ABSTRACT

Chronic inflammatory diseases often are accompanied by intense angiogenesis, supporting the destructive proliferation of inflammatory tissues. A model of inflammatory angiogenesis is the murine air pouch granuloma, which has a hyperangiogenic component. In this model, we explored the regulation of inflammatory angiogenesis using SB 220025, a specific inhibitor of human p38 mitogen-activated protein (MAP) kinase, with an IC50 value of 60 nM and 50- to 1000-fold selectivity vs. other kinases tested. In vivo, this compound reduced the lipopolysaccharide-induced production of tumor necrosis factor at an ED50 value of 7.5 mg/kg. In the inflammatory angiogenesis model, over the course of granuloma development, we observed elevated levels of interleukin-1β and tumor necrosis factor-α during the chronic inflammatory phase when intense angiogenesis occurs. SB 220025 at 30 mg/kg b.i.d. p.o. was able to greatly reduce the expression of these cytokines and inhibit angiogenesis by ~40%. To further study the effects of p38/CSBP MAP kinase inhibition in angiogenesis-dependent chronic inflammatory disease, SB 220025 was tested in murine collagen-induced arthritis. In this model, SB 220025 was able to prevent the progression of established arthritis. Thus, this p38/CSBP MAP kinase inhibitor, which can reduce inflammatory cytokine production and inhibit angiogenesis, is an effective treatment for chronic proliferative inflammatory disease.

Proliferating tissues require angiogenesis to support their growth, and thus diseases such as cancer and chronic inflammation are thought to be angiogenesis dependent (Folkman, 1995; Jackson, 1996). In the case of chronic inflammation, angiogenesis may be required not only to support the proliferation but also to allow the massive cellular infiltration associated with the chronically inflamed state. Angiogenesis is normally under very tight control. In the normal adult, the majority of the vasculature is stable, with endothelial cell turnover on the order of thousands of days. Nevertheless, these quiescent cells can rapidly switch to an angiogenic phenotype under certain conditions, as, for example, in wound healing. Once a new capillary bed is established, however, the endothelium normally returns to its quiescent state.

Understanding the signals that regulate angiogenesis is key to controlling it under pathological conditions. Growth factors such as VEGF and FGF are clearly able to induce angiogenesis and, in the case of VEGF, appear to be regulated by physiological signals like hypoxia. Hypoxia is not always necessary, however; some inflammatory mediators can potently induce angiogenesis in vivo even in the absence of hypoxia. Both IL-1β and TNF-α can induce angiogenesis in the normally avascular cornea (BenEzra et al., 1990; BenEzra and Mafitzir, 1996; Fajardo et al., 1992). These two cytokines have numerous activities, including upregulation of other cytokines, such as IL-8; upregulation of adhesion molecule expression; stimulation of matrix metalloproteinase expression; and increased prostaglandin production (Dinarello, 1991). Many of these activities may contribute to the angiogenic activity of these cytokines. Thus, inhibition of the activity of IL-1β and TNF-α can have an obvious benefit in angiogenesis-dependent inflammatory diseases. One means of inhibiting IL-1β and TNF-α activity is by decreasing their production. SB 220025 is a new compound belonging to the CSAID™ class of cytokine biosynthesis inhibitors (Cuenda et al., 1995; Lee et al., 1994), which act specifically on p38/CSBP MAP kinase to block a cascade, resulting in decreased production of IL-1β and TNF-α as well as other mediators, such as IL-6 and prostaglandins (Beyaert et al., 1996; Pouliot et
glycogen synthase. The 50-isoforms) and a peptide substrate (PLSRTLSVAAKK) derived from cells.

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was for 20 min at 37°C, and the phosphorylated peptide was isolated on phosphocellulose (p81) and counted as described above.

3-(N-morpholino)propanesulfonic acid, pH 6.5, 10 mM MgCl2, 0.9 mM EGTA, 1.1 mM CaCl2, 40 μg/ml 1-α-phosphatidylysersine, 1 μg/ml 1,3-diolein, 50 μM ATP (with 0.5 μCl of 32P) and 5 μg of peptide substrate. Incubation was for 20 min at 37°C, and the phosphorylated peptide was isolated on phosphocellulose (p81) and counted as described above.

PKA was assayed using the catalytic subunit of PKA (Sigma) and histone H2A as substrate. Reactions of 50 μl contained 50 mM 3-(N-morpholino)propanesulfonic acid, pH 6.5, 10 mM MgCl2, 50 μM ATP (0.5 μCl of 32P), 0.1 mg/ml histone and 1 μM cAMP. Incubation was for 20 min at 37°C, and the phosphorylated peptide was isolated on phosphocellulose (p81) and counted as described above.

PKC was assayed using rat brain cytosol (containing all PKC isozymes) and a peptide substrate (PLSRTLSVAAKK) derived from glycogen synthase. The 50-μl reactions contained 10 mM Tris, pH 7.5, 10 mM MgCl2, 0.9 mM EGTA, 1.1 mM CaCl2, 40 μg/ml 1-α-phosphatidyllysersine, 1 μg/ml 1,3-diolein, 50 μM ATP (with 0.5 μCl of 32P) and 5 μg of peptide substrate. Incubation was for 20 min at 37°C, and the phosphorylated peptide was isolated on phosphocellulose (p81) and counted as described above.

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rErk (p42/44) (Upstate Biotechnology) was assayed using T669 peptide (above) in the same reaction conditions used for p38.

LPS induction of TNF-α. The method of Olivera et al. (1992) was used for LPS induction of TNF-α. Balb/C mice (Charles River Labs, Wilmington, MA) were administered the test compound or vehicle (acidified tragacanth) 30 min before challenge with intraperitoneal injection of 25 μg of LPS (Escherichia coli, type W, 055:B5; Difco, Detroit, MI). After 120 min, blood was collected through exsanguination, and serum samples were used to measure TNF-α levels by ELISA (see below).

Murine air pouch granuloma. This model is based on the methods of Colville-Nash et al. (1995). Female Balb/C mice (20 ± 2 g) were used, and granulomatous tissue was induced in inanesthetized animals (Aerarane; Hanna Pharmaceutical Supply, Wilmington, DE) through the injection of 3 ml of air into the dorsal subcutaneous tissue on day −1, followed by the injection of 0.5 ml of 0.1% v/v croton oil (Sigma) in Freund’s complete adjuvant (Sigma) on day 0. The dosing regimen was started on day 0 and continued until day 5, in conscious animals, with the compound being solubilized in 0.2 ml of N,N-dimethyl acetamide (Sigma)/Cremophor EL (Sigma)/saline or water (10:10:80) (saline was used for intraperitoneal injections, whereas water was used for oral dosing). On the indicated days, the animals were anesthetized and warmed to 40°C for peripheral vasodilation. A vascular cast was made by the intravenous injection of 1 ml of solution of 10% carmine red/5% gelatin solution (Sigma). The animals were chilled to 2°C to 4°C for 3 hr before the removal of the granulomatous tissue.

The removed tissue was weighed, dried at 40°C for 3 days before digestion in 0.9 m of a 0.05 M phosphate buffer, pH 7.0, containing 12 U/ml papain (Sigma) and 0.33 g/liter N-acetyl-L-cysteine (Sigma) for 2 days at 56°C and solubilization of the carmine red with 0.1 ml of 5 mM NaOH. Samples were filtered (0.2 μm), and the carmine content was determined against a carmine standard curve read at 490 nm. Sample and standard values were determined using DeltaSoft ELISA analysis software (Biometallics, Princeton, NJ). The vascular index is the ratio of the milligram of carmine dye per gram of dry tissue. Cedarwood oil clearing was done as described by Colville-Nash et al. (1995), using disected granulomas containing a carmine vascular cast. These were fixed in ethanol and then incubated in cedarwood oil (Sigma) for >14 days, causing the tissue to become translucent and allowing the vasculature to be easily visualized via the carmine dye trapped within the vessels.

Cytokine ELISAs. Tissue extracts were made through homogenization of granulomas in 0.5 ml of 5 mM KH2PO4/0.1 g of wet tissue. IL-1β levels were determined using a Cytoscreen Immunoassay Kit (BioSource International, Camarillo, CA). TNF-α levels were determined using the following assay: plates were coated with hamster anti-murine TNF-α antibody (Genzyme, Cambridge, MA) for 2 hr at 37°C and washed and blocked with a casein/BSA solution (5 g/liter for each) for 1 hr at 37°C, and the samples were added and incubated at 4°C overnight. Plates were washed, and the secondary antibody rabbit anti-mouse TNF-α (Genzyme), was added for 2 hr at 37°C; the plates were washed, and the tertiary antibody goat anti-rabbit peroxidase conjugate (BioSource International, Camarillo, CA) was added for 2 hr at 37°C. The plates were then washed, and substrates OPD (Sigma) was added for 20 min at room temperature. The reaction was terminated with 25 μl of 0.1 M Na2O2, the absorbance was read at 460 nm. Sample values for both ELISAs are calculated using DeltaSoft ELISA analysis software (Biometallics Inc., Princeton, NJ).

Collagen-induced arthritis. The model was described previously (Griwold et al., 1988). Briefly, male DBA/1 LacJ mice (16–18 g; Jackson Labs, Bar Harbor, ME) were primed intradermally with 0.1 ml of an emulsion consisting of equal volumes of Freund’s complete adjuvant and bovine type II collagen at 2 mg/ml in 0.01 N acetic acid. After 21 days, the mice were given an intraperitoneal booster of 0.1 ml of 1 mg/ml bovine type II collagen in 0.01 N acetic acid without adjuvant. After the booster, mice were evaluated daily for incidence and severity of arthritis in their limbs. Scores of 0 to 4 for each limb were determined subjectively, in which 0 equals noninvolved and 4 equals the greatest severity of erythema and swelling. The maximum possible score for an arthritic mouse was 16 (4 points/limb). When the severity score reached 4, which was typically within 1–2 weeks of the collagen booster, mice were randomly assigned to vehicle or drug-treated groups, and oral dosing with SB 220025 or vehicle alone began. This day was designated day 0, and dosing continued for 10 days. Mice were evaluated, with no attention paid to their group, on days 7 and 10. All scoring was done by the same scorer as previously described (Griwold et al., 1988).

Results

SB 220025 selectively inhibits p38 MAP kinase. The pyrimidyl imidazole compounds as exemplified by SB 203580 have been previously demonstrated to specifically inhibit p38 (Cuenca et al., 1995; Lee et al., 1994; Young et al., 1997). SB 220025, a novel member of this structural class (fig. 1), was tested in a number of kinase assays to assess its use as a p38 inhibitor (table 1). This compound inhibited p38 phosphorylation of an EGFR peptide substrate with an IC50 value of 60 nM, which is 10-fold more potent than SB 203580 (IC50 = 0.6 μM; Cuenca et al., 1995). In selectivity assays, p38 inhibition by SB 220025 was >1000-fold selective over Erk (p42/p44 MAP kinase), 500-fold selective over PAK, >1000-fold selective over EGFR and 50-fold selective over PKC.
SB 220025 inhibits inflammatory cytokine production in vivo. To examine the in vivo efficacy of SB 220025, an acute model of LPS-induced TNF-α expression was used. SB 220025 at a range of doses from 3 to 50 mg/kg was given to mice orally 30 min before challenge with LPS. Serum TNF-α was measured by ELISA after 2 hr. This compound dose-dependently inhibited TNF-α production with an ED50 value of 7.5 mg/kg (fig. 2). Greater than 80% inhibition was obtained at 50 mg/kg. Thus, SB 220025 is an orally available, potent inhibitor of TNF-α synthesis.

SB 220025 inhibits angiogenesis in the murine air pouch granuloma model. The inflammatory cytokines TNF-α and IL-1β are potent inducers of angiogenesis. To test whether they are involved in the promotion of angiogenesis in chronic inflammation, we used the cytokine-suppressive p38/CSBP MAP kinase inhibitor SB 220025 in a model of inflammatory angiogenesis. The murine air pouch granuloma has been characterized as a chronic inflammatory progression with a profound angiogenic component (Colville-Nash et al., 1995). It provides a model in which modulation of angiogenesis in an inflammatory bed can be quantified. Granulomas were formed in a 3-ml dorsal subcutaneous air pouch by injection of 0.5 ml of Freund’s complete adjuvant and croton oil. Within 3 days, a cohesive granulomatous tissue encased the adjuvant mixture. The granulomas were evaluated by weight, histology and vascular index (mg of carmine dye/g of dry tissue), which was used to assess the extent of angiogenesis.

Using a range of doses, we analyzed the effect of SB 220025 on granuloma size and vascular index on day 6. This time point was chosen because it allows sufficient time for angiogenesis and the development of chronic inflammatory character but occurs before the onset of fibrotic features (Colville-Nash et al., 1995; Jackson et al., 1997). The compound caused a dose-dependent reduction in angiogenesis as measured by the vascular index of the granuloma (fig. 3). The maximum effect was a 44% reduction at 50 mg/kg. This is similar to the maximum effect we obtained with a positive control, the angiostatic steroid medroxyprogesterone (fig. 3), which was chosen for its well-documented antiangiogenic activity (Gross et al., 1981), lack of anti-inflammatory activity and consistent pharmacology in our experience with this model. Neither SB 220025 nor the angiostatic steroid had an effect on granuloma size (dry weight).

Effect of SB 220025 on the time course of angiogenesis. We evaluated the effect of the p38/CSBP inhibitor at several time points to determine whether its effects would be different at the various stages of inflammatory and angiogenic progression.
genic progression. SB 220025 was given orally, at an intermediate dose of 30 mg/kg twice a day starting on day 0, and granulomas were evaluated on days 3, 5, 7 and 14. Granuloma size remained fairly constant and was unaffected by the SB 220025 (fig. 4). The vascular index of the control group rose gradually from day 3 to 14, whereas the vascular index of the treated group remained constant. At day 3, the compound did not cause a significant reduction in vascular index compared with control; however, at days 5, 7 and 14, the vascular index was lowered significantly by SB 220025. Thus, the p38/CSBP MAP kinase inhibitor did not affect the initial burst of angiogenesis but did prevent the increase in angiogenesis that occurs after day 3.

Inflammatory cytokines such as IL-1β and TNF-α have been implicated in the pathogenesis of angiogenesis in chronic inflammation, and p38 inhibitors, such as SB 220025, have been demonstrated to inhibit the synthesis of these cytokines. We measured the levels of these cytokines over the course to granuloma development to determine whether the modulation of their expression by SB 220025 correlated with inhibition of angiogenesis. Cytokine levels were measured by ELISA using homogenates of granuloma tissue. TNF-α levels rose sharply, peaking at day 7 and dropping back down to moderate levels by day 14 (fig. 4). SB 220025 greatly reduced TNF-α levels at day 7. IL-β levels were also high in control granulomas, peaking at day 7, and as with TNF-α, the p38/CSBP MAP kinase inhibitor effectively blocked the increased IL-β expression. Thus, the ability of SB 220025 to block the sharp rise in TNF-α and IL-β between days 5 and 7 correlated well with the ability of the compound to prevent the increase in vascular index that occurs over the same time points.

Microscopic analysis angiogenesis in the granuloma. Angiogenesis in the granuloma was microscopically evaluated using cedarwood oil clearing. Figure 5 shows the vasculature of day 6 granulomas from both untreated and SB 220025-treated mice. The profound angiogenesis in the granuloma is demonstrated by the extensive vascular network in the control tissue. There was a striking reduction in the vasculature of the treated tissue. The fine capillaries seen in the control tissue were completely absent in the treated tissue, and only a few larger vessels remained visible.

Effect of SB 220025 on chronic inflammatory disease. The anti-inflammatory and antiangiogenic activities of SB 220025 suggest that it would provide an effective treatment in chronic inflammatory diseases such as rheumatoid arthritis, which has both inflammatory cytokine and angiogenic components. Thus, we tested SB 220025 in a chronic inflammatory disease model, murine collagen-induced arthritis. Mice were primed with bovine collagen, and 3 weeks later, the animals were given intraperitoneal injections of soluble collagen and monitored for the appearance of arthritis. Dosing began after arthritis was evident, usually between days 7 and 14 after collagen boost. The first day of dosing was designated day 0. Animals treated with SB 220025 (50 mg/kg p.o. b.i.d.) had no increase in severity of arthritis over 10 days, whereas the severity of arthritis in the control mice was increased at days 7 and 10 (fig. 6). Thus, the p38/CSBP MAP kinase inhibitor effectively blocked the progression of arthritis.

Discussion

Proinflammatory cytokines such as IL-1β and TNF-α have been shown to play a central role in many inflammatory processes (Dinarello, 1991). This study demonstrates the importance of IL-1β and TNF-α in chronic inflammatory angiogenesis and arthritis. Angiogenesis is a normal physiological response in wound healing, but in diseases such as rheumatoid arthritis and psoriasis, it can take on a pathological role. The association between angiogenesis and chronic inflammation has led to the hypothesis that angiogenesis is induced by inflammatory events. Indeed, it has been shown that IL-1β can induce angiogenesis in the normally avascular cornea (BenEzra et al., 1990; BenEzra and Maftzir, 1996; Fajardo et al., 1992). We evaluated the role of these cytokines in inflammatory angiogenesis in vivo by using a murine air pouch granuloma model. Both IL-1β and TNF-α levels in the granuloma tissue increased sharply over the first 7 days of granuloma formation, the same time period in which angiogenesis was very active.

We modulated the activity of IL-1β and TNF-α using the p38/CSBP inhibitor SB 220025, which inhibits their synthesis. This compound is more potent than the previously reported p38 inhibitor SB 203580. We observed an ED₅₀ value of 75 mg/kg for LPS-induced serum TNF-α production, which is twice as potent a value as that reported for SB 203580 (Badger et al., 1996). SB 220025 caused a significant dose-dependent decrease in the vascular density of the granuloma, and this correlated with decreases in IL-1β and TNF-α levels. The hypothesis is that decreasing IL1 and TNF-α levels resulted in inhibition of angiogenesis in an inflammatory tissue bed.

When we analyzed a time course of granuloma development, we observed that the control group granuloma size, as measured by dry weight, increased dramatically from day 0 to 3 and then was steady from day 3 to 14. In contrast, the control group vascular index increased steadily from day 3 to
Granuloma size was not decreased by inhibition of angiogenesis with SB 220025. This was not surprising because granuloma growth is not angiogenesis dependent in this air pouch model (Colville-Nash et al., 1995; Jackson et al., 1997). The model provides an in vivo system for the study of hyperrangiogenesis in an inflammatory tissue but is not a model of inflammatory disease. To test the effect of SB 220025 in a model of rheumatoid arthritis, an angiogenesis-dependent chronic inflammatory disease, we used murine collagen-induced arthritis. Using a therapeutic dosing regimen, in which dosing did not begin until there was evidence of arthritic joint disease, SB 220025 was able to prevent further increases in the severity of arthritis. Thus, an inhibitor of IL-1β/TNF-α synthesis and angiogenesis was a very effective treatment for arthritis. This agrees with other studies that demonstrated that TNF-α antibodies (Piguet et al., 1992) were an effective treatment for collagen arthritis and that the angiogenesis inhibitor AGM-1470 was able to reduce the severity of collagen-induced arthritis in rats (Peacock et al., 1992). Interestingly, in a study of other anti-inflammatory drugs (Griswold et al., 1988), the nonsteroidal anti-inflammatory drug ibuprofen was not particularly effective in this model, further suggesting that the anticytokine and antiangiogenic properties of SB 220025 are key to its antiarthritic activity.

Although inhibition of IL-1β and TNF-α synthesis is strongly implicated to be responsible for the antiangiogenic and antiarthritic activities of SB 220025, it is possible that inhibition of the synthesis of other cytokines also may be involved. Other factors, such as the inducible cyclooxygenase, IL-6, IL-8 and GM-CSF, also are regulated by p38/CSBP MAP kinase (Beyaert et al., 1996; Lee et al., 1988, 1989, 1993; Pouliot et al., 1997) and thus may be affected by SB 220025. However, IL-1β and TNF-α are reported to have more potent...
angiogenic activities than eicosanoids and these other cytokines, and the most effective antiangiogenic activity of SB 220025 on days 3 and 5 of granuloma development correlated well with inhibition of IL-1β and TNF-α synthesis. It is important to note that p38 inhibitors such as SB 203580 and SB 220025 also affect the signaling pathways of these cytokines and thus may work via inhibition of both cytokine synthesis and action (Badger et al., 1996; Cuenda et al., 1995). In addition, although it is a very selective inhibitor of p38 MAP kinase and we are unaware of any other activities that could account for its pharmacology, it is possible that SB 220025 may also inhibit an as-yet-unidentified kinase. Therefore, in vivo data should be interpreted with normal caution.

The association between inflammation and angiogenesis has long been observed, but until recently there has been little evidence to clearly demonstrate the link. This study shows that angiogenesis is dependent on inflammatory cytokines in a chronic inflammatory model. It is not clear whether inflammatory cytokines are involved in other angiogenesis-dependent processes, such as tumor growth, and this remains to be tested. From our studies and others (Badger et al., 1996), it is apparent that p38/CSBP MAP kinase inhibition should provide an effective treatment for chronic proliferative inflammatory diseases.

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