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p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor

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Tumour necrosis factor (TNF) is a pleiotropic cytokine, the activities of which include effects on gene expression, cell growth and cell death. The biological signalling mechanisms which are responsible for these TNF effects remain largely unknown. Here we demonstrate that the stress-responsive p38 mitogen-activated protein (MAP) kinase is involved in TNF-induced cytokine expression. TNF treatment of cells activated the p38 MAP kinase pathway, as revealed by increased phosphorylation of p38 MAP kinase itself, activation of the substrate protein MAPKAP kinase-2, and culminating in the phosphorylation of the heat shock protein 27 (hsp27). Pretreatment of cells with the highly specific p38 MAP kinase inhibitor SB203580 completely blocked this TNF-induced activation of MAPKAP kinase-2 and hsp27 phosphorylation. Under the same conditions, SB203580 also completely inhibited TNF-induced synthesis of interleukin (IL)-6 and expression of a reporter gene that was driven by a minimal promoter containing two NF-κB elements. However, neither TNF-induced DNA binding of NF-κB nor TNF-induced phosphorylation of its subunits was modulated by SB203580, suggesting that NF-κB is not a direct target for the p38 MAP kinase pathway. Interestingly, TNF-induced cytotoxicity was not affected by SB203580, indicating that p38 MAP kinase might be an interesting target to interfere selectively with TNF-induced gene activation.

Keywords: cytotoxicity/IL-6/MAP kinases/NF-κB/TNF

Introduction

Tumour necrosis factor (TNF) is a cytokine that elicits a large number of biological effects, including haemorrhagic necrosis of transplanted tumours, cytotoxicity, immunoregulation, cellular proliferation, antiviral responses and transcriptional activation of many genes (reviewed in Fiers, 1995). In particular, TNF plays an important role in the systemic inflammatory response syndrome (SIRS) and is a major mediator of toxic shock or sepsis. These pleiotropic effects considerably limit the use of TNF as an antitumour agent. TNF actions are initiated by binding to two distinct TNF receptors of 55 kDa (p55) and 75 kDa (p75), with p55 receptors mediating the majority of the TNF effects (reviewed in Vandenabeele *et al.*, 1995). The binding of TNF to its receptors leads to the activation of several second messengers, although the response is often cell type-dependent (reviewed in Beyaert and Fiers, 1994). Like other cytokine receptors, TNF receptors do not contain an intrinsic kinase domain. Nevertheless, exposure to TNF results in the rapid phosphorylation of several cellular target proteins, the small heat shock protein 27 (hsp27) being one of the most striking examples (Hepburn *et al.*, 1988; Guesdon *et al.*, 1993). Kinases that have been reported to become activated by TNF include protein kinase A (Zhang *et al.*, 1988), protein kinase C (Schütze *et al.*, 1989) and mitogen-activated protein (MAP) kinases ERK1 and ERK2 (Van Lint *et al.*, 1992; Vietor *et al.*, 1993). As these protein kinases have been implicated also in the mechanism of signal transduction by many other extracellular stimuli, it seems unlikely that the activation of these is sufficient to account for the distinctive pattern of activities that are induced by TNF. Therefore, there has been much interest in the role of novel TNF/interleukin (IL)-1/stress-activated protein kinases, such as a ceramide-activated protein kinase (Mathias *et al.*, 1991), β-casein kinase (Guesdon *et al.*, 1993), protein kinase C ζ (Müller *et al.*, 1995) and hsp27 kinase (Guesdon *et al.*, 1993; Freshney *et al.*, 1994; Rouse *et al.*, 1994). However, the physiological significance of their activation has so far not been characterized.

MAP kinases are important mediators of signal transduction from the cell surface to the nucleus. Until recently, ERK1 and ERK2 were the only cloned and well-characterized mammalian MAP kinases. However, the recent discovery of two other MAP kinase subtypes, the c-jun kinase (JNK) subfamily and p38/RK MAP kinase, reveals the existence of parallel MAP kinase cascades that can be activated independently and simultaneously (reviewed in Cano and Mahadevan, 1995). ERKs are activated predominantly by growth factors or phorbol ester (reviewed in Marshall, 1995), but activation by TNF or IL-1 has also been demonstrated (Van Lint *et al.*, 1992; Vietor *et al.*, 1993). In contrast, JNK and p38/RK MAP kinase are activated by inflammatory cytokines and cellular stresses such as heat shock, osmotic stress or ultraviolet (UV) light (Galcheva-Gargova *et al.*, 1994; Kyriakis *et al.*, 1994; Raingeaud *et al.*, 1995). The identification of distinct MAP kinase kinases which specifically activate ERK, JNK or p38/RK MAP kinases suggests the existence of independent signalling roles for these MAP kinase cascades (Dérijard *et al.*, 1995; Lin *et al.*, 1995). Mammalian p38 MAP kinase was originally identified in murine pre-B cells transfected with the LPS-complex receptor CD14 and in murine macrophages where it is activated in

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response to LPS (Han *et al.*, 1994). In parallel, p38 MAP kinase was also identified as a 'reactivating kinase' (termed RK), which activated MAPKAP kinase-2, which in turn phosphorylated hsp27 (Freshney *et al.*, 1994; Rouse *et al.*, 1994). The amino acid sequence of RK identified it as the mammalian homologue of the yeast osmosensing MAP kinase HOG1 (Brewster *et al.*, 1993), and the *Xenopus* kinase Mpk2 (Rouse *et al.*, 1994). CSBP1 and CSBP2 have recently been identified as human homologues of p38 MAP kinase (Lee *et al.*, 1994), and were found to interact with a novel class of pyridinyl imidazoles which were originally developed as inhibitors of the LPS-induced synthesis of IL-1 and TNF from preformed mRNA in monocytes (Young *et al.*, 1993). One of these drugs, termed SB203580, inhibited p38 MAP kinase with an IC_{50} of 0.6 μM, and even at 100 μM had no effect on the activities of 12 other protein kinases tested, including ERK2 and JNK. Moreover, SB203580 prevented the activation (Cuenda *et al.*, 1995) and phosphorylation (Ben-Levy *et al.*, 1995) of MAPKAP kinase-2 and the phosphorylation of hsp27 by cellular stresses, LPS and IL-1, but did not inhibit the kinase(s) lying upstream of p38 MAP kinase by growth factors (Cuenda *et al.*, 1995). These observations suggested that SB203580 might be useful for probing other physiological roles of the p38 MAP kinase pathway in the response of cells to cytokines. Here we report that the p38 MAP kinase pathway is activated in TNF-treated cells and demonstrate its crucial but remarkably selective role in gene induction, such as synthesis of IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF).

Results

TNF activates the p38 MAP kinase signal transduction pathway

p38 MAP kinase has been shown to be activated after phosphorylation by upstream kinases. Activated p38 MAP kinase then phosphorylates and activates MAPKAP kinase-2 and the cascade culminates in the phosphorylation of hsp27 (Rouse *et al.*, 1994; Cuenda *et al.*, 1995; Doza *et al.*, 1995). To demonstrate that TNF activates the p38 MAP kinase pathway in murine L929 cells, we first studied the effect of TNF on the phosphorylation of p38 MAP kinase in cells that were metabolically labelled with $^{32}P_i$. p38 MAP kinase was immunoprecipitated from L929 cell lysates and subjected to SDS–PAGE. As shown in Figure 1A, treatment with TNF for 15 min considerably increased the phosphorylation of a 38–40 kDa protein which was specifically recognized by the anti-p38 MAP kinase antiserum. Phosphorylation of a slightly faster migrating protein was also elevated, and this might represent a degradation product. As described previously (Cuenda *et al.*, 1995), pre-incubation of the cells with 10 μM SB203580 had no effect on the phosphorylation of p38 MAP kinase, demonstrating that the inhibitor does not interfere with the upstream activator(s) of p38 MAP kinase (data not shown). A TNF-induced increase in p38 MAP kinase phosphorylation was also observed in human U937 cells (data not shown).

To demonstrate further that TNF activates the p38 MAP kinase pathway, we examined the activity of MAPKAP kinase-2 in L929 and HeLa cells with or without pretreatment with 20 μM SB203580, followed by treatment with TNF for 15 min. Treatment with TNF caused a 4-fold increase in MAPKAP kinase-2 activity in L929 cells (Figure 1B, upper panel) and a 30-fold increase in HeLa cells (Figure 1B, lower panel), which was completely abolished by preincubation of the cells with SB203580.

TNF has previously been shown to increase hsp27 phosphorylation in several cell lines (Hepburn *et al.*, 1988; Guesdon *et al.*, 1993). To further confirm that SB203580 specifically blocks TNF-induced activation of p38 MAP kinase, we measured its effect on TNF-induced phosphorylation of hsp27 in U937 cells that were metabolically labelled with $^{32}P_i$. Prior incubation of U937 cells with 10 μM SB203580 completely suppressed the phosphorylation of hsp27 by TNF treatment (Figure 1C). The effect of SB203580 on the phosphorylation of hsp27 was highly specific because this drug had no effect on the phosphorylation state of any of the other major ^{32}P-labelled proteins in U937 cells that were resolved by one-dimensional SDS–PAGE (data not shown). hsp27 phosphorylation could not be tested in L929 cells, as these do not express the murine homologue hsp25.

p38 MAP kinase mediates TNF-induced expression of IL-6 and GM-CSF

To evaluate the biological significance of p38 MAP kinase activation by TNF, we tested the effect of SB203580 on TNF-induced IL-6 production in L929 cells and in HeLa cells. Cells were pretreated for 2 h with an increasing concentration of SB203580 (up to 10 μM), followed by an 18 h incubation with a range of TNF concentrations. SB203580 was present throughout the incubation period. TNF increased the levels of biologically active IL-6 in the cell supernatant in a dose-dependent manner (Figure 2A). This induction could be completely prevented by SB203580; 50% inhibition was observed at ~1 μM and inhibition was essentially complete at 10 μM. SB203580 was not toxic for the cells, even at 3-fold higher concentrations (data not shown). A similar inhibition of TNF-induced IL-6 production by SB203580 was observed in HeLa cells (data not shown).

TNF also stimulates the production of GM-CSF in L929 cells (Beyaert *et al.*, 1991). Again, SB203580 completely blocked the TNF-induced production of GM-CSF with similar potency to the inhibition of IL-6 production (Figure 2B). Similarly, the p38 MAP kinase inhibitor also inhibited TNF-induced GM-CSF production in the PC60 T cell hybridoma cell line (data not shown).

We and others previously demonstrated that TNF augments IL-6 production primarily by an increase in the rate of transcription (Walther *et al.*, 1988; Vandevoorde *et al.*, 1991). To investigate the level at which SB203580 inhibited IL-6 production in TNF-treated L929 cells, we performed a Northern analysis of total cytoplasmic RNA using a ^{32}P-labelled murine IL-6 probe. As compared with a very weak signal in untreated cells, IL-6 mRNA levels were significantly increased by 5 h TNF treatment. In contrast, only a very weak TNF effect could be observed in the presence of SB203580 (Figure 3). mRNA levels of GAPDH served as an internal control and were not changed by any treatment. In view of the results mentioned above and those obtained with reporter genes, to be described below, we can conclude that the p38 MAP

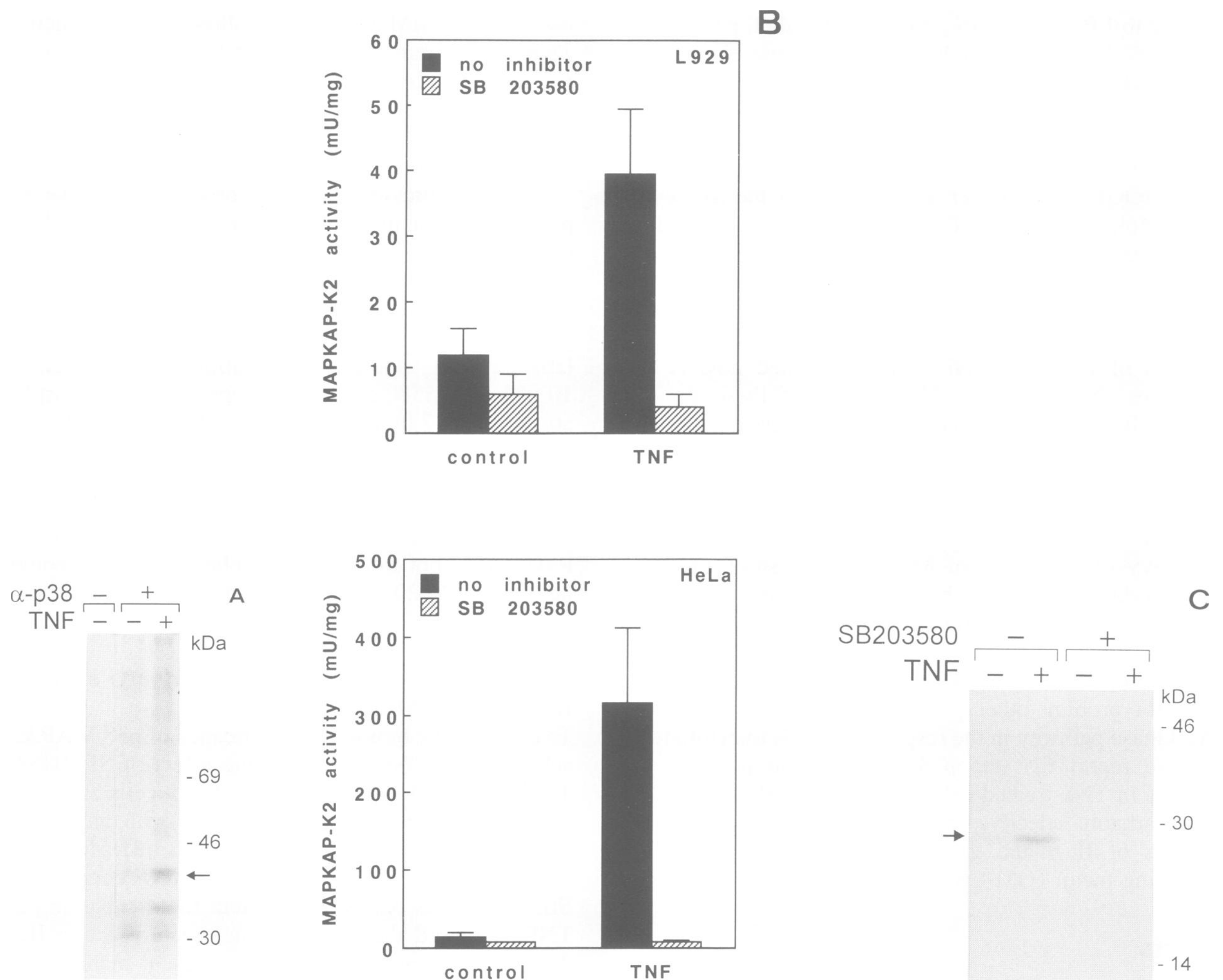

Fig. 1. TNF activates the p38 MAP kinase pathway. (**A**) TNF-induced phosphorylation of p38 MAP kinase. 5×10^5 ^{32}P-labelled L929 cells were either untreated (–) or treated (+) for 15 min with 5000 IU mTNF/ml. Phosphorylated p38 MAP kinase was detected by immunoprecipitation, 10% SDS–PAGE and autoradiography. The arrow indicates the position of p38 MAP kinase. (**B**) TNF-induced activation of MAPKAP kinase-2. L929 cells (upper panel) and HeLa cells (lower panel) were incubated for 60 min in the absence (filled bars) or presence (hatched bars) of 20 μM SB203580 and then stimulated for 15 min with 2000 IU TNF/ml in the continued presence or absence of SB203580. After lysis in the presence of protein phosphatase inhibitors (Rouse *et al.*, 1994), MAPKAP kinase-2 was immunoprecipitated and assayed as described in Materials and methods. (**C**) TNF-induced phosphorylation of hsp27. 5×10^5 ^{32}P-labelled U937 cells were incubated for 1.5 h with 10 μM SB203580 and stimulated with 5000 IU hTNF/ml for 30 min in the continuous presence of SB203580. hsp27 was immunoprecipitated from cell lysates, and analysed by 12.5% SDS–PAGE and autoradiography. The arrow indicates the position of hsp27.

kinase pathway modulates TNF-induced IL-6 production at the level of transcription.

Possible regulation of NF-κB activity by the p38 MAP kinase pathway

Activation of the transcription factor NF-κB has previously been shown to be indispensable for IL-6 gene induction by TNF or other stimuli (Libermann and Baltimore, 1990; Zhang *et al.*, 1990). Considering the modulation of IL-6 expression by the p38 MAP kinase pathway, we explored a possible role for p38 MAP kinase in the activation of NF-κB. In initial experiments we used L929 cells that were stably transfected with a reporter gene construct carrying two copies of the NF-κB-binding sequence in front of the chloramphenicol acetyltransferase (CAT) gene. This κB–CAT reporter gene construct has been shown to respond specifically to NF-κB activation (Pierce *et al.*, 1988; Lenardo and Baltimore, 1989; our own unpublished results). CAT expression in these transfectants was relatively low in uninduced cells (Figure 4), as was observed for the endogenous IL-6 gene. But the CAT gene was inducible after stimulation with TNF, resulting in a 5-fold stimulation after 6 h. Interestingly, treatment of the cells with 10 μM SB203580 almost completely inhibited TNF-induced CAT expression. A similar observation could be made in cells that were transfected with a CAT gene under control of the wild-type IL-6 promoter, although the TNF response was less pronounced; an IL-6 promoter or a κB–CAT reporter mutated in the κB site no longer responded to TNF (data not shown). These data suggest that the p38 MAP kinase pathway is required for transcriptional induction mediated by NF-κB.

NF-κB is not present in the nucleus of uninduced cells, but becomes activated after induction by TNF and then translocates to the nucleus (Rice and Ernst, 1993; Baeuerle and Henkel, 1994; Israël, 1995). To further explore the possible role of the p38 MAP kinase pathway in the TNF-induced activation of NF-κB, we studied the effect of

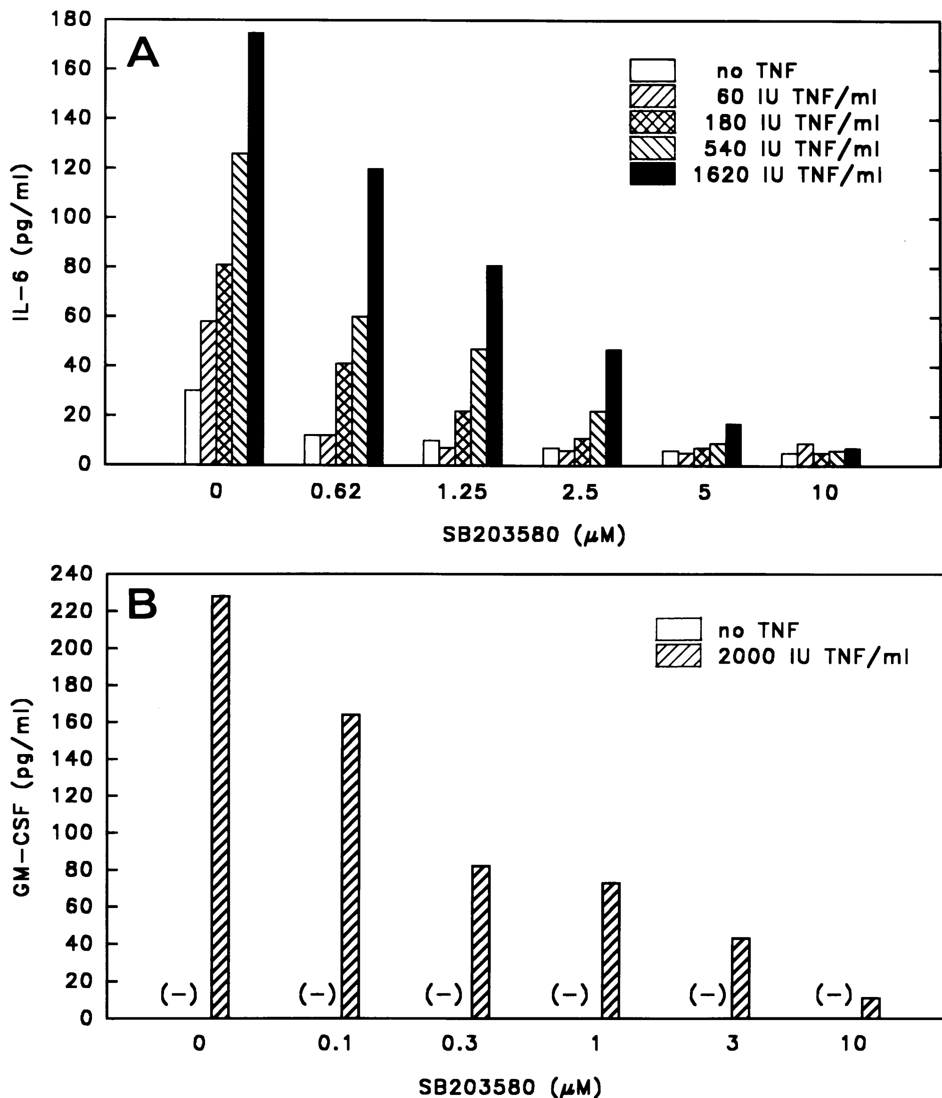

Fig. 2. TNF-induced expression of IL-6 and GM-CSF. 10^4 L929 cells were pretreated with varying concentrations of SB203580; 2 h later varying concentrations of mTNF were added. After a further 18 h incubation, supernatants were collected and biologically active IL-6 (**A**) and GM-CSF (**B**) were measured in a 7TD1 and Fdcp1 bioassay, respectively, as described previously (Beyaert *et al.*, 1991).

SB203580 on TNF-induced NF-κB DNA binding in an electrophoretic mobility shift assay (EMSA), using an NF-κB-specific ^{32}P-labelled oligonucleotide (Patestos *et al.*, 1993). An inducible protein–DNA complex was observed in nuclear extracts from TNF-treated L929 cells (Figure 5). Competition experiments with increasing amounts of unlabelled oligonucleotide inhibited the binding, confirming its specificity. Remarkably, L929 cells pretreated with 10 μM SB203580 revealed no significant decrease of TNF-induced NF-κB DNA binding, indicating that the p38 MAP kinase pathway does not interfere with release of NF-κB from IκB, nor with its nuclear translocation and DNA binding.

The activity of many transcription factors is controlled by phosphorylation of their transactivation domain (Karin, 1994). Also the DNA-binding subunits of NF-κB become phosphorylated after TNF treatment (Naumann and Scheidereit, 1994). So far, a role for NF-κB subunit phosphorylation in TNF-induced NF-κB activation has not been proven, but the recent demonstration that phosphorylation of a transactivation domain of the p65 subunit further augments the transactivation potential of activated nuclear NF-κB makes this rather likely (Schmitz *et al.*, 1995b). To investigate whether the p38 MAP kinase pathway might modulate the activity of nuclear NF-κB by changing the phosphorylation of its subunits, we tested whether inhibition of the p38 MAP kinase pathway with SB203580 had any effect on TNF-stimulated phosphorylation of p50 or p65 in L929 cells. Immunoprecipitation of p50 and p65 NF-κB subunits from ^{32}P-labelled L929 cells revealed that the phosphorylation of both subunits was enhanced after TNF treatment (Figure 6). In the case of p50, this was most pronounced in its p105 precursor. The faster-migrating phosphoprotein, that disappeared after TNF treatment, represents co-immunoprecipitated IκB that is degraded after TNF treatment (Henkel *et al.*, 1993). The identity of the other phosphoproteins that were co-immunoprecipitated, is still unknown. As apparent in Figure 6, neither the constitutive phosphorylation, nor the TNF-induced phosphorylation of any of the subunits was changed by prior incubation of the cells with 10 μM SB203580. Although it cannot be excluded that the p38

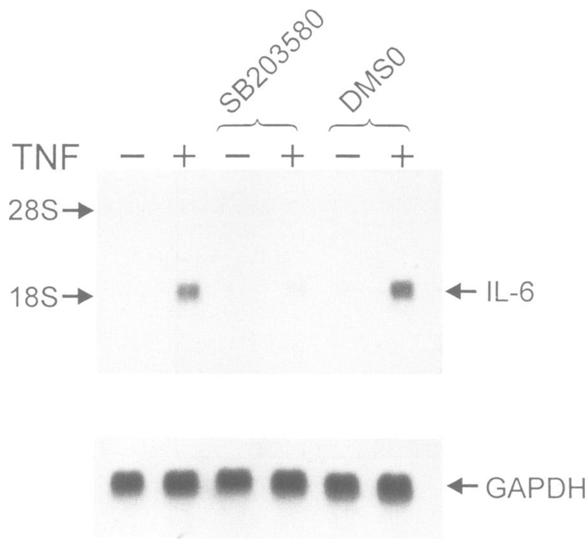

Fig. 3. Northern blot analysis of TNF-induced IL-6 expression. L929 cells were pretreated for 2 h with 10 μM SB203580 or 0.1% DMSO that was used as a solvent. Cells were then treated for 5 h with 5000 IU mTNF/ml in the continuous presence of SB203580 or DMSO. Total cytoplasmic RNA was extracted and 12 μg RNA was analysed by Northern blotting for the expression of IL-6 mRNA (upper panel). Repeated hybridization of the filters with a GAPDH-specific probe confirmed identical RNA loading in all lanes (lower panel). The migration positions of the rRNA species are also indicated.

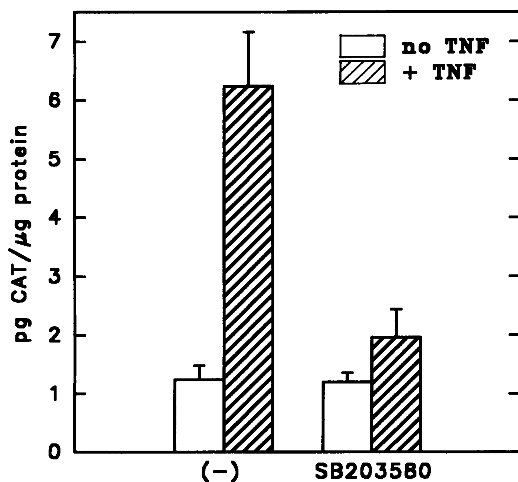

Fig. 4. Activation of an NF-κB CAT reporter gene by TNF is modulated by p38 MAP kinase. L929 cells stably transfected with an NF-κB CAT reporter plasmid (J16) were either untreated or treated for 2 h with 10 μM SB203580. Induction with 1000 IU mTNF/ml (hatched bars) was for 6 h. CAT expression in cell lysates was quantified by CAT–ELISA. Results are the mean of triplicate stimulations.

MAP kinase changes the phosphorylation pattern of certain specific amino acids which may have escaped detection, these results strongly suggest that the p38 MAP kinase pathway is not the main mediator of the TNF-induced phosphorylation of NF-κB subunits.

The p38 MAP kinase pathway is not involved in the cytotoxic effect of TNF in L929 cells

In order to further analyse the involvement of the p38 MAP kinase pathway in TNF signalling, we next asked the question whether this pathway was also involved in

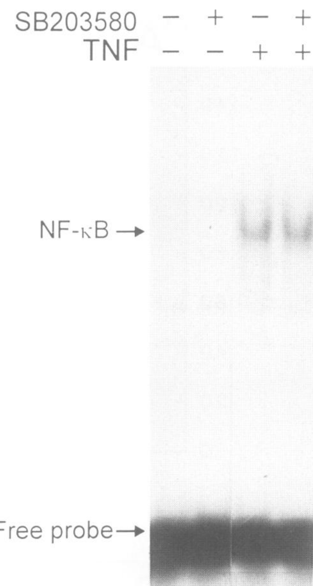

Fig. 5. TNF-induced formation of NF-κB/DNA complexes. 5 μg of nuclear extract obtained from L929 cells that were either untreated or pretreated for 2 h with 10 μM SB203580, followed by 45 min stimulation with 1000 IU mTNF/ml, were analysed in an EMSA with a ^{32}P-labelled oligonucleotide encompassing the κB-binding site of the IL-6 promoter.

the TNF-induced cytotoxic effect. TNF-induced cytotoxicity in L929 cells was tested in the absence or presence of SB203580 under exactly the same conditions as used for the inhibition studies on TNF-induced IL-6 production. As shown in Figure 7, SB203580 had no effect on TNF cytotoxicity, even at concentrations that were 60-fold higher than those required to inhibit TNF-induced IL-6 production. These results indicate that TNF-induced gene expression and TNF-induced cytotoxicity follow signalling pathways that differ at least partially, the p38 MAP kinase being required only for the former.

Discussion

In this paper we show that the recently identified p38 MAP kinase pathway is activated by TNF, and that this pathway is involved in the TNF-induced production of IL-6 and GM-CSF. This has been achieved by exploiting SB203580 as a highly specific inhibitor of p38 MAP kinase (Cuenda *et al.*, 1995; also see Introduction). TNF increased the phosphorylation (activation) of p38 MAP kinase which, in turn, promoted the activation of MAPKAP kinase-2 and thereupon the phosphorylation of hsp27. Both activation of MAPKAP kinase-2 and phosphorylation of hsp27 were prevented by SB203580, confirming their TNF-induced activation via the p38 MAP kinase pathway. This pathway was first described to be activated by cellular stresses and IL-1 (Freshney *et al.*, 1994; Rouse *et al.*, 1994; Cuenda *et al.*, 1995), but its biological roles had so far not been established. hsp27 has chaperone-like properties and acts as an inhibitor of actin polymerization (Miron *et al.*, 1991; Knauf *et al.*, 1994). There is evidence that phosphorylation of hsp27 may stimulate the polymerization of actin and so help to repair the actin microfilament network disrupted during cellular stress, thereby aiding cell survival (Lavoie *et al.*, 1993, 1995). However,

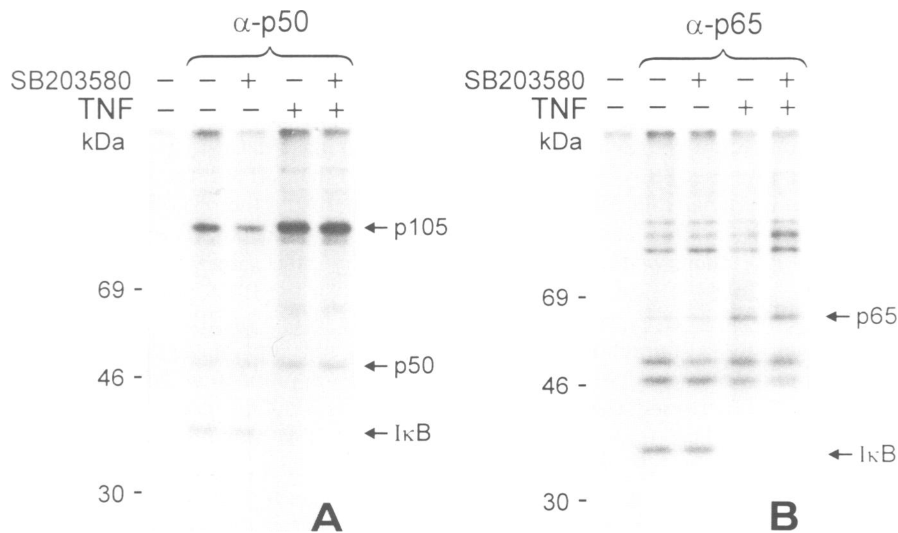

Fig. 6. TNF-induced phosphorylation of NF-κB subunits. 5×10^5 ^{32}P-labelled L929 cells were stimulated for 15 min with 5000 IU mTNF/ml in the absence or presence of 10 µM SB203580 which was given 2 h before TNF. Cell lysates were immunoprecipitated with an anti-p50 antiserum (**A**) or an anti-p65 antiserum (**B**), respectively, and analysed by 10% SDS–PAGE and autoradiography. The first lane of each panel represents a precipitation that was done in the absence of antiserum. The positions of p50, p105, p65 and co-immunoprecipitated IκB are indicated by an arrow.

the role of hsp27 phosphorylation remains controversial, because others have reported that the chaperone-like function of hsp27 and its ability to confer thermotolerance to cells are functions that are independent of its phosphorylation (Knauf *et al.*, 1994). The role of hsp27 in TNF signalling is also controversial. In one case, overexpression of hsp27 was reported to confer resistance to TNF (Mehlen *et al.*, 1995), but in other studies its overexpression failed to alter susceptibility to TNF (Jäättelä *et al.*, 1992; our own unpublished results). Additional studies will be needed to resolve these conflicting results. However, the fact that the TNF-responsive L929 cells used in the present study fail to express the murine homologue hsp25, even after TNF treatment, exclude a direct signalling role for murine hsp25 or human hsp27 phosphorylation in TNF action. Activation of p38 MAP kinase and MAPKAP kinase-2 by TNF is likely to result in the phosphorylation of additional substrates, but these remain to be identified.

An important activity of TNF is the regulation of gene expression, certainly if one considers the role of several TNF-induced gene products in TNF toxicity *in vivo*. One of the induced proteins is the pleiotropic cytokine IL-6, acting on the immune system, on the neuroendocrine system, and perhaps most important of all, one of the main inducers of the acute phase response (Van Snick, 1990). Our present observation that the p38 MAP kinase inhibitor SB203580 inhibits TNF-induced IL-6 and GM-CSF production without interfering with the cytotoxic effect of TNF is therefore of particular interest, since it implies a specific role for the p38 MAP kinase pathway in TNF-induced cytokine synthesis. In a previous study, closely related analogues of SB203580 were described to inhibit LPS-induced TNF and IL-1 synthesis in THP-1 monocytes (Lee *et al.*, 1994). Remarkably, the level at which p38 MAP kinase regulates cytokine synthesis seems to vary. In the case of LPS-mediated induction of TNF and IL-1, inhibition occurred at the translational level (Lee *et al.*, 1994). More specifically, inhibition of TNF

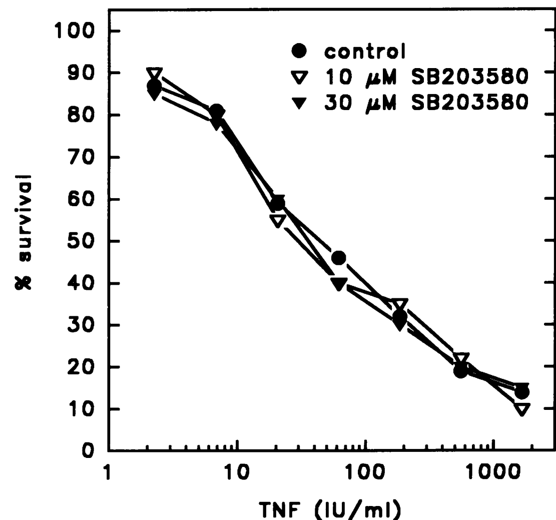

Fig. 7. TNF-induced cytotoxicity is not affected by SB203580. 3×10^4 L929 cells were stimulated for 18 h with a serial dilution of mTNF in the absence (●) or the presence of 10 µM (▽) or 30 µM (▼) SB203580 which was given 2 h before TNF. At the end of the incubation period, cell survival was analysed by an MTT assay (Tada *et al.*, 1986), and calculated as the percentage of the staining value of untreated cultures. SB203580 on its own had no effect on cell viability.

and IL-1 synthesis was shown to be due to inhibition of translation initiation that was regulated through the AUUUA repeated motif in the 3′-untranslated region of the mRNAs (Prichett *et al.*, 1995; and unpublished results). In contrast, our present results show that p38 MAP kinase modulates TNF-induced IL-6 synthesis already at the transcriptional level, since SB203580 almost completely inhibited IL-6 gene transcription. Moreover, TNF-induced activation of the IL-6 promoter or of a minimal promoter containing two NF-κB sites in front of a reporter gene followed by an SV40-derived 3′-untranslated region without AUUUA motifs, could still be prevented completely

by the p38 MAP kinase inhibitor. These results suggest a role for transcriptional activators as targets for the p38 MAP kinase pathway-mediated regulation of IL-6 gene expression.

In the case of the IL-6 promoter, a variety of so-called responsive elements has been proposed (Ray *et al.*, 1990). Previous studies have shown a crucial role for NF-κB as a transcriptional activator for IL-6 after TNF treatment (Libermann and Baltimore, 1990; Zhang *et al.*, 1990). Our present observation that the p38 MAP kinase inhibitor SB203580 still inhibited TNF-induced expression of a reporter gene that is driven by a minimal promoter containing only two κB-binding elements, suggests that p38 MAP kinase is required for NF-κB-mediated transcriptional activation. The most abundant form of NF-κB consists of p50 and p65 heterodimers. NF-κB is located in the cytoplasm of many cell types in an inducible form, in which the heterodimer is complexed to the inhibitory subunit IκB (Rice and Ernst, 1993; Baeuerle and Henkel, 1994; Israël, 1995). Stimulation of cells with TNF or a number of other stress- or infection-dependent triggers leads to rapid phosphorylation of IκB followed by proteolytic degradation (Henkel *et al.*, 1993). The released nucleophilic heterodimer then moves to the nucleus, where it binds to a specific DNA sequence and induces gene transcription. Based on these properties, activation of NF-κB has mostly been analysed by EMSA using a NF-κB-specific oligonucleotide as a probe. However, we could not observe any effect of SB203580 on the TNF-induced DNA binding of NF-κB, suggesting that the role of p38 MAP kinase in NF-κB activation, if any, is at another level. Enhancement of transcription involves in many cases phosphorylation of transactivation domains of transcription factors (Karin, 1994). TNF-induced phosphorylation of p50 and p65 subunits of NF-κB has already been demonstrated (Naumann and Scheidereit, 1994), but its function is still unclear. Although a p38 MAP kinase-controlled phosphorylation of NF-κB subunits might be involved in the modulation of TNF-induced NF-κB activation, immunoprecipitation of p50 and p65 from ^{32}P-labelled L929 cells failed to show a detectable effect of the p38 MAP kinase inhibitor SB203580 on the TNF-induced phosphorylation state of p65, p50 or its p105 precursor. It seems therefore more likely that the p38 MAP kinase pathway regulates TNF-induced IL-6 transcription through phosphorylation of another factor, for example one involved in the transactivation by NF-κB. In this context, it is worth mentioning that the transcription factor ATF-2 has recently been shown to be a substrate for p38 MAP kinase *in vitro* (Dérijard *et al.*, 1995; Raingeaud *et al.*, 1995), while phosphorylation of ATF-2 by another member of the MAP kinase family, *viz.* JNK, has been shown to increase its transcriptional activity (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). Moreover, ATF-2 and other family members can interact with NF-κB in a DNA-independent manner, leading to increased transcriptional activity (Du *et al.*, 1993; Kaszubska *et al.*, 1993), and are therefore possible candidates to mediate the p38 MAP kinase-regulated activation of NF-κB and IL-6 expression. However, although ATF-2 became phosphorylated after TNF treatment, its phosphorylation could not be inhibited by SB203580 (R.Beyaert *et al.*, unpublished observations). This indicates that *in vivo* ATF-2 is phosphorylated not by the p38 MAP kinase pathway, but by another TNF-activated kinase, possibly JNK, and makes an involvement of ATF-2 in the regulation of TNF-induced IL-6 synthesis by the p38 MAP kinase pathway rather unlikely. However, a role for some other ATF-2 family member cannot be excluded. Alternatively, the p38 MAP kinase pathway might modulate TNF-induced, κB enhancer-mediated transcription of IL-6 by interfering with the activity of basal transcription factors or the RNA polymerase complex. Note, however, that the effect of SB203580 is specific for induced genes and is not apparent at the level of total RNA synthesis. The observation that NF-κB is able to interact directly with the TATA-binding protein of the TFIID complex, supports this possibility (Kerr *et al.*, 1993). In addition, it has been shown that transcriptional stimulation through the activation domain of the p65 NF-κB subunit depends on the interaction between the TFIID complex and at least one co-activator, called PC1 (Schmitz *et al.*, 1995a). Further studies on the interactions between NF-κB, co-activators and general transcription factors should elucidate the mechanism by which the p38 MAP kinase pathway affects the activity of NF-κB-driven transcription.

Activation of the p38 MAP kinase pathway might be one of the earliest and possibly the central response after an insult causing stress to the body. Indeed, besides TNF, also IL-1, LPS, heat shock, UV radiation and hyperosmolarity have been shown to activate the p38 MAP kinase pathway (Han *et al.*, 1994; Rouse *et al.*, 1994). Until now, the therapeutic use of TNF has been limited by its many side effects, which often involve the induction of other cytokines, such as IL-6 and IL-1 (reviewed in Fiers, 1995). It is still unclear to what extent the signal(s) going to the nucleus and responsible for gene activation is (are) linked to cellular signals leading to cell death. The present finding that a highly specific inhibitor of the p38 MAP kinase pathway specifically inhibits TNF-induced cytokine expression without interfering with its cytotoxic effect on L929 cells, indicates that at least partially different signal transduction pathways are involved in these processes. Further studies are needed to establish whether TNF-induced synthesis of all deleterious mediators can be blocked by SB203580, and whether the drug does not affect the TNF-induced cytotoxicity in any cell line. If so, SB203580 might provide a new approach to increase the therapeutic potential of TNF as a direct anticancer agent.

Materials and methods

TNF and reagents

Recombinant mTNF and hTNF were produced in *Escherichia coli* and purified to at least 99% homogeneity (Tavernier *et al.*, 1987). The preparations had a specific activity of 1.4×10^8 and 2.3×10^7 IU/mg protein, respectively, as determined in a TNF cytolysis assay, and contained <4 ng endotoxin/mg protein. Reference mTNF (code 88/532) and hTNF (code 87/650) were obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). The pyridinyl imidazole SB203580 originated from SmithKline Beecham and was dissolved in DMSO; the characteristics of that product have been described previously (Lee *et al.*, 1994; Cuenda *et al.*, 1995). Anti-p38 MAP kinase antibodies raised in rabbits against the C-terminal 14 residues of the *Xenopus* p38 MAP kinase homologue XMpk2 (Rouse *et al.*, 1994) were generously provided by Dr A.Nebreda (Imperial Cancer Research Fund, Clare Hall Laboratories, Welwyn, UK). Anti-

hsp27 antibodies were from Stressgen (Victoria, BC, Canada). Anti-p50 (#1163) and anti-p65 (#1226) antibodies were generously provided by Dr N.Rice (NCI-Frederick Cancer Research and Development Center, Frederick, MD), and have been described elsewhere (Rice and Ernst, 1993). Antibodies that immunoprecipitate MAPKAP kinase-2 were raised in sheep in the Scottish Antibody Production Unit (Carluke, Ayrshire, UK) against the peptide MTSALATMRVDYEQIK corresponding to a sequence towards the C-terminus of human MAPKAP kinase-2 (Stokoe *et al.*, 1993).

Cell culture and cytokine assays

L929, HeLa and U937 cells were cultured as described (Beyaert *et al.*, 1991). IL-6 and GM-CSF were assayed on the basis of the proliferative response of 7TD1 and Fdcp1 cells, respectively (Beyaert *et al.*, 1991). In TNF cytotoxicity assays, cell viability was measured by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Tada *et al.*, 1986).

Northern blot analysis of IL-6 expression

Total cellular RNA was prepared with TRIzol reagent (Gibco BRL, Gaitersburg, MD) according to the manufacturer's instructions. RNA (12 μg) was subjected to electrophoresis in 1% formaldehyde agarose gels, transferred to a nylon membrane by capillary blotting and fixed by UV irradiation. Hybridization was carried out at 42°C in 50 mM Tris–HCl, pH 7.4, 40% formamide, 4× SSC (15 mM sodium citrate, 150 mM NaCl), 10× Denhardt's solution, 0.1% $Na_4P_2O_7$, 1% SDS and 200 μg/ml herring sperm DNA. The blots were washed to a stringency of 2× SSC and 0.1% SDS at 42°C, and then exposed to an X-ray film at –70°C. A 655 bp fragment of mIL-6 cDNA was labelled with $^{32}P_i$ using random primers (Boehringer, Mannheim, Germany), and used as a hybridization probe.

Metabolic cell labelling and immunoprecipitation

Cells in a 6-well plate were labelled for 2 h in 0.5 ml phosphate-free growth medium containing 200 μCi $^{32}P_i$/well and then treated with TNF for the indicated time periods. In the case of pretreatment with SB203580, the latter was given at the same time as $^{32}P_i$. At the end of the incubation period, cells were washed in phosphate-buffered saline and cell extracts prepared in lysis buffer consisting of 20 mM Tris–HCl, pH 7.3, 50 mM NaCl, 0.5% (w/v) NP-40, 0.5% sodium deoxycholate, 10 mM NaF, 1 mM Na_3VO_4, 180 μg/ml phenylmethylsulfonyl fluoride, 0.27 trypsin inhibitory units/ml aprotinin and 10 μg/ml leupeptin. Lysates were cleared by centrifugation at 10 000 *g* for 10 min and subjected to immunoprecipitation with the appropriate antisera for 2 h. Precipitates were collected by adsorption to Trisacryl GF-2000 protein A (Pierce, Rockford, IL) for 1 h at 4°C with continuous mixing. The adsorbed immune precipitates were washed four times with 1 ml modified RIPA buffer as described (Beyaert *et al.*, 1990). Adsorbed proteins were solubilized in SDS, boiled and analysed by SDS–PAGE.

Immunoprecipitation and assay of MAPKAP kinase-2

Lysates (20–30 μl containing 50 μg protein) were incubated on a shaking platform for 90 min at 0–4°C with 5 μl of protein G–Sepharose conjugated to 2 μg of MAPKAP kinase-2 antibody. The immunoprecipitates were washed twice with lysis buffer containing 0.5 M NaCl and twice with lysis buffer as such, and MAPKAP kinase-2 was then assayed using the peptide KKLNRTLSVA (Stokoe *et al.*, 1993). One unit of MAPKAP kinase-2 was the amount which incorporated 1 nmol of phosphate into the peptide substrate in 1 min.

CAT-reporter plasmid transfection and CAT-ELISA

The plasmid J16 containing two copies of the NF-κB binding site GGGACTTTCC upstream of a truncated c-*fos* promoter, including only 56 bp upstream from the transcription start site, and linked to the CAT gene, has been described previously (Pierce *et al.*, 1988; Lenardo and Baltimore, 1989). Stable co-transfection of L929 cells with the selection plasmid pSV2neo and a 10-fold excess of the reporter plasmid J16 was performed by the calcium phosphate procedure. Transfected cells were selected in 400 μg/ml G418 for 2 weeks, after which individual colonies were isolated and expanded. These clones were then characterized for CAT expression and TNF inducibility in a CAT-ELISA (5 Prime-3 Prime, West Chester, PA). Preparation of cell extracts and CAT-ELISA were performed according to the manufacturer's instructions, with the exception of a slightly modified blocking buffer (25 g/l casein, 0.3 M NaOH, adjusted to pH 7.0 with HCl) and the inclusion of a final washing step without Tween-20.

EMSA

Subconfluent cells in 6-well plates were stimulated with TNF and collected by scraping. After two washings in phosphate-buffered saline, nuclear extracts were prepared as described by Dignam *et al.* (1983). Equal amounts of protein (5 μg) were then incubated for 30 min with an NF-κB-specific ^{32}P-labelled oligonucleotide 5′-agctATGT*GGGATTTCCC*ATGAGCagct-3′ encompassing the κB motif (italics) from the mIL-6 gene (Tanabe *et al.*, 1988). Binding buffer consisted of 20 mM HEPES, pH 7.5, 60 mM KCl, 4% Ficoll 400, 2 mM DTT, 100 μg/ml poly(dI-dC) and 1 mg/ml bovine serum albumin. Samples were analysed by electrophoresis on a 4% native polyacrylamide gel that was run in 0.5× TBE buffer, pH 8.0. As a negative control, an oligonucleotide mutated in the NF-κB binding site by two point mutations, but otherwise identical with the wild-type fragment, was used; DNA/NF-κB complexes were only observed using the wild-type oligonucleotide.

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