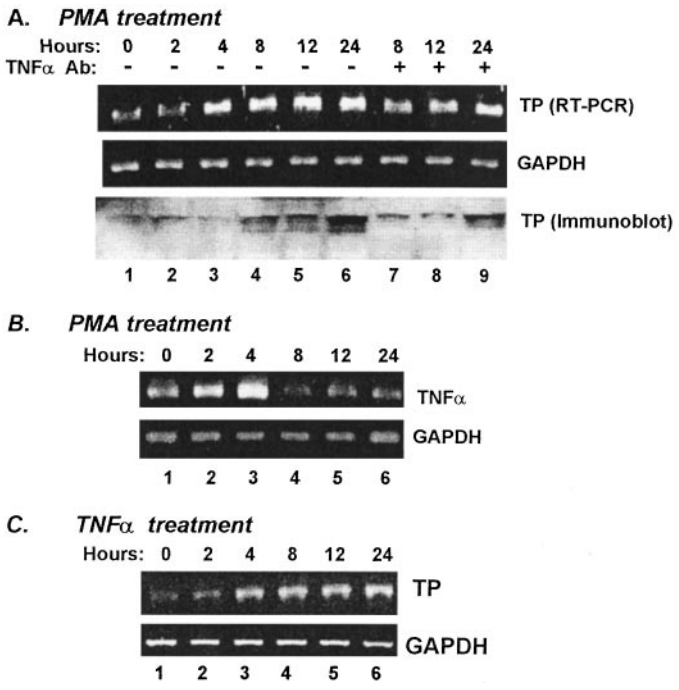

Fig. 1. Effect of PMA and TNFα on TP mRNA and protein levels in THP-1 cells. A, cells were treated with 20 nM PMA for the indicated times without (lanes 1–6) or with (lanes 7–9) a neutralizing anti-TNFα antibody (20 μg/ml). RNA was extracted and analyzed by RT-PCR using primers specific for TP or GAPDH using conditions described under *Materials and Methods*. Samples were run on agarose gels and stained with ethidium bromide. Preliminary studies confirmed that band intensities were proportional to the amount of cDNA used in the PCR reactions, using RNA from both control and treated cells. For the immunoblot, protein was extracted, fractionated on 10% SDS-polyacrylamide gels, and incubated with an anti-TP antibody (1:500), as described under *Materials and Methods*. Membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:3000), washed, and incubated with enhanced chemiluminescence reagent to visualize proteins by chemiluminescence. B, TNFα and GAPDH mRNA levels were determined in cells treated with 20 nM PMA for the indicated times. C, expression of TP and GAPDH mRNA in cells treated with 20 ng/ml TNFα for the indicated times was determined.

TNFα Increased TP Levels in THP-1 Cells. In our previous study, we found that TNFα increased both TP mRNA and enzyme activity in colon cancer WiDr cells (Zhu et al., 2002). We next determined whether TNFα had a similar effect on TP in THP-1 cells. When the cells were incubated with 20 ng/ml TNFα for various times, TP mRNA was found to be induced as early as 2 h and kept a sustained elevation during 24 h of incubation (Fig. 1C). TNFα also increased TP enzymatic activity; although the extent of increase was iden-

TABLE 1
Induction of TP activity by PMA and TNFα
THP1 cells were treated with PMA (20 nM), TNFα (20 ng/ml), or vehicle (control) for 24 or 48 h, as indicated. Cell extracts were prepared and TP activity analyzed as described under *Materials and Methods*. Data are presented as mean ± S.E.M. of four experiments

	TP Activity	
Treatment	24 h	48 h
	pmol/mg/min	
Control	1287 ± 302	1252 ± 598
PMA	1580 ± 195	2146 ± 604*
TNFα	2140 ± 302*	2200 ± 209*

* Significantly different from control, $P < 0.05$.

tical to that seen with PMA, it began at a time point earlier than that observed with PMA treatment (Table 1).

TNFα-Neutralizing Antibody Blocked the PMA-Induced Increase in TP mRNA. The observed increases in TP expression with PMA and TNFα, coupled with the increase in TNFα also seen with PMA treatment, suggested that the induction of TP expression during THP-1 differentiation might have been mediated by an autocrine effect of TNFα. To test this hypothesis, THP-1 cells were cotreated with PMA and a TNFα-neutralizing antibody. As Fig. 1A illustrates, the anti-TNFα antibody (20 μg/ml) decreased the TP mRNA and protein levels at by 55 to 85% at time points up to 12 h and by 15 to 35% at 24 h (lanes 7–9) compared with the cells treated with PMA alone and examined at the same time points (lanes 4–6). Note that the inhibitory effect of the anti-TNFα antibody was not complete, particularly at 24 h, suggesting that a portion of the effect of PMA on TP expression occurred independently of the effect of the concomitant increase in TNFα expression. On the other hand, the antibody may not have been able to fully neutralize the TNFα at the later time point.

Role of TNF-R2, TNFα Signaling Pathways, and NFκB Transcription Factor in the TNFα Induction of TP mRNA. The cellular actions of TNFα are mediated by two cell surface receptors, TNF-R1 and TNF-R2, both of which are expressed on THP-1 cells undergoing differentiation (Glaser et al., 1999). To determine which TNFα receptor(s) might be involved in the regulation of TP expression, THP-1 cells were treated with antibodies specific for the p55 TNF-R1 or the p75 TNF-R2, both with and without concurrent treatment with TNFα. As Fig. 2 shows, when used alone, the TNF-R2 antibody (lane 5), induced a 3-fold greater increase in TP mRNA levels than the TNF-R1 antibody (lane 3), compared with untreated cells (lane 1). There was no additional increase in TP mRNA levels observed when the TNF-R2 antibody was used in combination with TNFα (lane 6), suggesting that the TNF-R2 antibody is acting as an agonist and that it activated the same pathways as TNFα. A role for TNF-R1 cannot be completely ruled out based on this experiment, however, because the TNF-R1 antibody alone caused a modest increase in TP expression (lane 3), and a modest attenuation of the TNFα-induced increase in TP mRNA (lane 4).

The anti-inflammatory actions of TNFα can be antagonized by nonsteroidal anti-inflammatory drugs (NSAIDs). The PMA-induced increase in TP expression was found to be inhibited in a concentration-dependent manner by aspirin (70% and 100% inhibition at 5 and 10 mM, respectively), but not by the NSAID indomethacin (Fig. 3). Although aspirin and indomethacin share the ability to inhibit cellular cyclooxygenases, they differ in that aspirin can also inhibit the activation of the transcription factor NFκB, whereas indomethacin does not. To determine whether NFκB was involved in the TNFα-induced increase in TP mRNA, THP-1 cells were cotreated with TNFα and either a cell-permeable NFκB inhibitor peptide (SN50) at a concentration of 18 μM or a control inactive peptide (SN50M) that has two altered amino acids. SN50 contains the nuclear translocation sequence of NFκB and has been shown to prevent its translocation into the nucleus (Lin et al., 1995). As shown in Fig. 4, SN50 had no effect when used alone (lane 3) but blocked 85% of the TNFα-induced increase in TP mRNA levels (lane 4). The control peptide SN50M had no effect on TP mRNA levels in control- or TNFα-treated cells (lanes 5 and 6).

PMA-Induced NFκB Binding Activity in THP-1 Monocytes Cells. To obtain further evidence supporting a role for NFκB in PMA-induced TP expression, an electrophoretic mobility shift assay was used to evaluate the expression of NFκB-DNA binding activity in PMA-treated THP-1 cells. PMA induced an increase in a labeled complex (Fig. 5,

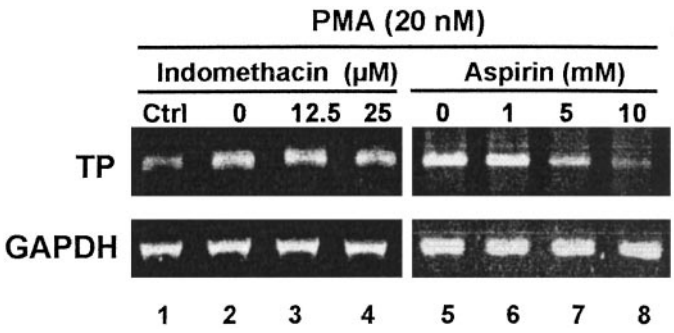

Fig. 3. Aspirin blocked PMA-induced TP mRNA in human monocyte THP-1 cells. The cells were cotreated with 20 nM PMA and 0, 1, 5, and 10 mM aspirin (lanes 5–8) or 0, 12.5, and 25 μM indomethacin (lanes 2–4) for 24 h. Cells in lane 1 (Ctrl) were treated only with vehicle (i.e., no FPMA or NSAID). RNA was extracted and analyzed by RT-PCR using primers specific for TP or GAPDH using conditions described under *Materials and Methods*.

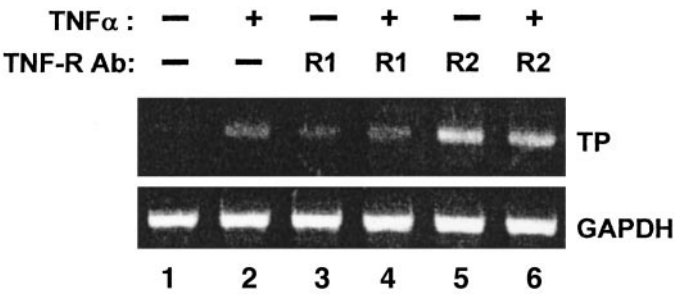

Fig. 2. Effect of TNF-R1 and TNF-R2 antibodies on TP mRNA levels in TNFα-treated THP-1 cells. THP-1 cells were treated for 24 h with 20 ng/ml TNFα (lanes 2, 4, and 6) in the absence (lanes 1 and 2) or presence of neutralizing anti-TNF-R1 antibody (20 μg/ml; lanes 3 and 4) or anti-TNF-R2 antibody (20 μg/ml; lanes 5 and 6). The incubations with the antibodies were begun 2 h before the addition of the TNFα. RNA was extracted and analyzed by RT-PCR using primers specific for TP or GAPDH, using conditions described under *Materials and Methods*.

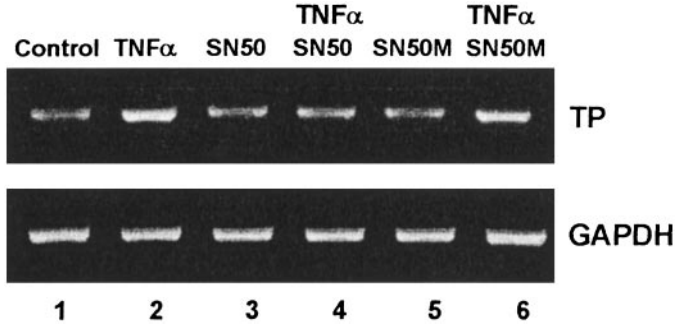

Fig. 4. NFκB inhibitor peptide SN50 blocked TP mRNA expression in TNFα-treated THP-1 cells. The cells were treated for 24 h with 20 ng/ml TNFα (lanes 2, 4, and 6) in the presence of 18 μM SN50 (lane 4) or an inactive control peptide (SN50M; lane 6). The effects of the peptides on TP expression in the absence of TNFα were also determined (lanes 3 and 5). RNA was extracted and analyzed by RT-PCR using primers specific for TP or GAPDH, using conditions described under *Materials and Methods*.

lane 2) whose association with NFκB was demonstrated by its loss in the presence of excess unlabeled NFκB oligomer (lane 5), and its loss and concurrent appearance of a supershifted band in the presence of an anti-p65 NFκB (Rel A) antibody (lane 6). In agreement with their effects on TP mRNA levels, aspirin blocked the PMA-induced increase in the NFκB complex (lane 3), whereas indomethacin did not (lane 4).

Discussion

In this study, we found that the TNFα and TP genes were both induced during PMA-mediated differentiation of monocytic THP-1 cells. Upon the addition of PMA, an increase in TNFα mRNA was first observed at 2 h, was further elevated at 4 h, and declined to baseline by 8 h; this was accompanied by an increase in TP mRNA levels beginning at 4 h and reaching maximal expression at 12 h. The PMA-induced TP increase was partially blocked by anti-TNFα antibody, with the largest inhibition at 8 and 12 h and a lesser effect observed at 24 h. In addition, exogenous TNFα also increased the TP mRNA and protein levels in THP-1 cells. Together, these results strongly suggest that the PMA induction of TP gene is partly mediated by the autocrine action of synthesized TNFα. Consistent with our findings here, similar mechanisms of autocrine regulation by TNFα have been found in the expression of other genes in macrophages. TNFα induced matrix metalloproteinase, a matrix-degrading enzyme, in an autocrine manner in THP-1 cells (Robinson et al., 2002). During the induction of monocyte/macrophage differentiation, TNFα and plasminogen activator inhibitor type-1 (PAI-1) gene expression was activated, and the synthesized TNFα up-regulated and prolonged, in an autocrine manner, the synthesis of PAI-1 (Lopez et al., 2000). Autocrine regulation is not always stimulatory on gene expression; for example, in adipocytes, TNFα was shown to inhibit the expression of leptin in an autocrine manner (Yamaguchi et al., 1998). Furthermore, the TNFα antibodies blocked only ~50% of the PMA-induced increase in TP, leaving open the possibility that there are other mechanism(s) operative in inducing TP in PMA-treated THP-1 cells.

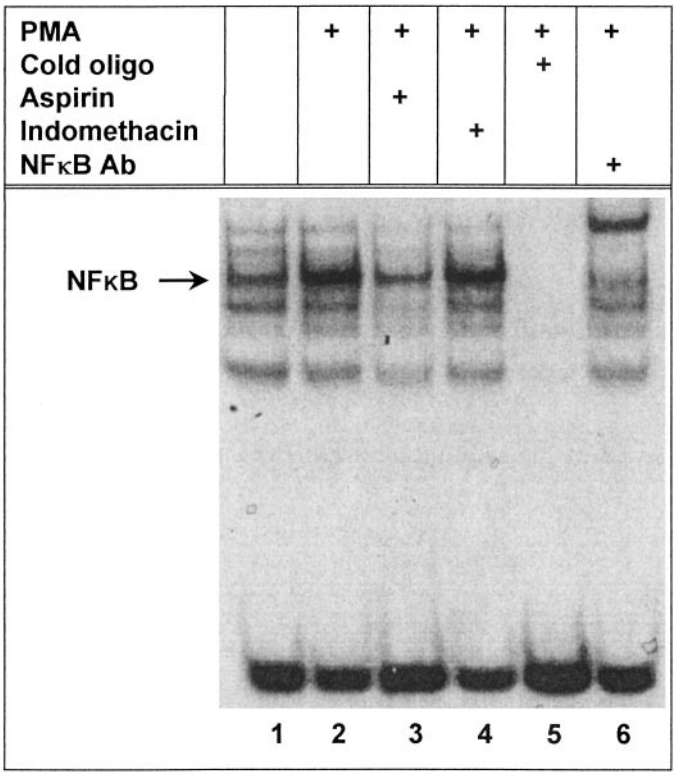

Fig. 5. PMA induced NFκB binding activity in human monocyte THP-1 cells. Nuclear extracts were prepared from control cells (lane 1) and from cells treated with 20 nM PMA (lanes 2–6), as described under *Materials and Methods*. The effects of PMA in combination with aspirin (5 mM) or indomethacin (25 μM) (lanes 3 and 4, respectively) were also determined. Binding reactions contained 6 μg of nuclear proteins and 1 μg each of poly(dI-dC)-poly(dI-dC) and poly(dG-dC)-poly(dG-dC) in the presence of 10 fmol of [γ-^{32}P]ATP end-labeled double-stranded NFκB consensus oligonucleotides, (5′-AGTTGAGGGGACTTTCCCAGGC-3′). Oligonucleotide competition experiments were performed in the presence of 50-fold excess of unlabeled NFκB oligonucleotides (lane 5). For supershift experiments, nuclear extracts were preincubated with 2 μl of polyclonal anti-NFκB antibody (lane 6). The binding reactions were analyzed by polyacrylamide gel electrophoresis and visualized by autoradiography, as described under *Materials and Methods*. The location of a NFκB-specific band is indicated by an arrow.

TNFα promotes angiogenesis in part through its ability to up-regulate the expression of various angiogenic factors. TNFα-dependent gene induction is mainly mediated by two cell surface receptors, TNF-R1 and TNF-R2; the 55-kDa TNF-R1 is widely expressed on most cell types, whereas expression of the 75-kDa TNF-R2 has been found to be restricted to hematopoietic and endothelial cells (Hohmann et al., 1989; Brockhaus et al., 1990). Only limited studies have examined the roles of the specific receptors in TNFα-induced angiogenesis. These investigations suggested a proangiogenic role for TNF-R2, based on its ability to activate EtK/BmX, an endothelial/epithelial tyrosine kinase involved in TNFα-induced angiogenesis, and an antiangiogenic effect for TNF-R1, based on the effect on wound healing of its loss in TNF-R1 knockout mice (Mori et al., 2002; Pan et al., 2002). Our findings that stimulation of TNF-R2 strongly induced expression of the angiogenic factor TP were consistent with these observations. Although the effect of the TNF-R1 antibody on TP expression seemed to be modest at best, its role in TNFα-mediated TP expression cannot be completely discounted, because both TNFα and its receptors can occur in soluble forms and as integral membrane proteins at the cell surface or in the Golgi apparatus and thus may vary in their responsiveness to the antibodies. Furthermore, there are data to suggest that both receptors can contribute to the same cellular response as a consequence of the "passing" of ligand from one receptor type to the other, suggesting that neither receptor alone is sufficient to mediate a particular effect (Tartaglia et al., 1993). In support of the last observation and of relevance to our studies was the finding that deletion of either of the TNF receptors abolished TNF-induced activation of NFκB in macrophages (Mukhopadhyay et al., 2001). Other studies, however, suggested that TNF-R1 predominates in the activation of NFκB by TNFα (McFarlane et al., 2002).

The NFκB family of transcription factors mediate cellular responses to a broad range of extracellular stimuli, including those that are immunological, proinflammatory, and stress-related (Baldwin, 1996; Ainbinder et al., 2002). Transactivation of NFκB has been considered to serve a critical role in the induction of expression of many genes by TNFα (Karin M, 1999). Consequently, NFκB controls the expression of a large number of genes, including cytokines, adhesion molecules,

cell cycle regulators, and pro- and antiapoptotic factors (Pahle, 1999; Ainbinder et al., 2002). Sequence analysis of the TP promoter suggests there are at least six sites in the region from 900 to 1200 nucleotides upstream from the TP transcription start site with potential consensus sequences for NFκB binding (Zabel et al., 1991), including sites that are near previously identified SP1 binding sites (Zhu et al., 2002). In addition to our findings with TP, members of the NFκB family have been implicated in other TNFα-dependent gene induction events, including the induction of angiogenic molecules. For example, NFκB signaling blockade significantly inhibited expression in vitro and in vivo of the proangiogenic molecules VEGF, interleukin-8, and matrix metalloproteinase-9 and hence decreased neoplastic angiogenesis (Huang et al., 2001). VEGF-R2 (flk-1/KDR) expression has also been shown to be induced by TNFα, and this was found to be mediated through NFκB in combination with a cAMP response element-binding protein and histone acetylases (Illi et al., 2000). Furthermore, NFκB was involved in the regulation of E-selectin and vascular cell adhesion molecule-1; the soluble forms of these proteins induced angiogenesis (Koch et al., 1995; Boyle et al., 1998). Thus our data implicating TNFα and NFκB in the regulation of TP expression were consistent with the role both play in angiogenesis and suggest that TP is part of a broad family of genes activated under a number of pro-inflammatory conditions.

We found that the effect of PMA on TP expression could be blocked by aspirin. Aspirin and other cyclooxygenase (COX) inhibitors reduce the risk of cancer development in humans and suppress tumor growth in animal models (Moorghen et al., 1988; Gridley et al., 1993; Reddy et al., 1993; Thun et al., 1993; Giovannucci et al., 1994; Sandler et al., 2003). Although the underlying mechanisms are not fully understood, one of their anticancer activities seems to involve inhibition of tumor angiogenesis, which has been shown to be modulated by inhibition of the COXs (Leahy et al., 2000; Dempke et al., 2001). Other studies suggest there may be additional mechanisms involved, however. Using selected HCT-116 colon carcinoma cells that lacked both COX-1 and COX-2 to study in vitro angiogenesis, it was found that aspirin (but not all other NSAIDS) still effectively inhibited endothelial cell tube formation in a coculture assay (Tsujii et al., 1998). These investigators proposed that there might be a COX-independent mechanism mediating aspirin's antiangiogenic effect. In a related observation, aspirin, but not indomethacin or dexamethasone, was found to inhibit the activation of the NFκB pathway. Data suggested that this effect of aspirin was caused by its inhibition of an IκB kinase, thereby preventing the latter from phosphorylating IκB (Kopp and Ghosh, 1994; Grilli et al., 1996; Yin et al., 1998). In the absence of its phosphorylation, IκB is not degraded and can therefore continue to sequester NFκB and prevent its translocation to the nucleus.

Although the precise mechanism by which NFκB modulates TP gene expression in macrophages is unclear, the present study showed that aspirin inhibited both activation of NFκB binding activity and PMA-induced TP expression in THP-1 cells. Thus, aspirin probably suppressed PMA induction of the TP gene in the monocytes by preventing activation of NFκB. In support of this conclusion were the observations that indomethacin, which as noted above lacks the ability to inhibit the activation of the NFκB pathway, did not block TP expression, whereas a peptide inhibitor of NFκB translocation did. Because the TNFα gene is itself subject to regulation by NFκB (Shackelford et al., 1997; Steer et al., 2000; Sugita et al., 2002), it was also possible that aspirin did not directly affect TP transcription; rather, it may have inhibited an NFκB-mediated effect on TNFα. The role of the IκB kinases in these actions, as well as the potential interactions of NFκB with other transcription factors known to regulate TP, remain to be determined. Furthermore, the extent to which the respective induction and inhibition of TP by TNFα and aspirin contributes to the pro- and anti-inflammatory and pro- and antiangiogenic actions of these agents is also not known. Given the documented elevated expression of TP in a number of pro-inflammatory conditions and its association with the pathological progression of these diseases, further exploration of the role of TP in the pathogenesis, and the effect of its inhibition on the clinical course of these diseases, would be warranted.

Acknowledgments

We thank Michelle Lenzi for her excellent technical assistance.

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