RESEARCH ARTICLE

Calpeptin Prevents Malignant Pleural Mesothelioma Cell Proliferation via the Angiopoietin-1/Tie-2 System

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Abstract

Malignant pleural mesothelioma (MPM), an aggressive malignant tumor of mesothelial origin associated with asbestos exposure, shows a limited response to conventional chemotherapy and radiotherapy. Therefore, the overall survival of MPM patients remains very poor. Progress in the development of therapeutic strategies for MPM has been limited. We recently reported that the calpain inhibitor, calpeptin exerted inhibitory effects on pulmonary fibrosis by inhibiting the proliferation of lung fibroblasts. In the present study, we examined the preventive effects of calpeptin on the cell growth of MPM, the origin of which is mesenchymal cells, similar to lung fibroblasts. Calpeptin inhibited the proliferation of MPM cells, but not mesothelial cells. It also prevented 1) the expression of angiopoietin (Ang)-1 and Tie-2 mRNA in MPM cells, but not mesothelial cells and 2) the Ang-1-induced proliferation of MPM cells through an NF-kB dependent pathway, which may be the mechanism underlying the preventive effects of calpeptin on the growth of MPM cells. These results suggest potential clinical use of calpeptin for the treatment of MPM.

Keywords: Calpeptin - mesothelioma - cell proliferation - Ang-1/Tie-2 mRNA

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor of mesothelial origin associated with asbestos exposure, and shows a limited response to conventional chemotherapy and radiotherapy (Wagner et al., 1960; Selikoff et al., 1980; Robinson et al., 2005; Robinson and Lake, 2005). Although the multi-target antifolate pemetrexed was recently approved as a first-line agent in combination with cisplatin for the treatment of MPM, the overall survival of MPM patients remains very poor (Vogelzang et al., 2003) with a median survival duration of 8-18 months (Nowak et al., 2002). In several centers, potentially curative surgery combined with some form of adjuvant therapy has been performed. Despite extensive research for the development of MPM treatments, progress in effective therapeutic and preventive strategies against MPM has been limited, and, thus, novel treatments are urgently needed (Sterman et al., 1999).

Calpain, a calcium-dependent intracellular cysteine protease, plays an important role in some cellular processes such as cell proliferation, differentiation, and apoptosis (Goll et al., 2003). Pulmonary fibrosis is a progressive and lethal pulmonary disease characterized by the proliferation of lung fibroblasts and the deposition of extracellular matrix materials. Idiopathic pulmonary fibrosis is the most common type with a prevalence of 16-18 per 100,000 people, leading to a high fatality rate (>50% 5-year mortality rate) due to eventual respiratory failure (Coultas et al., 1994; Gross and Hunninghake , 2001; American Thoracic Society/European Respiratory Society, 2002). We previously reported the important roles of IL-6 and TGF- β1 by demonstrating the preventive effects of two substances (ATRA and Thalidomide) on pulmonary fibrosis (Tabata et al., 2006; Tabata et al., 2006; Tabata et al., 2007). Moreover, we recently reported that the calpain inhibitor Calpeptin histologically prevented bleomycin-induced lung fibrosis in mice (Tabata et al., 2010). Calpeptin decreased the expression of IL-6, TGF-β1, angiopoietin-1, and collagen type Iα1 mRNA in mouse lung tissues. In vitro studies showed that Calpeptin reduced 1) the production of IL-6, TGF-β1, angiopoietin-1, and collagen by lung fibroblasts, and 2) the IL-6-dependent proliferation and angiopoietin-1-dependent migration of cells, which may be one of the mechanisms underlying the inhibitory effects of Calpeptin on pulmonary fibrosis.

In the present study, we examined whether Calpeptin exerts suppressive effects on the cell proliferation of MPM, the origin of which is mesenchymal cells, similar to lung fibroblasts.

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Materials and Methods

Cell culture

The human malignant pleural mesothelioma (MPM) cell lines H28 (epithelioid), H2052 (sarcomatoid), H2452 (biphasic), and MSTO-211H (biphasic) and the human mesothelial cell line MeT-5A were purchased from the American Type Culture Collection (Rockville, MD). These cells were cultured in RPMI 1640 (Sigma Chemical Co., St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum in a humidified incubator with 5% CO_2 at 37°C. Calpeptin (Calbiochem, San Diego, CA) was diluted in DMSO and added to the growth medium to yield the final DMSO solvent concentration<0.01% (v/v). As a control, the same concentration of DMSO was added to cells. This final concentration of DMSO had no significant effects on these cells.

In some experiments, the cells were stimulated with recombinant human angiopoietin-1 (Ang-1) (R&D Systems, Oxford, UK) and were preincubated with the NF-κB inhibitor and proteasome inhibitor MG-132 (5 μM, Calbiochem, San Diego, CA), p38MAPK inhibitor SB203580 (10 μM, Calbiochem), or ERK1/2 inhibitor PD98059 (25 μM, Calbiochem) for 60 minutes as previously described (Tabata et al., 2006).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using TaqMan Gene expression products as previously described (Tabata et al., 2006). 18SrRNA served as an endogenous control (Applied Biosystems).

Cell proliferation assay

A cell proliferation assay was performed as previously described (Tabata et al., 2006). Cell Counting Kit-8 (Dojindo, Tokyo, Japan) was used to measure the growth of cells.

Measurement of NF-κB p65

Nuclear extracts were prepared and protein concentrations in nuclear extracts were measured as previously described (Tabata et al., 2006). Nuclear NF-κB p65 was detected using an ELISA Kit (BioSource, Sigma, and Active Motif, respectively).

Statistical analysis

Results are presented as the mean ± SD. Statistical analyses were performed using the Bonferroni/Dunn multiple comparisons test.

Results

Inhibitory effects of Calpeptin on the proliferation of MPM cells and MeT-5A cells

We examined the effects of Calpeptin on the growth of MPM cells and MeT-5A cells. The addition of Calpeptin had a preventive effect on the proliferation of all MPM cells such as MSTO-211H (biphasic) (A), H2052 (sarcomatoid) (B), H2452 (biphasic) (C), and H28 (epithelioid) cells (D) in a dose-dependent manner (Figure 1A-D). The maximum preventive effect was observed at 100 nM Calpeptin (9% decrease [p<0.01], 10% decrease [p<0.01], 13% decrease [p<0.01], and 9% decrease [p<0.01], respectively); whereas, lower concentrations (1 nM or 10 nM) of Calpeptin had markedly weaker effects than those of 100 nM. The viability of MPM cells and MeT-5A cells was not affected by 100 nM Calpeptin (data not shown). On the other hand, Calpeptin had no effect on the proliferation of MeT-5A (Figure 1E).

Effects of Calpeptin on Ang-1and Tie-2 expression in MPM cells and MeT-5A cells

We previously reported the mRNA expression of Ang-1, Ang-2, and Tie-2 in MPM cells and MeT-5A cells using a real -time RT-PCR analysis (Tabata et al., 2010). Ang-1 mRNA was detected in H2052 and MSTO-211H cells. However, Ang-1 mRNA expression was not detected in H28, H2452, or Met-5A cells. On the other hand, Tie-2 mRNA was detected in H2452, MSTO-211H, and MeT-5A cells. MPM and MeT-5A cells did not express Ang-2 mRNA. The protein levels of Ang-1

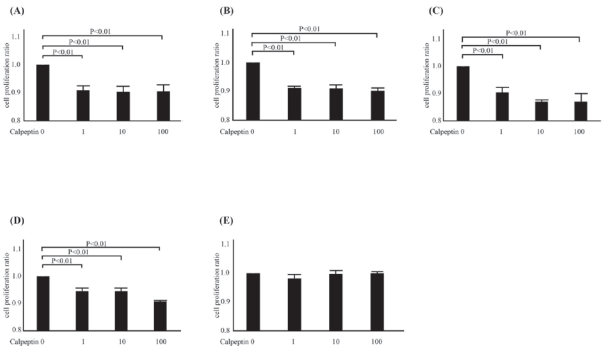

Figure 1. Inhibitory effects of Calpeptin on the Proliferation of MPM Cells and MeT-5A Cells. MPM cells such as MSTO-211H (A), H2052 (B), H2452 (C), H28 (D), and MeT-5A cells (E) were cultured with or without Calpeptin (1-100nM) for 48 hours and cell proliferation was assayed. All results are indicated as the mean ± SD of three separate experiments

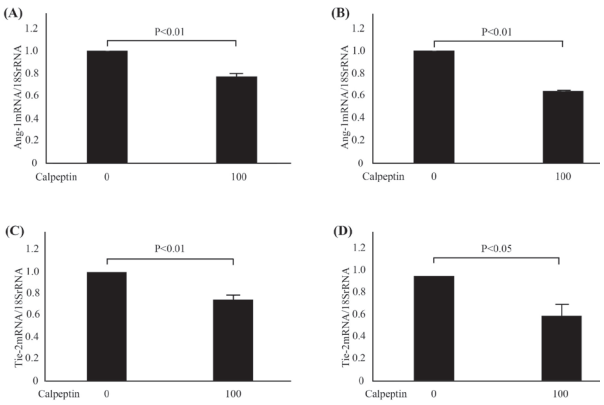

Figure 2. Inhibitory Effects of the Calpain Inhibitor Calpeptin on the Ang-1/Tie-2 System. MPM cells were cultured in the presence or absence of 100 nM Calpeptin for 18 hours and real-time RT-PCR was performed to investigate changes in Ang-1 mRNA levels in MSTO-211H (A) and H2052 cells (B), and Tie-2mRNA levels in MSTO-211H (C) and H2452 cells (D). All results are indicated as the mean ± SD of three separate experiments

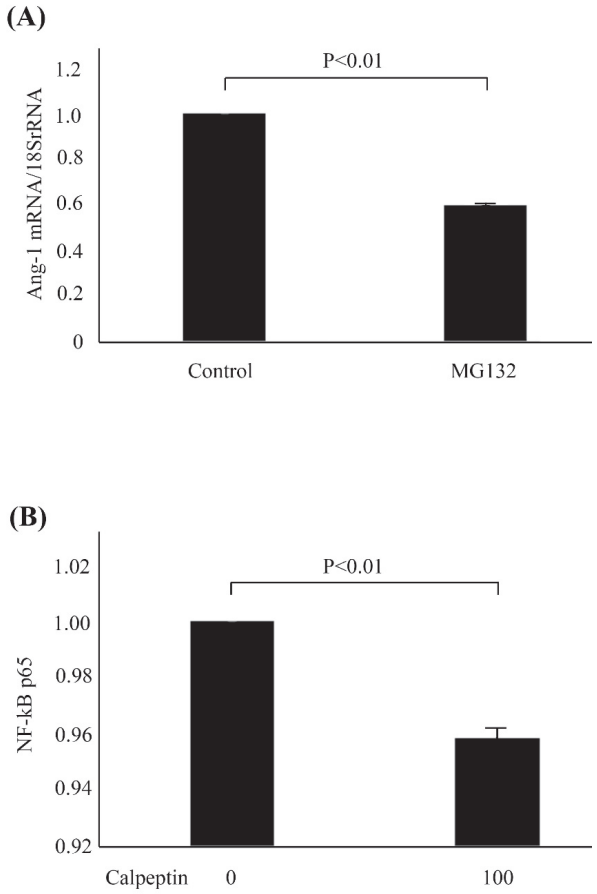

Figure 3. Involvement of NF-κB in the Suppressive Effect of the Calpain Inhibitor Calpeptin on Ang-1 mRNA Expression in MSTO-211H Cells. A) MSTO-211H cells were cultured in the presence or absence of 5 μM MG-132 for 7 hours. Real-time RT-PCR was performed to examine changes in Ang-1 mRNA levels. (B) The activities of NF-κB were analyzed as described in the Materials and Methods. MSTO-211H cells were treated with or without 100 nM Calpeptin for 1 hour and the amount of NF-κB p65 in nuclear protein extracts was analyzed. Results are indicated as the mean ± SD of three separate experiments in triplicate

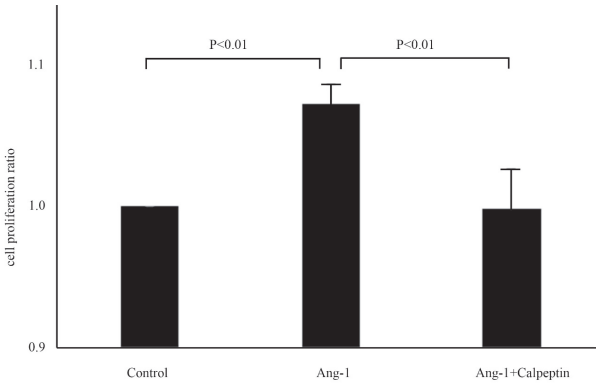

Figure 4. Effects of the Calpain Inhibitor Calpeptin on the Ang-1-Mediated Proliferation of MSTO-211H Cells. MSTO-211H MPM cells were cultured in the presence of 100 ng/ml Ang-1 with or without 100 nM Calpeptin for 48 hours, and cell proliferation was assayed. Results are indicated as the mean ± SD of three separate experiments in triplicate

and Ang-2 in cells were measured by ELISA. H2052 and MSTO-211H cells produced Ang-1 (2384.4±372.6 and 140.9±22.1 pg/ml, respectively), whereas H28, H2452, and MeT-5A cells did not. On the other hand, none of the cells examined produced Ang-2 (data not shown). We herein examined the effects of Calpeptin on Ang-1/Tie-2 expression in MPM cells and MeT-5A cells. Figure 2A, B shows that the Ang-1mRNA/18SrRNA ratio was decreased in MSTO-211H (A) and H2052 cells (B) by the treatment with 100 nM Calpeptin after 18 hours ($p<0.01$ and $p<0.01$, respectively). Figure 2C, D shows that the Tie-2 mRNA/18SrRNA ratio was decreased in MSTO-211H (C) and H2452 (D) cells by the treatment with 100 nM Calpeptin after 18 hours ($p<0.01$ and $p<0.05$, respectively). However, the addition of 100 nM Calpeptin had no effect on the Tie-2 mRNA/18SrRNA ratio in MeT-5A cells (data not shown).

Involvement of NF-κB in the suppressive effect of Calpeptin on Ang-1 mRNA expression

In order to elucidate the mechanism underlying the suppressive effect of Calpeptin on Ang-1 expression in MSTO-211H cells, we examined whether any kinases are required for this process. The pretreatment of cells with a NF-κB inhibitor led to a decrease in Ang-1 mRNA levels ($p<0.01$, Figure 3A), suggesting that NF-κB plays an important role in the production of Ang-1. We then showed that the levels of nuclear NF-κB p65 in cells were reduced in the presence of Calpeptin ($p<0.01$, Figure 3B). Inhibitors of p38MAPK and ERK1/2 had no effect on Ang-1 mRNA levels in cells (data not shown).

Effects of Calpeptin on the Ang-1/Tie-2-mediated proliferation of MPM cells

In our previous study, we showed that the addition of Ang-1 stimulated MSTO-211H cell growth in a dose -dependent manner and reached a plateau at a concentration of 100 ng/ml (Tabata et al., 2010), MSTO-211H cells secreted Ang-1, and Ang-1 induced the proliferation of these cells in an autocrine manner. In the present study, we examined the effects of Calpeptin on Ang-1-mediated cell proliferation. We found that Calpeptin inhibited the Ang-1-induced proliferation of MSTO-211H cells ($p<0.01$) (Figure 4).

Discussion

We previously reported that Calpeptin prevented bleomycin-induced lung fibrosis in mice (Tabata et al., 2010). MPM is a malignant transformation of mesothelial cells, which originate from mesenchymal cells, similar to lung fibroblasts. Therefore, we examined the relationship between Calpeptin and the growth of MPM cells and mesothelial cells. Our results demonstrated that Calpeptin prevented the proliferation of MPM cells, but not mesothelial cells in a dose -dependent manner.

We then examined the cellular suppressive mechanism of Calpeptin on the proliferation of MPM cells. Ang-1 and Ang-2, which are counteracting ligands for the endothelial -specific receptor, tyrosine kinase Tie-2 are important regulators of blood vessel growth, maturation, and

function. Ang-1 promotes angiogenesis. In contrast, Ang-2 antagonizes Ang-1 (Thurston et al., 2000; Yancopoulos et al., 2000; Jain, 2003).

We previously showed the production of Ang-1 in 2 out of 4 MPM cells, and the expression of mRNA of Tie-2 in 2 of these cells. Met-5A cells expressed Tie-2 mRNA, but did not produce Ang-1. MSTO-211H cells expressed the mRNA of Ang-1 and Tie-2. In order to clarify the involvement of Ang-1 in MPM tumor growth, we previously demonstrated that Ang-1 induced the proliferation of MSTO-211H cells and H2452 cells with the expression of Tie-2 in a dose-dependent manner. On the other hand, Ang-1 had no effect on the proliferation of H2052 or H28 cells, without the expression of Tie-2, suggesting the important role of Tie-2 in Ang-1-induced proliferation (Tabata et al., 2010).

We previously demonstrated the inhibitory effects of Calpeptin on the expression of Ang-1, one of important key cytokines in pulmonary fibrosis (Tabata et al., 2007). We herein showed that Calpeptin suppressed the mRNA expression of Ang-1 and the Ang-1 receptor, Tie-2 in MPM cells, and also prevented Ang-1-dependent cell proliferation, suggesting that Calpeptin exerts inhibitory effects on MPM cell proliferation via an "Ang-1/Tie-2 autocrine and/or paracrine mechanism" in MPM cells and Ang-1-producing cells, such as fibroblasts (Tabata et al., 2007) and pericytes (Jain, 2003).

In order to elucidate the cellular mechanisms underlying the regulation of Ang-1 expression, we used several inhibitors such as ERK, JNK, p38MAPK, and NF-κB (Sullivan et al., 2005). We identified an important role for NF-κB in this process (Figure 3A, B). These results suggest one mechanism whereby Calpeptin reduces the expression of Ang-1 and Ang-1 induces the proliferation of MPM cells through an NF-κB-dependent pathway (Figure 4). Moreover, a recent study reported that asbestos-induced oncogenesis was associated with the activation of NF-κB (Yang et al., 2006); therefore, the suppressive effects of Calpeptin on the activation of NF-κB may be beneficial for the prevention of MPM tumor growth.

Previous studies demonstrated that increased vascular formation in MPM is associated with a poor prognosis (Edwards et al., 2003; Robinson and Lake, 2005). Ang-1 is one of the major regulators of blood vessel growth (Thurston et al., 2000; Yancopoulos et al., 2000; Jain, 2003). Therefore, the suppressive effects of Calpeptin on the expression of Ang-1 in MPM cells may play an important role in the development of MPM.

The inhibitory effects of Calpeptin on cell proliferation are not derived from its toxic effects because it did not influence the viabilities of any of the cells examined in this study, as demonstrated by trypan blue staining (data not shown).

In summary, we herein show the preventive effects of Calpeptin on the proliferation of MPM cells, but not mesothelial cells, for which one of the possible mechanisms is the inhibition of Ang-1 -induced MPM cell proliferation through an NF-kB dependent pathway. Although the precise cellular suppressive mechanism of Calpeptin in the proliferation of MPM cells has not been fully elucidated, our results suggest the potential of the clinical use of Calpeptin for the treatment of MPM.

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