p38 Mitogen-Activated Protein Kinase Is Required for the Antitumor Activity of the Vascular Disrupting Agent 5,6-Dimethylxanthenone-4-acetic Acid

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ABSTRACT

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a potent vascular disrupting agent, selectively destroys established tumor vasculature, causing a rapid collapse in blood flow that ultimately leads to inhibition of tumor growth. Here, we demonstrate that p38 MAPK is critically involved in DMXAA-induced cytoskeleton reorganization in endothelial cells and tumor necrosis factor-α (TNF-α) production in macrophages, both of which were essential for DMXAA-induced vascular disruption. Inhibition of p38 mitogen-activated protein kinase (MAPK) significantly attenuated DMXAA-induced actin cytoskeleton reorganization in human umbilical vein endothelial cells and TNF-α production in macrophages. In vivo, p38 MAPK inhibition attenuated the immediate reduction in tumor blood flow induced by DMXAA treatment (<30 min) by inhibiting actin cytoskeleton reorganization in tumor vascular endothelial cells and blunted the long-lasting (>4 h) DMXAA-induced shutdown of the tumor vasculature by inhibiting intratumoral TNF-α production. These results indicate that p38 MAPK plays a critical role in DMXAA-induced endothelial cell cytoskeleton reorganization and TNF-α production, thus regulating DMXAA-induced antitumor activity.

Introduction

The tumor vasculature represents a promising target for cancer therapy. Vascular disrupting agents (VDAs) are a new class of potential antitumor drugs that selectively destroy the established tumor vasculature, shut down blood supply, deprive tumors of oxygen and nutrition, and cause extensive tumor necrosis (Chaplin and Dougherty, 1999; Thorpe et al., 2003; Lippert, 2007). There are two types of VDAs: small molecules and ligand-directed agents. Currently, most small molecule VDAs are tubulin inhibitors, such as combretastatin A-4P and N-acetylcoccolin-O-phosphate (ZD6126); the remainder are synthetic flavonoids, including flavone acetic acid and 5,6-dimethylxantheonone-4-acetic acid (DMXAA) (Cai, 2007).

DMXAA, also known as ASA404, is a flavone acetic acid derivative that acts as an effective VDA. DMXAA causes a rapid and remarkable collapse in blood flow within 30 min after administration and leads to complete vascular shutdown later (Tozer et al., 2005). Clinical studies have confirmed the antivascular effect, demonstrating an increase in tumor vascular permeability (Zhao et al., 2005), changes in tumor perfusion (Murata et al., 2001), and increased plasma concentrations of the serotonin metabolite 5-hydroxyindole acetic acid (Zhao et al., 2002b). DMXAA is thought to exert its antitumor effects through both induction of apoptosis in tumor vascular endothelial cells (Ching et al., 2002) and an indirect vascular effect involving production of a spectrum of cytokines and chemokines, including tumor necrosis factor-α (TNF-α) (Wang et al., 2004), interferon-β (Shirey et al., 2011), interferon-inducible protein 10 (Cao and Ching, 2001), and inducible nitric oxide synthase (Peng et al., 2011). It has been reported that TNF-α is an important cytokine involved in mediating the antitumor effects of DMXAA (Ching et al., 1999). In situ hybridization studies of the murine colon 38 tumor have shown that the extent of DMXAA-induced TNF-α synthesis is greater in tumors than in the spleen, liver, or serum (Joseph et al., 1999). More importantly, previous results have demonstrated that mice lacking genes for TNF or...
TNF receptor-1 (Ching et al., 1999) exhibit a partially diminished tumor-regression response to DMXAA treatment.

DMXAA completed Phase I clinical trials in New Zealand and the United Kingdom, showing good tolerance (Rustin et al., 2003). A Phase II clinical trial that tested DMXAA in combination with carboplatin and paclitaxel against non-small-cell lung cancer demonstrated a greater tumor response rate, a reduction in median time to tumor progression, and improved median survival (McKeage et al., 2009). However, a Phase III trial in 2010 showed no survival benefit of DMXAA combination therapy in patients with late-stage lung cancer. Given the effectiveness of DMXAA as a VDA, efforts to elucidate novel mechanisms of action of this agent and explore new administration approaches remain of paramount importance.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases involved in regulating a variety of cellular processes, including inflammation, cell growth and survival, and cellular differentiation (Chang and Karin, 2001). Three kinds of MAPKs have been identified: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Of these, p38 MAPK seems to be involved in the production of cytokines by stimulated monocytes (Saklatvala, 2004). Inhibitors of p38 MAPK have been reported to block lipopolysaccharide-induced TNF-α production at both transcriptional (Manthey et al., 1998) and post-transcriptional (Young et al., 1993) levels. p38 MAPK has also been shown to regulate actin microfilament dynamics via activation of mitogen-activated protein kinase-activated protein kinase (MAPKAPK) 2/3 and phosphorylation of heat shock protein 27 (Freshney et al., 1994; Rouse et al., 1994) in endothelial cells, resulting in stress fiber formation. Previous research has also provided evidence that p38 MAPK is necessary for combretastatin A4-induced endothelial cell blebbing (Kanthou and Tozer, 2002) and is involved in the activation of DMXAA (Zhao et al., 2007). It has been reported that MAPKs, especially p38 MAPK, play an important role in regulation of both TNF-α and interleukin (IL)-6 protein production induced by DMXAA in murine macrophages at the post-transcriptional level (Sun et al., 2011). However, the direct roles of p38 MAPK in the in vivo antitumor activity of DMXAA, especially in the DMXAA-induced tumor vascular shutdown, are still not known.

In this study, we examined the role of p38 MAPK in the antitumor activity of DMXAA. In vitro, p38 MAPK was critically involved in DMXAA-induced actin cytoskeleton reorganization in human umbilical vein endothelial cells (HUVECs) and TNF-α production in macrophages. Likewise, in vivo inhibition of p38 MAPK significantly attenuated the tumor vascular shutdown by inhibiting the rapid DMXAA-induced endothelial cell cytoskeleton reorganization and subsequent TNF-α production. These results demonstrate that p38 MAPK is required for DMXAA-induced disruption of the tumor vasculature.

**Materials and Methods**

**Reagents.** DMXAA and N-[(2R)-3,4-difluoro-2-(2-fluoro-4-iodophenylamino)-benzamide (PD0325981) were synthesized at Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). 4,4-Fluorophenyl-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190), 1,9-pyrazoloanthrone (SP600125), Hoechst 33342, and monoclonal antibodies specific for β-tubulin were obtained from Sigma-Aldrich (St. Louis, MO). Endothelial cell growth supplement was purchased from Invitrogen (Carlsbad, CA). Monoclonal antibodies to phospho-ERK1/2, ERK1/2, phospho-p38 MAPK, p38 MAPK, phospho-MAPKAPK-2, and phospho-myosin light chain (MLC) were from Cell Signaling Technology (Danvers, MA). Alexa Fluor 594-conjugated phalloidin was from Invitrogen. Mouse TNF-α neutralizing antibody and normal goat IgG were from R&D Systems Europe (Abingdon, Oxfordshire, UK).

**Cell Lines.** The murine macrophage cell line, Ana-1, was obtained from the Cell Bank of the Shanghai Institute for Biological Sciences, Chinese Academy of Science. Ana-1 was cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum. HUVECs were isolated from umbilical cord veins using collagenase, as described previously (Jaffe et al., 1973). In brief, cells were detached by treatment with 0.25% type I collagenase, seeded on gelatin-coated culture plates, and maintained in M199 medium containing 10% fetal bovine serum, 20 μg/ml endothelial cell growth supplement, and 50 μg/ml heparin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. HUVECs were passaged 2 to 6 times before using in experiments.

**Endothelial Cell Morphological Examination.** Changes in endothelial cell shape were examined using proliferating HUVECs. HUVECs were plated in six-well plates preincubated with 1% gelatin. Cells were grown for at least 24 h before treatment. Photographs were taken using an Olympus fluorescence microscope (Olympus America, Inc., Center Valley, PA). For quantitation of cell area, images were acquired from randomly selected areas of slides and then were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD).

**F-Actin Staining.** HUVECs were plated on glass coverslips 24 h before performing experiments. After DMXAA treatment with or without inhibitors, as indicated in the text, cells were washed with phosphate-buffered saline (PBS) and then fixed for 20 min in 4% paraformaldehyde/PBS. Alexa Fluor 594-conjugated phalloidin (diluted 1:100 in 2% bovine serum albumin/PBS) and 4',6-diamidino-2-phenylindole (0.1 μg/ml) were used to stain F-actin and nuclei, respectively. After extensive washes, cells were mounted and visualized by fluorescence microscopy.

**Western Blotting.** After drug treatment, cells were washed twice with ice-cold PBS and lysed in SDS sample buffer. Cell lysates containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. After blocking in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20, membranes were incubated at 4°C overnight with the appropriate primary antibodies and then were exposed to secondary antibodies for 2 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA).

**TNF-α Determination.** Removed tumor tissues were homogenized in culture medium using a tissue homogenizer. The homogenates were centrifuged (2000 g, 30 min, 4°C), and supernatants were normalized in culture medium using a tissue homogenizer. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. After blocking in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20, membranes were incubated at 4°C overnight with the appropriate primary antibodies and then were exposed to secondary antibodies for 2 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA).

**RNA Interference.** Ana-1 cells were transfected with small interfering RNA (siRNA) duplexes (100 nM) against p38 using the TurboFect siRNA transfection reagent (Fermentas, St. Leon-Roth, Germany), as described by the manufacturer. The cells were then allowed to recover for 72 h before stimulation. The siRNA sequence targeting mouse p38a (NM_011951), synthesized by Genepharm (Shanghai, China), spans nucleotides 481 to 499 (target sequence, 5'-GAC TGT GAG CTC AAG ATT C-3') and is specific for mouse p38 MAPK according to a Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information database. The sense p38-siRNA sequence was 5'-GAC UGU GAG CUC AAG AUU Ctt-3', and the antisense sequence was 5'-GAA UCU UGA GCU CAC...
DMXAA induces morphological changes and stimulates actin stress fiber formation via p38 MAPK activation in endothelial cells. A and B, HUVECs were exposed to 500 μM DMXAA for 30 min with or without pretreatment with 20 μM SB202190, 20 μM PD0325901, or 20 μM SP600125 for 30 min. Cells were photographed under an inverted microscope. The average cell-covered area was quantified from four randomly sampled microscope fields using Image-Pro Plus 6.0. C, HUVECs were pretreated with vehicle, 20 μM SB202190, or 20 μM PD0325901 for 30 min and then were exposed to 100 μM DMXAA for 30 min. F-actin was stained with fluorescein isothiocyanate-phalloidin. Cells were photographed under a fluorescence microscope. D, HUVECs were exposed to 100 μM DMXAA for the indicated periods of time. E, HUVECs were preincubated with or without 20 μM SB202190 for 30 min before DMXAA exposure. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. Data shown are representative of three independent experiments.

Results

p38 MAPK Activation Participates in DMXAA-Induced Morphological Changes and Actin Stress Fiber Formation in HUVECs. Because endothelial cells lining tumor blood vessels are the main targets for VDAs, such as combretastatin A4-P, we were interested in examining whether DMXAA had an effect on endothelial cell morphology. HUVECs rapidly rounded up and retracted from each other after treatment with 500 μM DMXAA for 5 min (Fig. 1A), morphological changes that lasted for up to 4 h (data not shown). To investigate the signaling pathways involved in these morphological changes, we pretreated cells with inhibitors of ERK (PD0325901), p38 (SB202190), or JNK (SP600125) before exposing them to DMXAA. Only pretreatment with SB202190 reversed DMXAA-induced morphological changes, determined by HUVEC morphology and the time course of changes in HUVEC cell area; PD0325901 and SP600125 were without effect (Fig. 1, A and B). These results suggest that p38 MAPK, but not ERK or JNK, is required for the antitumor activity of DMXAA.
critically involved in mediating the effects of DMXAA on HUVEC morphology.

Cell morphological changes are associated mainly with alterations in the cell cytoskeleton, including actin and microtubules. Accordingly, we next examined whether the HUVEC cytoskeleton was affected by DMXAA treatment. Compared with control cells, there was a significant thickening of actin bundles and an increase in the formation of stress fibers across HUVECs after treatment with 100 μM DMXAA for 30 min (Fig. 1C), whereas microtubules were not affected (data not shown). Furthermore, pretreatment with the p38 MAPK kinase inhibitor SB202190, but not the ERK1/2 inhibitor PD0325901, significantly attenuated DMXAA-induced actin cytoskeleton reorganization (Fig. 1C), indicating that p38 MAPK is required for the DMXAA-induced actin cytoskeleton reorganization that leads to changes in HUVEC morphology.

As expected, DMXAA significantly stimulated the activation of p38 MAPK, but not ERK1/2, as early as 5 min in HUVECs (Fig. 1D). MLC, which regulates actin stress fiber assembly (Smith et al., 1988), was also phosphorylated at Thr18 and Ser19 after treatment with DMXAA for 15 min (Fig. 1D). Furthermore, pretreatment with the p38 MAPK inhibitor SB202190 significantly inhibited DMXAA-induced phosphorylation of both p38 MAPK and MLC (Fig. 1E), suggesting that DMXAA-stimulated p38 MAPK activation is upstream of MLC phosphorylation in HUVECs.

**p38 MAPK Activation Is Essential for TNF-α Production in Ana-1 Murine Macrophages after DMXAA Exposure.** Several studies have shown that TNF-α, secreted from macrophages, is an important regulator of the antitumor activity of DMXAA. Thus, we tested whether p38 MAPK was also activated in murine Ana-1 macrophages during DMXAA treatment. As shown in Fig. 2A, p38 MAPK activation in Ana-1 cells was observed as early as 30 min after DMXAA treatment (Fig. 2A). MAPKAPK-2, a downstream effector of p38 MAPK, was also phosphorylated 30 min after exposure to DMXAA (Fig. 2A). These results raise the possibility that DMXAA stimulation of p38 MAPK activation is not cell-type-specific. In addition, ERK1/2 phosphorylation was also slightly increased after DMXAA treatment for 30 min in Ana-1 macrophages (Fig. 2A).

To explore whether DMXAA-induced p38 MAPK activation was related to TNF-α production in Ana-1 cells, we pretreated cells with the p38 MAPK inhibitor SB202190 before exposure to DMXAA. Figure 2B shows that DMXAA-stimulated TNF-α production was significantly reduced (~60%) by SB202190 pretreatment for 30 min (Fig. 2B) and was slightly affected by pretreatment with the ERK1/2 inhibitor PD0325901 (~20%), but was unaffected by the JNK inhibitor SP600125 (data not shown), suggesting that MAPKs, especially p38 MAPK, participate in DMXAA-induced TNF-α production in Ana-1 cells.

To confirm the role of p38 MAPK in TNF-α production, we used a more specific approach, using siRNA against p38 MAPK. As shown in Fig. 2C, transfection of Ana-1 cells with p38 siRNA significantly reduced p38 MAPK protein expression as well as DMXAA-induced TNF-α production, confirming that p38 MAPK is a critical mediator of DMXAA-induced TNF-α production in macrophages.

**p38 MAPK Activation Is Critically Involved in DMXAA-Induced Tumor Vascular Disruption In Vivo.** The role of p38 MAPK in mediating DMXAA-induced antitu-
Tumor activity was examined in vivo using an established Lewis lung cancer model in C57 mice. Tumor fragments were implanted in C57 mice and allowed to grow to 10 mm in diameter before DMXAA administration. Consistent with previous reports (Joseph et al., 1999), treatment of C57 mice with DMXAA at a dose of 20 mg/kg induced a time-dependent production of intratumoral TNF-α, which increased from undetectable levels at 30 min and 2 h to reach significant levels at 4 h (Fig. 3A). In contrast, increased levels of p38 MAPK phosphorylation in tumor tissues were detected as early as 30 min after DMXAA treatment (Fig. 3B), indicating that DMXAA-stimulated p38 MAPK activation precedes TNF-α production in tumor tissues.

To investigate the vascular disrupting effect of DMXAA, we visualized blood-perfused vessels in tumor cryosections as revealed by surrounding Hoechst 33342-labeled cells. Consistent with previous reports, tumor blood flow was markedly reduced as early as 30 min after DMXAA administration compared with normal saline treatment (Fig. 4A). Considering that the TNF-α level was not affected within 30 min after DMXAA administration (Figs. 3A and 4B), these results clearly exclude the possibility that intratumoral TNF-α production is responsible for the rapid early stage of DMXAA-induced tumor blood flow reduction.

The aforementioned in vitro results demonstrate that DMXAA induces rapid actin cytoskeleton reorganization in endothelial cells, an effect that is mediated by p38 MAPK activation. Accordingly, we next used the p38 MAPK inhibitor SB202190 to examine whether p38 MAPK participated in the rapid reduction in tumor blood flow induced in vivo by DMXAA. As shown in Fig. 4, A and B, SB202190 pretreatment significantly attenuated the DMXAA-induced reduction in tumor blood flow without affecting intratumoral TNF-α production. Given that endothelial cells lining tumor blood vessels are the main targets for VDAs, these results suggest that DMXAA-induced, p38 MAPK-mediated rapid reorganization of the cytoskeleton in endothelial cells may be responsible for the rapid reduction of tumor blood flow.

We next addressed the role of p38 MAPK in the later, sustained component of DMXAA-induced blood flow reduction. SB202190 pretreatment dramatically reversed the reduction in tumor blood flow induced by 4-h DMXAA administration and inhibited DMXAA-induced p38 MAPK phosphorylation (Fig. 4, A and C). Because a large amount of intratumoral TNF-α has been produced at this time point (Figs. 3A and 4B), we determined whether SB202190 inhibited TNF-α production in tumor tissues as it did in Ana-1 macrophage cells. As expected, SB202190 significantly inhibited the induction of intratumoral TNF-α production by 4-h DMXAA treatment (Fig. 4B), indicating that TNF-α production is also mediated by p38 MAPK. To further test the role of TNF-α production in the late-stage (4 h) vascular-disrupting effect of DMXAA, we used an anti-TNF-α neutralizing antibody. The anti-TNF-α antibody, which effectively neutralized intratumoral TNF-α (Fig. 5B), partially reversed the reduction in tumor blood flow induced by DMXAA treatment for 24 h (Fig. 5A), whereas a nonspecific IgG did not, raising the possibility that intratumoral TNF-α production plays an essential role in maintaining the DMXAA-induced reduction in tumor blood flow. Collectively, the results of in vivo experiments suggest that DMXAA-induced tumor vascular disruption may be a two-step process: 1) a rapid reduction in tumor blood flow induced by endothelial cell cytoskeleton reorganization and 2) a sustained decrease in blood flow maintained by intratumoral TNF-α production. In both cases, p38 MAPK is the key regulator and thus mediates DMXAA-induced disruption of the tumor vasculature.

p38 MAPK Activation Is Required for DMXAA-Induced Tumor Necrosis In Vivo. Finally, we determined the fate of tumor cells after DMXAA treatment. Mice bearing Lewis lung cancer tumors were treated with 20 mg/kg DMXAA for 24 h, after which tumor tissue was fixed, sectioned, and stained with hematoxylin and eosin. The necrotic area of tumors was expressed as percentage of total area. As shown in Fig. 6, A and B, extensive necrotic areas within tumors were observed in mice treated with DMXAA for 24 h; in contrast, untreated mice showed large areas of viable cells without significant necrosis. SB202190 treatment alone had no effect on cell viability in tumor tissues; however, it remarkably decreased DMXAA-induced tumor necrosis, demonstrating the critical role of p38 MAPK in DMXAA-induced necrosis in vivo.

**Discussion**

DMXAA is a unique VDA with a mechanism distinct from that of other known VDAs, such as the microtubule-disrupting agent combretastatin A4-P and its derivatives. In this study, we explored the mechanism by which DMXAA disrupts the tumor vasculature. We found that DMXAA induced actin cytoskeleton reorganization in endothelial cells and TNF-α production in macrophage cells, with both effects being mediated by p38 MAPK. More importantly, these in vitro activities were translated into the in vivo antitumor effects of...
In this study, we suggest for the first time that DMXAA-induced tumor vascular disruption consists of at least two stages: an early stage (<30 min) in which tumor blood flow is rapidly reduced because of endothelial cell cytoskeleton reorganization and a late stage marked by a sustained decrease in blood flow mediated by intratumoral TNF-α production. Notably, both effects contribute to DMXAA-induced vascular disruption. Most importantly, p38 MAPK plays an essential role in both DMXAA-induced endothelial cell cytoskeleton reorganization and TNF-α produc-

Fig. 4. Inhibition of p38 MAPK attenuates both early and late-stage blood flow reduction after DMXAA administration in vivo. A, C57 mice bearing Lewis lung carcinomas were treated with DMXAA (20 mg/kg) for 30 min or 4 h with or without coadministration of SB202190 for 30 min and then were perfused in vivo with Hoechst 33342 (800 μg/mouse). One minute later, tumor tissues were harvested and cryosectioned. Images of tumor tissue cryosections were obtained by fluorescence microscopy. Fluorescence intensity was quantified in eight randomly selected microscope fields using Image-Pro Plus 6.0. B, tumor tissues were homogenized, and TNF-α in the supernatant after centrifugation was assayed by ELISA and expressed as picograms per gram of tissue. Data are presented as mean ± S.E.M. (**, p < 0.01, n = 5). C, Western blot of tumor tissue extract using antibodies specific for phospho-p38 MAPK and total p38 MAPK. C57 mice bearing Lewis lung carcinomas were treated with DMXAA (20 mg/kg) for 4 h with or without coadministration of SB202190.
tion and is thus a critical mediator of the antitumor activity of DMXAA.

Endothelial cells lining tumor blood vessels are the main targets for VDAs, such as combretastatin A4-P and its derivatives. Combretastatin A4-P induces rapid microtubule depolymerization, which stimulates MLC phosphorylation and thus induces actin cytoskeleton reorganization and endothelial cell contraction, leading to a rapid increase in vascular resistance and reduced tumor blood flow in vivo (Galbraith et al., 2001). In the current study, we also suggest that the rapid shutdown of tumor blood flow induced by DMXAA results from its action on the endothelial cell cytoskeleton. This conclusion is based on several lines of evidence. First, DMXAA induced rapid actin stress fiber formation and cytoskeleton reorganization in HUVECs through p38 MAPK activation. Second, previous investigations of the pharmacokinetics of DMXAA in colon 38 tumor-bearing mice (Zhao et al., 2002a) showed that after administration of DMXAA at a dose of 25 mg/kg, the maximal plasma concentration of DMXAA was 530 μg/mL, which is sufficient for DMXAA to induce rapid actin cytoskeleton reorganization in endothelial cells. Third, the rapid reduction in tumor flow induced by DMXAA occurred much earlier than the intratumoral production of TNF-α, and the rapid blood flow reduction was evident as early as 30 min after DMXAA administration, a time at which TNF-α production could not be detected. Thus, although we do not have direct evidence that DMXAA induces rapid endothelial cell cytoskeleton reorganization in vivo, these observations collectively indicate that the rapid shutdown of tumor blood flow induced by DMXAA results from the effect of DMXAA on endothelial cell cytoskeleton, as is the case for combretastatin A4. This may also be why DMXAA has been explored as a VDA.

A number of early studies reported that, in vivo, DMXAA induces a prompt and irreversible shutdown of blood flow that is not recovered by 24 h, whereas the disruption in blood flow induced by combretastatin A4 disruption recovers within 24 h (Siim et al., 2003). This suggests that although DMXAA and other antitubulin VDAs, including combretastatin A4, share stimulation of actin cytoskeleton reorganization and morphological change as common mechanisms for inducing the rapid shutdown of tumor blood flow, the mechanisms underlying the sustained reduction in blood flow are different. Our study indicated that the DMXAA-induced sustained reduction of tumor blood flow is associated with significant intratumoral TNF-α production. More importantly, the DMXAA-induced sustained shutdown of tumor blood flow was blocked by inhibiting TNF-α production using a neutralizing TNF-α antibody or by pretreating with a p38 MAPK inhibitor. Given that TNF-α itself has been reported to destroy the tumor vasculature, blocking blood flow and inducing hemorrhage in tumors, and thus acting as a VDA (Zhang et al., 2009), we were not surprised to find that DMXAA exhibits a stronger and longer-lasting tumor vessel-disrupt-

![Fig. 5. TNF-α maintains the decreased blood flow during late-stage DMXAA-induced vascular disruption. A, mice were treated with DMXAA for 24 h with coadministration of an anti-TNF antibody (120 μg/mouse) or IgG (120 μg/mouse) and then were perfused with Hoechst 33342. Photographs of tumor tissue cryosections were obtained by fluorescence microscopy. Fluorescence intensity was quantified in eight randomly selected microscope fields using Image-Pro Plus 6.0. B, tumor tissues were homogenized, and TNF-α in the supernatant after centrifugation was assayed by ELISA and expressed as picograms per gram of tissue. Data are presented as mean ± S.E.M. (*, p < 0.05, n = 5).](https://jpet.aspetjournals.org/content/p38.2017.715.715.full)
Plus 6.0 (H11569/H11569) identified from seven randomly sampled microscope fields using Image-Pro

A, images were photographed under an inverted microscope. B, the tumor tissue was fixed, sectioned, and stained with hematoxylin and eosin. A, images were photographed under an inverted microscope. B, the average necrotic area, expressed as percentage of total area, was quantified from seven randomly sampled microscope fields using Image-Pro Plus 6.0 (**, p < 0.01).

**Fig. 6. Inhibition of p38 MAPK attenuates DMXAA-induced tumor tissue necrosis.** Lewis lung cancer tumors were implanted in C57 mice and allowed to grow to 10 mm in diameter. The mice were then treated with DMXAA at a dose of 20 mg/kg. Tumors were removed after 24 h, and tumor tissue was fixed, sectioned, and stained with hematoxylin and eosin. A, images were photographed under an inverted microscope. B, the average necrotic area, expressed as percentage of total area, was quantified from seven randomly sampled microscope fields using Image-Pro Plus 6.0 (**, p < 0.01).

In this report, we demonstrated that p38 MAPK activation was involved in both cytoskeleton reorganization in endothelial cells and TNF-α production in macrophages; however, the mechanism by which p38 MAPK regulates actin cytoskeleton reorganization and TNF-α production remains unclear. Our results suggest that in endothelial cells, DMXAA-induced p38 MAPK activation leads to phosphorylation of MLC, triggering the formation of actin stress fibers. Alternatively, p38 MAPK activation via MAPKAPK-2/3 may induce phosphorylation of the actin polymerization modulator heat shock protein 27 to regulate actin microfilament dynamics (Guay et al., 1997). In addition, the fact that p38 MAPK regulated both DMXAA- and TNF-α production in macrophages raises the possibility that there may be a connection between actin cytoskeleton reorganization and TNF-α production in macrophages. Indeed, it was found that cytochalasin D pretreatment attenuated p38 MAPK activation induced by DMXAA (data not shown).

Four p38 MAP kinase isoforms (α, β, γ, δ) have been identified, sharing approximately 60% homology. p38α and p38β are ubiquitously expressed, p38γ is predominantly expressed in skeletal muscle, and p38δ is found in the lung, kidney, testis, pancreas, and small intestine (Kaminska, 2005). It has been reported that murine macrophages express both p38α and p38δ, but only p38α mediates lipopolysaccharide-induced inflammatory cytokine (such as TNF-α, IL-6, soluble TNF receptor type II, and CXCL16) expression (Tang et al., 2006). Other reports also demonstrate that p38α, but not p38β, was the major p38 isoform involved in the immune response in vivo (Beardmore et al., 2005; O’Keefe et al., 2007). The siRNA used in our study is designed to specifically target p38α (NM_011951). The p38α siRNA significantly inhibited DMXAA-induced TNF-α production in murine macrophage Ana-1 cells, an extent similar to the p38 MAPK inhibitor SB202190, which is known to inhibit both p38α and p38β, suggesting p38α is the major p38 MAPK isoform in mediating the pharmacological effect of DMXAA.

Consistent with the results reported previously (Sun et al., 2011), ERK1/2 was also activated in Ana-1 cells after DMXAA treatment, and it participated in TNF-α production as p38 MAPK did (Fig. 2). However, the reduction of TNF-α production induced by ERK1/2 inhibitor PD0325901 was much less than that of p38 MAPK inhibitor SB202190 (20 versus 60%), and more importantly, ERK1/2 was not involved in DMXAA-induced actin cytoskeleton reorganization in HUVECs (Fig. 1). Furthermore, in endothelial cells, the morphological change induced by DMXAA was not reversed by pretreatment with 20 μM JNK inhibitor SP600125 (Fig. 1A), and in macrophage Ana-1 cells, DMXAA-induced TNF-α production was not affected by SP600125 pretreatment. Thus, we believe that p38 MAPK, but not ERK1/2 or JNK, is the key factor mediating actin cytoskeleton reorganization in tumor vascular endothelial cells and TNF-α production in macrophages, both of which contribute to DMXAA antitumor activity.

At present, p38 MAPK inhibitors are mainly used clinically as anti-inflammatory agents. Because p38 MAPK plays such an essential role in regulating the antitumor activity of DMXAA, and because p38 MAPK inhibition significantly suppresses DMXAA-induced tumor vascular disruption and antitumor activity, we urge caution in the clinical use of p38 MAPK-inhibiting drugs in patients being treated with DMXAA or its derivatives, in order to avoid significantly reducing the antitumor activity of DMXAA.

In summary, both endothelial cell actin cytoskeleton reorganization and intratumor TNF-α production are critically involved in the DMXAA-induced shutdown of tumor blood flow. More importantly, both effects are regulated by p38 MAPK. These conclusions expand our knowledge about the mechanisms of DMXAA and may be of great importance for the clinical application and further development of this agent.

**Authorship Contributions**

**Participated in research design:** Wu, Quan, and Lou.

**Conducted experiments:** Wu, Xu, and Li.

**Contributed new reagents or analytic tools:** Hu.

**Performed data analysis:** Wu, Quan, and Lou.

**Wrote or contributed to the writing of the manuscript:** Wu, Quan, and Lou.

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