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Sulfasalazine Down-Regulates the Expression of the Angiogenic Factors Platelet-Derived Endothelial Cell Growth Factor/Thymidine Phosphorylase and Interleukin-8 in Human Monocytic-Macrophage THP1 and U937 Cells

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ABSTRACT

Platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) and interleukin-8 (IL-8) are angiogenic factors produced by tumor infiltrating macrophages. Here, we show that prolonged exposure of human monocytic/macrophage THP1 and U937 cells to sulfasalazine, an anti-inflammatory drug and inhibitor of nuclear factor-κB (NF-κB), resulted in down-regulation of PD-ECGF/TP and IL-8 (mRNA, protein and activity) along with elimination of their induction by tumor necrosis factor-α and interferon-γ. Concomitantly, sulfasalazine-exposed cells were markedly resistant to 5′-deoxyfluorouridine, the last intermediate of capecitabine requiring activation by PD-ECGF/TP. This is the first report suggesting that disruption of NF-κB-dependent signaling pathways can provoke a marked and sustained down-regulation of macrophage-related angiogenic factors. However, this may also negatively affect capecitabine efficacy.

Angiogenesis, the formation of new vasculature, plays a pivotal role in several pathological conditions, including neoplastic and inflammatory diseases such as rheumatoid arthritis (RA) (Folkman, 1995; Daly et al., 2003). Angiogenesis is assumed to be regulated by the balance of pro- and anti-angiogenic factors produced by all the cell types involved. If the balance shifts toward the proangiogenic state, the formation of new vasculature is induced, as is the case in the majority of cancers and RA (Hanahan and Folkman, 1996). Infiltrating macrophages have been recognized as producers of either pro- or antiangiogenic factors, although the pro-pathological state usually prevails (Burmester et al., 1997; Bingle et al., 2002). Proangiogenic factors produced by macrophages include interleukin-8 (IL-8) (Koch et al., 1992) and platelet-derived endothelial cell growth factor (PD-ECGF) (Takahashi et al., 1996). Both factors were found to be up-regulated in cervical cancer (Fujimoto et al., 2002) and RA (Waguri et al., 1997), suggesting a common coregulatory pathway.

IL-8 is a so called CXC chemokine, containing three amino acid residues Glu-Leu-Arg (ELR-motif) preceding the first conserved cysteine indicative of its proangiogenic action and is expressed in both cancer and RA (Belperio et al., 2000). PD-ECGF is identical to the enzyme thymidine phosphorylase (TP; E.C. 2.4.2.4) and catalyzes the catabolism of thymidine (TdR) into thymine and deoxyribose-1-phosphate (Brown and Bicknell, 1998). In a cancer chemotherapeutic setting, TP is essential for the final activation of the 5-fluorouracil (5-FU) prodrug capecitabine by catalyzing the conversion of 5′-deoxyfluorouridine (5′-DFUR) to 5-FU (Ackland and Peters, 1999). PD-ECGF/TP is overexpressed in a variety of solid tumors (Miwa et al., 1998) and is positively correlated with microvessel density (Toi et al., 1995) and poor prognosis (Takebayashi et al., 1996; Matsumura et al., 1998). Similarly, elevated PD-

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ABBREVIATIONS: RA, rheumatoid arthritis; IL-8, interleukin-8; PD-ECGF/TP, platelet-derived endothelial cell growth factor/thymidine phosphorylase; TP, thymidine phosphorylase; TdR, thymidine; 5-FU, 5-fluorouracil; 5′-DFUR, 5′-deoxyfluorouridine; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; SSZ, sulfasalazine; NF-κB, nuclear factor-κB; ECL, enhanced chemiluminescence; TNFR, tumor necrosis factor receptor; PE, phycoerythrin; IFN-γR, interferon-γ receptor; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

ECGF/TP has been observed in serum and synovial fluids of RA patients (Waguri et al., 1997).

It is well recognized that PD-ECGF/TP can be up-regulated by cytokines such as tumor necrosis factor-α (TNF-α), IL-1, and interferon-γ (IFN-γ) (Eda et al., 1993), various chemotherapeutic agents, including taxanes, cyclophosphamide, and mitomycin C (Sawada et al., 1998), and X-ray radiation (Sawada et al., 1999). The latter two may induce PD-ECGF/TP indirectly via up-regulation of TNF-α or IFN-γ (Blanquicett et al., 2002).

Sulfasalazine (SSZ) is a slow-acting anti-inflammatory drug commonly used in the second line treatment of RA and inflammatory bowel disease (Brooks, 2001). Its anti-inflammatory properties have been ascribed to the inhibition of the release of the proinflammatory cytokine TNF-α through the inhibition of the activation of the nuclear transcription factor NF-κB (Wahl et al., 1998), which controls, among various genes, the transcription of TNF-α. Beyond its anti-inflammatory action, SSZ has been assigned an antiangiogenic potential, however, a mechanistic basis for this effect was not revealed.

In this study, we show that exposure of the human monocytic/macrophage cell lines THP1 and U937 to SSZ results in a complete down-regulation of PD-ECGF/TP and IL-8. Furthermore, these cells did no longer respond to stimuli (TNF-α or IFN-γ) that normally induce PD-ECGF/TP and IL-8 in parental cells. This is the first report on a drug that provokes a marked down-regulation of the proangiogenic factors PD-ECGF/TP and IL-8 via a mechanism that is associated with defective signaling via the TNF-α or IFN-γ receptor pathways and altered NF-κB protein expression (signaling). These data also indicate that aberration in NF-κB signaling may affect PD-ECGF/TP-mediated activation of 5-FU prodrugs.

Materials and Methods

Chemicals. RPMI 1640 medium and fetal calf serum were obtained from Cambrex Bio Science (Verviers, Belgium). SSZ and 5′-DFUR were purchased from Sigma-Aldrich (St. Louis, MO). Hybond ECL nitrocellulose membranes, Hyperfilm ECL, and ECL (Plus) detection kit were obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Polyclonal goat anti-human PD-ECGF antibodies were obtained from R&D Systems (Abingdon, UK), and the secondary antibody was peroxidase-conjugated rabbit anti-goat from DakoCytomation Denmark A/S (Glostrup, Denmark). Monoclonal antibodies for NF-κB/p65 and NF-κB /p105/p50 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; sc-8008 and sc-8414, respectively); for this detection, a peroxidase-conjugated goat anti-mouse secondary antibody was used (DakoCytomation Denmark A/S). Labeled antibodies against TNF receptor I [fluorescein (fluorescein isothiocyanate), TNFRI] and TNF receptor II [phycoerythrin (PE), TNFRII] were from R&D systems and anti-IFN receptor (CD119) (PE, IFN-γR) was from BD Biosciences (San Jose, CA). RNAzol was obtained from Campro Scientific (Veenendaal, The Netherlands); Moloney murine leukemia virus reverse transcriptase was from Promega (Madison, WI); deoxynucleotides (dNTPs), random hexamers, and *Taq* polymerase from Pharmacia Biotech (Roosendaal, The Netherlands). IL-8 ELISA kits, TNF-α, and IFN-γ were purchased from Sanquin (Amsterdam, The Netherlands). All other chemicals were of analytical grade.

Cell Lines and Exposure to Sulfasalazine. THP1 and U937 human monocytic/macrophage cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin at 37°C in 5% CO_2 in a fully humidified atmosphere. Cells were seeded at an initial density of 3×10^5/ml and were exposed to SSZ for a period of 3 months to mimic the slow action of SSZ, which achieves its optimal activity after a period of 6 to 12 weeks (Brooks, 2001). Since peak plasma levels of RA patients receiving a daily dose of 2 to 3 g of SSZ can reach levels of 1 mM, whereas steady-state plasma levels of SSZ can reach 0.1 mM (Baggott et al., 1992; Smedegard and Bjork, 1995), we exposed cells to intermediate concentrations of 0.4 to 0.6 mM SSZ, which caused a moderate antiproliferative effect (IC_{50}–IC_{75}) in previously unexposed cells. Cell cultures were refreshed twice weekly. Cells stably growing in 0.6 mM SSZ, designated THP1/SSZ and U937/SSZ, were used for further characterization. The SSZ-exposed cells showed no change in cell doubling time, nor did they display any apparent morphological changes. Where indicated, cells were pretreated for 24 h with 150 U/ml IFN-γ or 20 ng/ml TNF-α before seeding for the thymidine phosphorylase activity assay. Culturing continued for an additional 24 h after which samples were harvested to assess IL-8 production.

Growth Inhibition Assays. Antiproliferative effects of TNF-α, 5′-DFUR, doxorubicin, and methotrexate were analyzed by plating 1.25×10^5 cells in individual wells of a 24-wells plate containing 1 ml of medium/well and up to 50 μl of the drug solution. Inhibition of cell growth was determined after 72-h drug exposure followed by viable cell counting using a hemocytometer and trypan blue exclusion. The drug concentration required to inhibit cell growth by 50% compared with control growth is defined as IC_{50}.

Thymidine Phosphorylase Activity Assay. TP catalyzes the phosphorolysis of thymidine. TP activity was measured in intact cells by seeding 5×10^5 cells (4 ml/well, six-well plate) in the presence of a final thymidine concentration of 0.5 mM. Samples (125 μl) were taken at selected time points up to 4 h. Samples were deproteinized by the addition of 40% trichloroacetic acid, neutralized, and analyzed for thymine formation by high-performance liquid chromatography as described previously (Laurensse et al., 1988; van Triest et al., 2000). For each time point in the enzyme activity curves, a correction was made for volume and cell loss due to previous sampling. The reaction was linear over the entire incubation period (4 h). TP activity is given as nanomoles of thymine produced per 10^6 cells per hour.

mRNA Analysis. RNA was extracted from 5×10^6 cells by the RNAzol method, examined for DNA contamination, and reverse transcribed using random hexamers as described by the manufacturer with minimal modifications (De Bruin et al., 2003).

Oligonucleotide primers used for reverse transcription-PCR of TP, IL-8, and β-actin were those described previously (De Bruin et al., 2003; Jauneau et al., 2003). Briefly, cDNA samples were amplified in a MJ Research PTC-2000 apparatus (Biozym, Landgraaf, The Netherlands) with 1-min steps of denaturation at 94°C, primer annealing at 58°C, and elongation at 72°C for 35 cycles starting with a hot start at 94°C. PCR products were separated by 120-V electrophoresis for 2 h on 2% agarose gel containing 0.1 μg/ml ethidium bromide. The assay has been optimized such that both target and reference genes are in the exponential phase of amplification (Rots et al., 2000; De Bruin et al., 2003; van der Wilt et al., 2003).

Western Blot Analysis. For determination of TP protein expression, logarithmic growing cells were harvested, and cell pellets were lysed in a lysis buffer (1% Triton X-100, 150 mM Tris HCl, pH 7.6, and 5 mM EDTA), sonicated, and centrifuged for 10 min at 14,000*g* at 4°C. Protein content was assayed using the Bio-Rad assay (Bio-Rad, Hercules, CA). Thirty micrograms of protein of each sample was separated on a 12.5% SDS-PAG and electroblotted onto a Hybond ECL nitrocellulose membrane. Membranes were incubated overnight at room temperature in a blocking buffer containing: 1% bovine serum albumin, 1% milk powder in 10 mM Tris HCl, pH 8.0, 0.15 M NaCl, and 0.05% Tween 20). The membranes were then incubated with the primary antibody (goat anti-human PD-ECGF at a 1:1000 dilution), followed by incubation with horseradish peroxidase-conju-

gated rabbit anti-goat antibody (1:2000). Nuclear and cytoplasmic fractions were prepared using the NE-PER nuclear and cytoplasmic extraction reagents from Pierce Chemical (Rockford, IL), according to manufacturer's protocol. Thirty micrograms of nuclear and cytoplasmic fractions was separated on a 10% SDS-PAG; after electoblotting, the membrane was probed with NF-κB/p65 or NF-κB/p105/p50 antibodies (mouse anti-human at a 1:500 dilution) followed by peroxidase-conjugated goat anti-mouse antibody (1:3000). Proteins were visualized using the enhanced chemiluminescence detection kit (ECL Plus) and Hyperfilm.

IL-8 ELISA. Cellular release of IL-8 was analyzed by ELISA and was performed according to manufacturer's protocol with minimal modifications (lower limit of detection, 5 pg/ml). Samples were taken from the same wells in which the TP activity assay was performed. For comparison, a similar set of wells was tested without adding TdR.

Expression of TNFRI, TNFRII and IFN-γR. THP1, THP1/SSZ, U937, and U937/SSZ cells were labeled with fluorescein isothiocyanate/PE-conjugated antibodies against TNFRI, TNFRII, and IFN-γR or their appropriate isotype controls. Subsequently, the cells were analyzed by flow cytometry using a fluorescence-activated cell sorting-calibur flow cytometer (BD Biosciences, Erembodegem-Aalst, Belgium). Data analysis was performed using Cellquest software package.

Statistics. The one-tailed, paired Student's *t* test was used to determine the difference in TP activity and IL-8 production, in untreated controls versus IFN-γ- or TNF-α-treated cells.

Results

Characterization of SSZ-Exposed Cells. Relative to parental cells, the THP1/SSZ and U937/SSZ variants displayed a 2- and 3.3-fold increase in the IC_{50} for SSZ, respectively. After the phenotypic characterization of SSZ-exposed THP1 and U937 cells, we observed a marked resistance to 5′-DFUR and TNF-α in these SSZ-treated variants (Table 1). For THP1/SSZ and U937/SSZ cells, no IC_{50} was reached at the highest concentration of 5′-DFUR and TNF-α tested; these cell lines displayed relative 5′-DFUR resistance levels of >138.8- and >89-fold, respectively. Furthermore, these cell lines were >34.5- and >40-fold resistant to TNF-α, respectively. This resistant phenotype was retained when SSZ-exposed cells were grown in the absence of SSZ for at least 1 month, suggesting that the observed effects were relatively stable (data not shown). To address whether these phenotypic changes relate to SSZ-induced targeting of NF-κB, we also determined the growth inhibitory effects of two chemotherapeutic drugs, the folate antagonist methotrexate and doxorubicin, mechanistically unrelated to TNF-α and 5′-DFUR. No significant differences for parental THP1 versus THP1/SSZ and parental U937 cells versus U937/SSZ were observed in growth inhibitory potential of methotrexate (IC_{50} of 9.3 ± 1.6 versus 10.1 ± 2.9 nM, and 7.3 ± 2.9 versus 8.3 ± 0.7 nM, respectively; mean ± S.D. of three experiments) and doxorubicin (IC_{50} of 20.4 versus 21.6 nM, and 12.9 versus 10.6 nM, respectively; mean of two experiments).

PD-ECGF/TP and IL-8 mRNA and PD-ECGF/TP Western Blot Analysis. A plausible explanation for the 5′-DFUR resistance in SSZ-exposed cells could be down regulation of PD-ECGF/TP expression/activity since TP activity is required for the conversion of 5′-DFUR to 5-FU (De Bruin et al., 2003). In this respect, TNF-α resistance may also have implications for alterations in PD-ECGF/TP expression/activity since TNF-α is a known positive regulator/inducer of PD-ECGF/TP (Eda et al., 1993). To investigate this and the possible coregulation of IL-8, another macrophage-produced angiogenic factor, PD-ECGF/TP and IL-8 gene expression was determined. In THP1/SSZ and U937/SSZ cells, PD-ECGF/TP mRNA levels were below the limit of detection (Fig. 1A). Likewise, a marked down-regulation of IL-8 mRNA was observed in THP1/SSZ and U937/SSZ cells (Fig. 1A). Consequently, Western blot analysis revealed that PD-ECGF/TP protein was below the level of detection (Fig. 1B).

Regulation of PD-ECGF/TP Activity and IL-8 Production. To determine a potential mechanism of down-regulation and coregulation of PD-ECGF/TP and IL-8, wild-type THP1 and U937 cells as well as SSZ-exposed cells were incubated with cytokines known to induce and possibly regulate PD-ECGF/TP (IFN-γ and TNF-α) or IL-8 (TNF-α) (Fig. 2, A and B). In THP1/SSZ and U937/SSZ cells exposed to SSZ for 3 months, TP-activity was not measurable. Remarkably, when THP1 cells were exposed to 0.6 mM SSZ for 1 week and 2 weeks, a modest decrease in TP activity (29 and 37%, respectively) could be already observed, suggesting that the decline in TP activity is rather gradual, which may be correlated with the slow action of SSZ.

IFN-γ and TNF-α significantly up-regulated TP activity in THP1 cells 2.1- and 1.5-fold, respectively. Similarly, TP activity was significantly increased in U937 cells after stimulation with IFN-γ (2.8-fold) and TNF-α (1.7-fold). In both parental cell lines, there was a significantly increased TP

TABLE 1

Cell growth inhibitory effects (IC_{50}) values of the fluoropyrimidine 5′-DFUR and TNF-α in the THP1 and U937 monocytic cell lines and their sulfasalazine-exposed variants THP1/SSZ and U937/SSZ

Data are the mean ± S.E.M. of three separate experiments.

Cell Line	5′-DFUR	RR[a]	TNF-α	RR
	μM		*ng/ml*	
THP1	1.8 ± 0.5		2.9 ± 0.1	
THP1/SSZ	>250	>138.8	>100	>34.5
U937	2.8 ± 0.2		2.3 ± 0.7	
U937/SSZ	>250	>89	>100	>40

[a] RR is the relative resistance (SSZ-exposed variant IC_{50})/(parental IC_{50}).

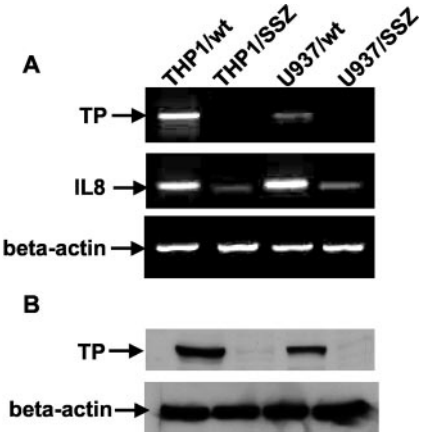

Fig. 1. A, representative agarose gel of separated reverse transcription-PCR products of TP, IL-8, and β-actin, showing the complete loss of PD-ECGF/TP and the marked decrease of IL-8 mRNA in the SSZ-exposed cells. Even higher cDNA concentrations in the PCR reaction did not lead to any detectable bands for PD-ECGF/TP, in line with a complete disappearance of PD-ECGF/TP expression. B, Western blot of PD-ECGF/TP showing the markedly diminished PD-ECGF/TP protein expression in the SSZ-exposed cells. β-Actin was included as a loading control.

activity after IFN-γ treatment compared with TNF-α-treated cells. Effects of TNF-α or IFN-γ on IL-8 release were consistent with the effects on TP; a 1.8- and 1.6-fold increase in IL-8 release from THP1 cells treated with IFN-γ or TNF-α, respectively. IL-8 production in U937 cells increased 1.6- and 1.2-fold after treatment with IFN-γ and TNF-α, respectively. There was no significant difference in IL-8 production in parental cells used for the TP activity assay, which were incubated with TdR for 24 h, compared with cells that were incubated for 24 h in the absence of TdR (data not shown). Strikingly, no detectable TP activity or IL-8 release was observed in THP1/SSZ and U937/SSZ cells. Moreover, both TNF-α and IFN-γ failed to increase TP activity and IL-8 release from SSZ-exposed cells.

TNFRI, TNFRII, and IFN-γR Expression and NF-κB/p65, NF-κB/p105/p50 Western Blot Analysis. SSZ-exposed cells were resistant to TNF-α, and IFN-γ or TNF-α could no longer induce PD-ECGF/TP and IL-8 in THP1/SSZ and U937/SSZ cells. To examine whether this apparently aberrant signaling is possibly due to alterations in the levels of cytokine receptors, TNFRI, TNFRII, and IFN-γR expression was assessed using flow cytometry. All three receptors were expressed on the surface of THP1 and U937 cells, although there was a heterogeneous expression of TNFRII on THP1 cells. No major change in IFN-γR expression was observed; however, a decrease in TNFRI levels and a complete down-regulation of TNFRII was noted in SSZ-exposed cells (Fig. 3A).

Since SSZ targets the NFκB signaling pathway, possible changes were analyzed by studying three NF-κB family members, p65, and p105/p50, at the level of protein expression. In the SSZ-exposed cells, there was a down regulation of p105 in the cytoplasm, accompanied by a down-regulation of p50 in the nuclear fraction compared with parental cells. There were no apparent differences in the cytoplasmic p65. Surprisingly, however, whereas parental cells contain no detectable levels of p65 in the nucleus, SSZ-exposed cells displayed a constitutive presence of substantial p65 levels in the nucleus (Fig. 3B).

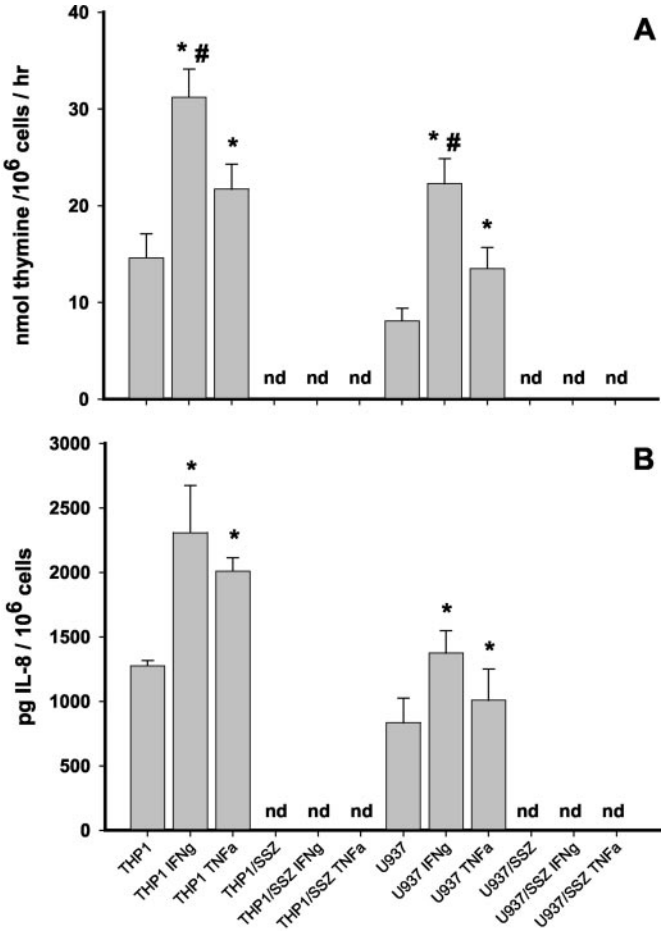

Fig. 2. TP activity and IL-8 production in parental and SSZ-exposed cells after incubation with various cytokines. Cells were treated for 24 h with IFN-γ (150 U/ml) or TNF-α (20 ng/ml), and then seeded at a density of 0.5×10^6 cells/4 ml, samples were taken over a 4-h period to determine the TP-activity, followed by another period of 24 h at which point the samples were taken for IL-8 determination. A, TP activity for the control and treated, parental, and SSZ-exposed cells. Note no TP activity is detected in the exposed cells. B, the same cells used to measure the TP activity were cultured for an additional 24 h to assess the IL-8 production. There was no measurable IL-8 production in the SSZ-exposed cells. *, $P < 0.05$ for control versus IFN-γ or TNF-α treatment. Results are the means of three separate experiments ± S.E.M. #, $P < 0.05$ for IFN-γ versus TNF-α treatment. nd, not detectable.

Discussion

In this article, we describe the marked down-regulation of two macrophage produced proangiogenic factors, PD-ECGF/TP and IL-8, in THP1 and U937 monocytic cell lines after exposure to SSZ. In addition, SSZ-exposed cells displayed resistance to TNF-α. Importantly, consistent with the down-regulation of PD-ECGF/TP was the high level of resistance to 5′-DFUR, which requires a prior activation to 5-FU by PD-ECGF/TP to elicit its cytotoxic activity. Thus, prior pretreatment with SSZ may provoke resistance to capecitabine, a prodrug for 5′-DFUR and ultimately 5-FU.

The exact mechanism of regulation of PD-ECGF/TP gene expression is yet unknown; however, the promoter contains several Sp1 transcription factor binding sites (i.e., GC-box) postulated to contribute both to the basal and TNF-α-inducible expression (Zhu et al., 2002). Furthermore, interferon-mediated signaling via the signal transducer and activator of transcription family of transcription factors occurs at two potential binding sites; the IFN-stimulated response element (Schwartz et al., 1998) and the γ-activated sequence (Goto et al., 2001). Since PD-ECGF/TP and IL-8 transcripts and proteins were absent or markedly reduced in SSZ-exposed cells, we investigated known pathways that can induce the expression of these factors. IFN-γ is a potent inducer of PD-ECGF/TP expression, whereas TNF-α induces both PD-ECGF/TP (Eda et al., 1993) and IL-8 gene expression (Hoffmann et al., 2002). Consistent with previous studies (Eda et al., 1993; Goto et al., 2001), we observed that IFN-γ and TNF-α were able to induce PD-ECGF/TP expression in both parental THP1 and U937 cells. IL-8 was also inducible by both cytokines in parental cell lines. IFN-γ and TNF-α seem to be common stimuli for PD-ECGF/TP and IL-8 expression in parental cells. Although IFN-γ is not commonly associated with IL-8 induction, it has been described previously that IL-8 induction can occur through a post-transcriptional mechanism (Bosco et al., 1994), whereas PD-ECGF/TP induction by IFN-γ is thought to be mediated directly via STAT1 signaling (Goto et al., 2001). However, in the SSZ-

exposed cell lines, PD-ECGF/TP and IL-8 were neither expressed nor induced by these cytokines.

Together, these results suggest aberrant signaling and regulatory pathway(s) in the SSZ-exposed cells, which was further reinforced by the down-regulation of TNFRI and TNFRII expression in SSZ-exposed cells. The expression of IFN-γR was largely unchanged for both SSZ-exposed cell lines, indicating that the putative defect would presumably lie downstream of the receptor. Although TNFRI is associated with cell death, it has been shown that TNFRII can exert an effect on cell death via TNFRI (Gupta, 2002). Furthermore, it has been described that TNFRII is more frequently a subject to regulation (Gupta, 2002). As such, the down-regulation of TNFRI and the loss of TNFRII could explain the resistance to TNF-α-induced cell death in the SSZ-exposed cells (Table 1). The down-regulation of both TNF receptors could also function as part of an efficient anti-inflammatory response mechanism exerted by SSZ, inhibiting the action of TNF-α. The present study suggests that TNF receptors and their ligands may play a role in the basal level of PD-ECGF/TP and IL-8 expression, and exposure to SSZ may disrupt these signaling pathways. The down-regulation of angiogenic factors is another property of SSZ in antirheumatic and related inflammatory diseases. Of note, treatment of (chronic) inflammatory bowel disease with SSZ, conferred at least some protection against the development of colon cancer in these patients (Ryan et al., 2003). The described down-regulation of angiogenic factors might play a role in this chemoprotective trait of SSZ.

SSZ has been shown to act as an NF-κB inhibitor, by blocking the phosphorylation of IκBα (Wahl et al., 1998). The present study reveals that prolonged inhibition of the NF-κB pathway signaling by SSZ ultimately resulted in an antiangiogenic response, illustrated by PD-ECGF/TP and IL-8 down-regulation. Our present data suggest that impaired NF-κB transcriptional activation may be the underlying mechanism for this effect. In particular, an enhanced nuclear accumulation of NF-κB/p65 was noted in the SSZ exposed cells compared with the parental cells; however, one of its heterodimerization partners necessary for transcriptional activity, NF-κB/p50, was not up-regulated (Perkins, 2000). This stoichiometric imbalance can possibly impair the formation

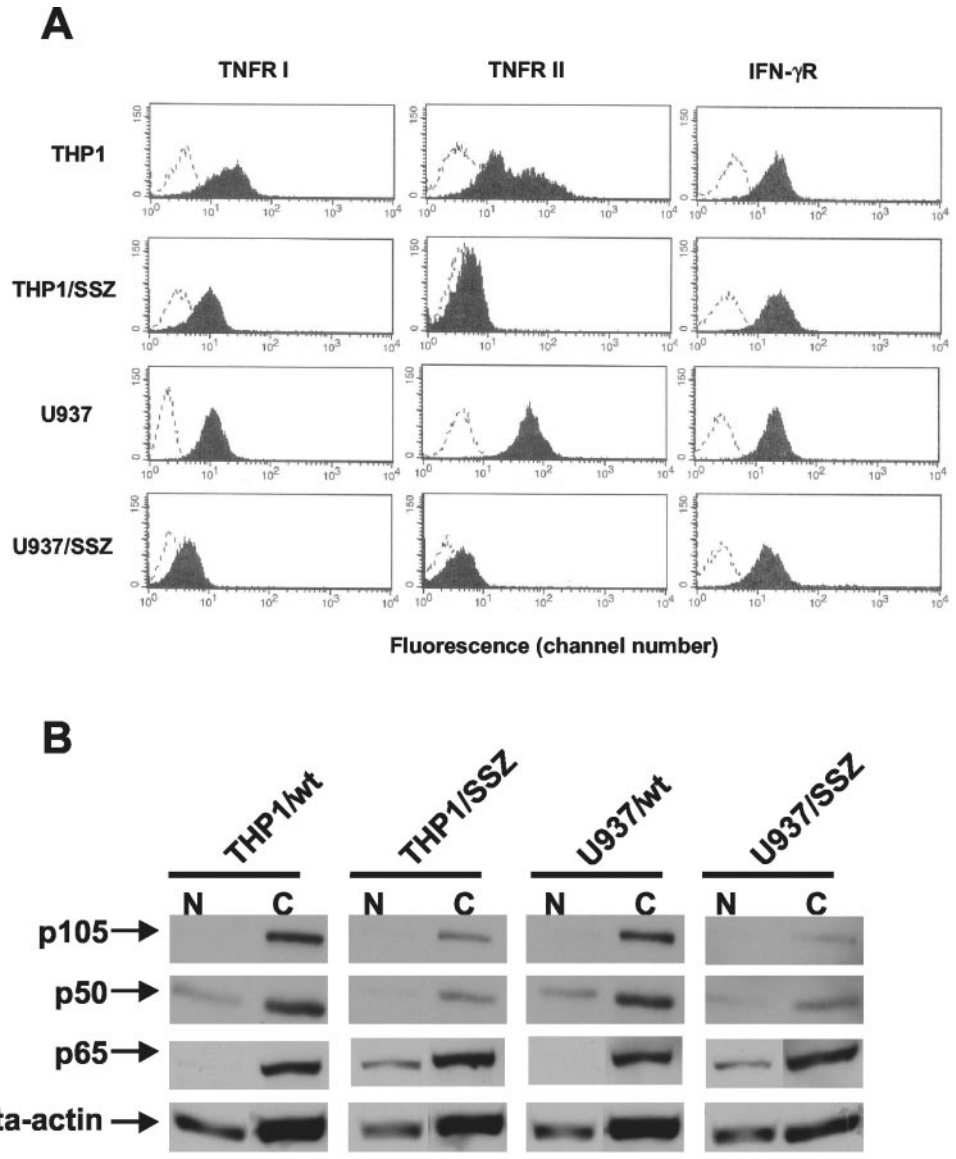

Fig. 3. A, representative picture of a flow cytometry experiment assessing the expression of IFN-γR, TNFRI, and TNFRII in parental and SSZ-exposed THP1 and U937 cells. Results show that IFN-γR was expressed at similar levels, TNFRI was down-regulated and there was a complete loss of TNFRII. The mean fluorescence (specific signal minus isotype control signal) for TNFRII decreased from 36 to 2 and 60 to 3 for THP1 and U937 compared with their SSZ-exposed counterparts, respectively. Dotted line, isotype control (autofluorescence); black fill, specific signal. B, Western blot of NF-κB family members p105/50 and p65 in cytoplasmic and nuclear fractions of parental and SSZ-exposed THP1 and U937 cells. Results show a minor decrease in cytoplasmic p105 and a decrease of nuclear p50 in the SSZ-exposed cells compared with parental cells. No apparent differences in cytoplasmic p65 levels were observed. In contrast, constitutive p65 expression was found in the nuclear fractions of SSZ-exposed cells, which was absent in the parental cells. The p105/50, p65, and β-actin are all from one representative experiment and blotted on three separate gels; from each gel the bands were rearranged to better compare the nuclear and cytoplasmic content.

of transcriptionally active p65/p50 heterodimers. This will necessarily result in a transcriptional dysfunctioning of the NF-κB pathway. NF-κB directly regulates IL-8, and the TNFRs use NF-κB pathway in their signal transduction. No direct involvement of NF-κB has been shown in relation to PD-ECGF/TP expression, although in a recent article, Zhu and Schwartz (2003) suggested that NF-κB and TNFRII may be involved in the regulation of PD-ECGF/TP gene expression.

Although this study is focused primarily on the role of PD-ECGF/TP in angiogenesis, the outcome of this study may have major implications for the chemotherapeutic efficacy of the 5-FU prodrug capecitabine (Miwa et al., 1998). Currently, capecitabine is the most widely prescribed oral drug for the treatment of colorectal cancer, but it requires activation by PD-ECGF/TP. Since exposure to SSZ down-regulates TP activity, this leads to resistance to capecitabine (Table 1). In numerous studies, PD-ECGF/TP has been shown to colocalize with tumor-associated macrophages (Takahashi et al., 1996; Toi et al., 1999). Consequently, PD-ECGF/TP from macrophages can be responsible for increased 5-FU accumulation and enhanced efficacy, due to a bystander effect (Evrard et al., 1999). However, when prior treatment with NF-κB interacting drugs down-regulates PD-ECGF/TP, this may negatively affect the efficacy of capecitabine. Therefore, these patients should be carefully screened for their TP level in macrophages and tumor cells before receiving capecitabine. In addition, future studies should also evaluate TP expression in macrophages of patients with RA treated with SSZ for a prolonged period.

To our knowledge this is the first example of a treatment (i.e., SSZ) that results not only in the down-regulation of PD-ECGF/TP but also in a complete inhibition of pathways known to up-regulate PD-ECGF/TP. The results were observed in two independent monocytic/macrophage cell lines. We propose that the down-regulation of PD-ECGF/TP and IL-8 may be the consequence of a nonfunctional (auto)crine loop due to changes in TNF-receptor expression and downstream signaling pathways of both IFN-γ and TNF-α receptors, in combination with an altered NF-κB pathway. Hence, NF-κB and TNF(R)-mediated signaling pathways warrant further attention as targets for future optimization of antiangiogenic therapy, focusing on their role in the functioning of macrophages and the expression of PD-ECGF/TP and IL-8.

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References

Ackland SP and Peters GJ (1999) Thymidine phosphorylase: its role in sensitivity and resistance to anticancer drugs. *Drug Resist Updat* **2:**205–214.
Baggott JE, Morgan SL, Ha T, Vaughn WH, and Hine RJ (1992) Inhibition of folate-dependent enzymes by non-steroidal anti-inflammatory drugs. *Biochem J* **282:**197–202.
Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, and Strieter RM (2000) CXC chemokines in angiogenesis. *J Leukoc Biol* **68:**1–8.
Bingle L, Brown NJ, and Lewis CE (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* **196:**254–265.
Blanquicett C, Gillespie GY, Nabors LB, Miller CR, Bharara S, Buchsbaum DJ, Diasio RB, and Johnson MR (2002) Induction of thymidine phosphorylase in both irradiated and shielded, contralateral human U87MG glioma xenografts: implications for a dual modality treatment using capecitabine and irradiation. *Mol Cancer Ther* **1:**1139–1145.
Bosco MC, Gusella GL, Espinoza-Delgado I, Longo DL, and Varesio L (1994) Interferon-gamma upregulates interleukin-8 gene expression in human monocytic cells by a posttranscriptional mechanism. *Blood* **83:**537–542.
Brooks CD (2001) Sulfasalazine for the management of juvenile rheumatoid arthritis. *J Rheumatol* **28:**845–853.
Brown NS and Bicknell R (1998) Thymidine phosphorylase, 2-deoxy-D-ribose and angiogenesis. *Biochem J* **334:**1–8.
Burmester GR, Stuhlmuller B, Keyszer G, and Kinne RW (1997) Mononuclear phagocytes and rheumatoid synovitis. mastermind or workhorse in arthritis? *Arthritis Rheum* **40:**5–18.
Daly ME, Makris A, Reed M, and Lewis CE (2003) Hemostatic regulators of tumor angiogenesis: a source of antiangiogenic agents for cancer treatment? *J Natl Cancer Inst* **95:**1660–1673.
De Bruin M, Van Capel T, Van der Born K, Kruyt FA, Fukushima M, Hoekman K, Pinedo HM, and Peters GJ (2003) Role of platelet-derived endothelial cell growth factor/thymidine phosphorylase in fluoropyrimidine sensitivity. *Br J Cancer* **88:**957–964.
Eda H, Fujimoto K, Watanabe S, Ura M, Hino A, Tanaka Y, Wada K, and Ishitsuka H (1993) Cytokines induce thymidine phosphorylase expression in tumor cells and make them more susceptible to 5′-deoxy-5-fluorouridine. *Cancer Chemother Pharmacol* **32:**333–338.
Evrard A, Cuq P, Ciccolini J, Vian L, and Cano JP (1999) Increased cytotoxicity and bystander effect of 5-fluorouracil and 5′-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. *Br J Cancer* **80:**1726–1733.
Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1:**27–31.
Fujimoto J, Aoki I, Toyoki H, Khatun S, and Tamaya T (2002) Clinical implications of expression of ets-1 related to angiogenesis in uterine cervical cancers. *Ann Oncol* **13:**1598–1604.
Goto H, Kohno K, Sone S, Akiyama S, Kuwano M, and Ono M (2001) Interferon gamma-dependent induction of thymidine phosphorylase/platelet-derived endothelial growth factor through gamma-activated sequence-like element in human macrophages. *Cancer Res* **61:**469–473.
Gupta S (2002) A decision between life and death during TNF-alpha-induced signaling. *J Clin Immunol* **22:**185–194.
Hanahan D and Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86:**353–364.
Hoffmann E, Dittrich-Breiholz O, Holtmann H, and Kracht M (2002) Multiple control of interleukin-8 gene expression. *J Leukoc Biol* **72:**847–855.
Jauneau AC, Ischenko A, Chan P, and Fontaine M (2003) Complement component anaphylatoxins upregulate chemokine expression by human astrocytes. *FEBS Lett* **537:**17–22.
Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, and Strieter RM (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science (Wash DC)* **258:**1798–1801.
Laurensse EJ, Pinedo HM, and Peters GJ (1988) A sensitive non-radioactive assay for pyrimidine nucleoside phosphorylase using reversed-phase high performance liquid chromatography. *Clin Chim Acta* **178:**71–78.
Matsumura M, Chiba Y, Lu C, Amaya H, Shimomatsuya T, Horiuchi T, Muraoka R, and Tanigawa N (1998) Platelet-derived endothelial cell growth factor/thymidine phosphorylase expression correlated with tumor angiogenesis and macrophage infiltration in colorectal cancer. *Cancer Lett* **128:**55–63.
Miwa M, Ura M, Nishida M, Sawada N, Ishikawa T, Mori K, Shimma N, Umeda I, and Ishitsuka H (1998) Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. *Eur J Cancer* **34:**1274–1281.
Perkins ND (2000) The Rel/NF-kappa B family: friend and foe. *Trends Biochem Sci* **25:**434–440.
Rots MG, Willey JC, Jansen G, Van Zantwijk CH, Noordhuis P, DeMuth JP, Kuiper E, Veerman AJ, Pieters R, and Peters GJ (2000) mRNA expression levels of methotrexate resistance-related proteins in childhood leukemia as determined by a standardized competitive template- based RT-PCR method. *Leukemia* **14:**2166–2175.
Ryan BM, Russel MG, Langholz E, and Stockbrugger RW (2003) Aminosalicylates and colorectal cancer in IBD: a not-so bitter pill to swallow. *Am J Gastroenterol* **98:**1682–1687.
Sawada N, Ishikawa T, Fukase Y, Nishida M, Yoshikubo T, and Ishitsuka H (1998) Induction of thymidine phosphorylase activity and enhancement of capecitabine efficacy by taxol/taxotere in human cancer xenografts. *Clin Cancer Res* **4:**1013–1019.
Sawada N, Ishikawa T, Sekiguchi F, Tanaka Y, and Ishitsuka H (1999) X-Ray irradiation induces thymidine phosphorylase and enhances the efficacy of capecitabine (xeloda) in human cancer xenografts. *Clin Cancer Res* **5:**2948–2953.
Schwartz EL, Wan E, Wang FS, and Baptiste N (1998) Regulation of expression of thymidine phosphorylase/platelet-derived endothelial cell growth factor in human colon carcinoma cells. *Cancer Res* **58:**1551–1557.
Smedegard G and Bjork J (1995) Sulphasalazine: mechanism of action in rheumatoid arthritis. *Br J Rheumatol* **34 (Suppl 2):**7–15.
Takahashi Y, Bucana CD, Liu W, Yoneda J, Kitadai Y, Cleary KR, and Ellis LM (1996) Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis: role of infiltrating cells. *J Natl Cancer Inst* **88:**1146–1151.
Takebayashi Y, Akiyama S, Akiba S, Yamada K, Miyadera K, Sumizawa T, Yamada Y, Murata F, and Aikou T (1996) Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phosphorylase, in human colorectal carcinoma [see comments]. *J Natl Cancer Inst* **88:**1110–1117.
Toi M, Inada K, Hoshina S, Suzuki H, Kondo S, and Tominaga T (1995) Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. *Clin Cancer Res* **1:**961–964.
Toi M, Ueno T, Matsumoto H, Saji H, Funata N, Koike M, and Tominaga T (1999) Significance of thymidine phosphorylase as a marker of protumor monocytes in breast cancer. *Clin Cancer Res* **5:**1131–1137.

van der Wilt CL, Kroep JR, Loves WJ, Rots MG, van Groeningen CJ, Kaspers GJ, and Peters GJ (2003) Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver. *Eur J Cancer* **39:**691–697.

van Triest B, Pinedo HM, Blaauwgeers JL, van Diest PJ, Schoenmakers PS, Voorn DA, Smid K, Hoekman K, Hoitsma HF, and Peters GJ (2000) Prognostic role of thymidylate synthase, thymidine phosphorylase/platelet-derived endothelial cell growth factor and proliferation markers in colorectal cancer. *Clin Cancer Res* **6:**1063–1072.

Waguri Y, Otsuka T, Sugimura I, Matsui N, Asai K, Moriyama A, and Kato T (1997) Gliostatin/platelet-derived endothelial cell growth factor as a clinical marker of rheumatoid arthritis and its regulation in fibroblast-like synoviocytes. *Br J Rheumatol* **36:**315–321.

Wahl C, Liptay S, Adler G, and Schmid RM (1998) Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. *J Clin Investig* **101:**1163–1174.

Zhu GH, Lenzi M, and Schwartz EL (2002) The Sp1 transcription factor contributes to the tumor necrosis factor-induced expression of the angiogenic factor thymidine phosphorylase in human colon carcinoma cells. *Oncogene* **21:**8477–8485.

Zhu GH and Schwartz EL (2003) Expression of the angiogenic factor thymidine phosphorylase in THP-1 monocytes: induction by autocrine tumor necrosis factor-alpha and inhibition by aspirin. *Mol Pharmacol* **64:**1251–1258.

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