Anti-angiogenic Effects of Dimethyl Sulfoxide on Endothelial Cells

Keiichi Koizumi, Yasuo Tsutsumi,* Yasuo Yoshioka, Masaki Watanabe, Takayuki Okamoto, Yohei Mukai, Shinsaku Nakagawa, and Tadanori Mayumi

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan. Received May 14, 2003; accepted June 30, 2003

Dimethyl sulfoxide (DMSO) has anti-inflammatory and analgesic properties and is the only intravesical agent approved by the FDA for the treatment of interstitial cystitis. While it is known that DMSO has numerous biological effects on cell differentiation and alteration of cell-surface carbohydrate structures, the anti-inflammatory mechanism of DMSO has been unclear. Therefore, further investigation of DMSO in terms of inflammation therapy is needed. This study assessed the in vitro anti-angiogenic effects of DMSO on human aorta endothelial cells to clarify one of the mechanisms of its anti-inflammatory activity. DMSO did not affect expression of E-selectin on endothelial cells in the presence of TNF-alpha. Furthermore, DMSO effectively inhibited capillary tube formation; this mechanism would be due to suppression of matrix metalloproteinase-2 (MMP-2) production. These results provide useful knowledge about the anti-inflammatory effects of DMSO and the regulatory mechanism of MMP-2.

Key words dimethyl sulfoxide; angiogenesis; matrix metalloproteinase-2; E-selectin; tumor necrosis factor-alpha

Dimethyl sulfoxide (DMSO), a by-product of the wood industry, has been used extensively as commercial solvents and pharmaceutical agents. Many researchers have studied it for a variety of applications, with respect to pain, inflammation, interstitial cystitis, and tumors. Dimethyl sulfoxide (DMSO) has anti-inflammatory and analgesic properties and is the only intravesical agent approved by the FDA for the treatment of interstitial cystitis. Yet DMSO shows numerous biological effects on cell differentiation, and alteration of cell-surface carbohydrate structures, but it is not clear its anti-inflammatory potency. As a result, this has limited the clinical application of DMSO. Additionally, DMSO is safer, less expensive, and at least as effective alternative for a variety of problems for which other less effective and more costly treatments are used. Therefore, further investigation of DMSO is needed to allow its use as an anti-inflammatory agent as well as an anti-tumor agent.

Angiogenesis plays a crucial role in many pathological conditions, including inflammation, tumors, the course of wound healing, oncogenesis, and diabetic retinopathy. In these instances, the new blood vessels are used as an oxygen and nutrition supply route and as an elimination pathway for metabolic decomposition products. During angiogenesis, endothelial cells need to divide, migrate, invade the extracellular matrix, and form capillary structures from preexisting blood vessels. Therefore, an in-depth understanding of the mechanism of DMSO for endothelial cells and angiogenesis should allow the development of successful therapies for inflammation, tumors, and the like. In tumor therapy, selective inhibition of tumor angiogenesis and/or destruction of tumor vascular-network appear to be a more attractive approach for controlling neoplasms than direct antitumor therapies.

There are few reports about effects of DMSO for endothelial cells and angiogenesis. The present study sought to investigate the effects of DMSO for endothelial cells and angiogenesis in an attempt to improve therapy by DMSO. First, the morphology and expression of E-selectin on endothelial cells in the presence of DMSO was examined. The influence of DMSO on the ability of endothelial cells to form tubes was also examined. DMSO inhibited capillary tube formation and this mechanism would be due to suppression of MMP production.

**MATERIALS AND METHODS**

**Cell Culture** Human aorta endothelial cells (HAEC) were supplied by Kurabo Inc. (Osaka, Japan) and maintained in HuMedia-EG 2 (Kurabo Inc., Osaka, Japan). Subconfluent cells were used between the 3rd and 4th passage for this study.

**Morphological Analysis** HAECs were cultured in the absence or presence of DMSO (1—3%) on 96 well plates for 9 d. Morphological changes were studied by phase-contrast microscopy.

**Cell ELISA** HAECs (2×10⁴ cells/well) were cultured on 96 well plates. Cells were incubated with fresh medium containing 50 ng/ml TNF-alpha in the absence or presence of DMSO for 6 h and then washed with PBS. Cells were incubated with anti-human E-selectin antibody (Seikagaku Co., Tokyo, Japan) as the primary antibody for 1 h and then washed. They were then incubated for 1 h with HRP-goat anti-mouse immunoglobulin (DAKO JAPAN Co., Ltd., Kyoto, Japan) and washed. Stained cells were allowed to react with TMB+ (DAKO JAPAN Co., Ltd.) and the enzyme reaction was stopped using H₂SO₄. The solution was read on a microplate reader at 450 nm.

**Tube Formation Assay** Disruption of in vitro tube formation was conducted on 48-well plates that had been coated with 200 μl/well Matrigel. HAEC was detached from confluent plates with 0.5% tripasin and plated at 3×10⁴ cells/well in 0.5 ml of DMEM-F12 containing 0.5% fetal calf serum and DMSO. Endothelial cell tubes were allowed to form overnight at 37 °C. Cells were then observed under a microscope.

**Gelatin Zymography** After subconfluent HAECs were cultured with DMSO for 24 h, the supernatants were collected after centrifuging to remove the debris and stored at −20 °C until used as the conditioned media. They were mixed with non-reducing sample buffer and electrophoresed at 4 °C on a
7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% gelatin. After electrophoresis, the gels were rinsed twice in 50 mM Tris–HCl containing 2.5% Triton X-100, 5 mM CaCl₂. One millimole of ZnCl₂ and 0.05% NaN₃ were used to remove SDS, and then gels were incubated with reaction buffer (50 mM Tris–HCl, 5 mM CaCl₂, 1 mM ZnCl₂, and 0.05% NaN₃) at 37 °C for 24 h. The gels were then stained with 0.1% Coomassie brilliant blue solution and destained until the lytic bands were clearly visible. Gelatinolytic activity was quantified by densitometric analysis using a Master Scan Gel Analysis System (Scanalytics, Billerica, MA, U.S.A.).

RESULTS AND DISCUSSION

DMSO relieves pain and also has well-documented action with respect to a variety of illnesses. In fact, research has suggested that DMSO prevents experimental hepatic fibrosis induced by dimethyl nitrosamine, lipopolysaccharide-induced synovitis, and the growth of leukemia. However, the clinical application of DMSO as an anti-inflammatory agent has thus been attempted, but the mechanism of its anti-inflammatory action is not clear. It is one of the most studied but least understood pharmaceutical agents. Therefore, further investigation must be performed to develop safer and more effective therapy with DMSO. The current research assessed the effects of DMSO on endothelial cells and angiogenesis. Angiogenesis is a prominent feature of many physiological and pathological processes, including inflammation, tumor growth, wound healing and luteinization. Angiogenesis is mediated by substances produced by lymphocytes and tumor cells, and many previous studies have demonstrated that tumor growth depends upon it. Angiogenesis is also critical for inflammation and tumor growth since vasculature supplies some nutrients and mediates removal of waste.

To examine the effects of DMSO on endothelial cells, culturing of HAECs was first performed in DMSO (0—3%) for 9 d. As the DMSO concentration increased, morphological changes in HAEC became apparent. In the absence of DMSO, the HAECs were observed to be in the characteristic cobblestone formation of vascular endothelial cells (Fig. 1a). In 1 or 2% DMSO, the HAECs became elongated and spread to a greater extent (Figs. 1b, c). In 3% of DMSO, the morphology of the HAECs changed from a cobblestone formation to an extension formation, as seen with fibroblast cells (Fig. 1d). These effects of DMSO were reversible. The HAECs recovered their cobblestone formation from fibroblast cell formation after depletion of DMSO (data not shown). The DMSO was not toxic as evidenced by MTT assay (data not shown).

E-selectin is expressed constitutively at a low level in endothelial cells, and this expression is increased with stimulation by inflammatory agents such as Tumor Necrosis Factor-alpha (TNF-alpha) secreted by monocytes. E-selectin is expressed on capillary endothelium during acute and chronic inflammation. Types of this inflammation include immune complex-dependent acute lung injury, cardiac allografts, rheumatoid arthritis, and skin inflammation. In inflammation, the interaction between E-selectin on endothelial cells and their ligands on lymphocytes is crucial for the penetration of lymphocytes into tissue. In fact, anti-E-selectin antibody displayed an anti-inflammatory effect. To investigate the mechanism of the anti-inflammatory effects of DMSO, the expression of E-selectin on endothelial cells that had been stimulated with inflammatory agents was examined in the presence of DMSO (Fig. 2). HAECs were stimulated with TNF-alpha at various concentrations for 5 h as a model of endothelial cells in inflammation tissue. Expression of the E-selectin on HAECs was confirmed in proportion to the concentration of TNF-alpha. In the presence of TNF-alpha, DMSO did not significantly affect E-selectin expression in endothelial cells.

HAECs placed on the basement membrane matrix Matrigel undergo capillary-like tube formation, mimicking certain steps in angiogenesis such as migration and differentiation. This model is frequently used for screening of anti-angiogenesis agents. The tube-forming activity of HAECs on Matrigel in the presence of DMSO was assessed to determine whether the DMSO could influence in vitro angiogenesis (Fig. 3). Cells plated on Matrigel in the absence of DMSO displayed a typical baseline tube formation. In the presence of DMSO, tubes were disrupted and this effect depended on the concentration of DMSO. Cells treated with
DMSO formed small clumps and few intact tubes were observed. The DMSO was not toxic as evidenced by MTT assay (data not shown). Next, the mechanism of inhibition of tube formation in HAECs by DMSO was examined.

Matrix metalloproteinase-2 (MMP-2), a member of the matrix metalloprotease family, is secreted by vascular endothelial cells and plays a crucial role in degrading the extracellular matrix during tube formation and angiogenesis.17) MMP-2 secretion involves the degradation of type 4, 5, 7, and 10 collagens and fibronectin, which are constituents of the basement membrane. This degradation facilitates the intrusion of endothelial cells across such structures, an important step for the sprouting of neostructures. HAECs were tested for the presence and activity of MMP-2 by gelatin zymography (Fig. 4). Unexposed cells produced an MMP-2 gelatinolytic band with an apparent relative molecular mass of 72 kDa. However, after exposure to DMSO, it declined significantly at 2% and further at 3%. These results indicated that inhibition of tube formation in endothelial cells by DMSO may result from a decrease in MMP-2 production on endothelial cells. Many researchers have shown that inhibitors of MMPs can inhibit angiogenesis.18,19) Tumor angiogenesis is reduced in mice in which the gene for MMP-2 has been removed. Therefore, the current data offers new possibilities with respect to the application of DMSO in tumor therapy.

DMSO has an ability to penetrate through membranes. From 70 to 90% has been found to be the most effective strength across the skin and bladder. Seventy percent DMSO has been used most widely as an topical analgesic, and previously 90% DMSO was used for therapy of arthritis and DMSO relieved joint pain and increased range of joint motion. Therefore DMSO could show inhibitory action of MMP-2 \textit{in vivo}.

In this study, we showed one of the possible anti-inflammatory mechanism of DMSO. DMSO may inhibit the formation of vascular networks in inflammatory tissues, through the inhibit of MMP-2 production. These results will promote to apply DMSO for inflammatory therapy.

Acknowledgements Keiichi Koizumi, Yasuo Tsutsumi, and Yasuo Yoshioka contributed equally to the work. This work was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists, a Grant-in-Aid for Cancer Research and for Scientific Research from the Japanese Ministry of Education, Science, Sports, and Culture, and by Health Sciences Research Grants for Research on Health Sciences from the Japanese Ministry of Health and Welfare.

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