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Original Article

Calpeptin prevents angiopoietin-1-induced proliferation of non-small cell lung cancer A549 cells

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Abstract: Objective: Lung cancer is a leading cause of cancer-related death worldwide. Most lung cancers are non-small cell lung cancer (NSCLC). The overall survival of NSCLC patients with advanced stage or metastatic lesions remains very poor. Therefore, the development of novel treatments for NCSLC is clinically needed. Previous studies reported a relationship between calpain, a calcium-dependent intracellular cysteine protease, and tumorigenesis. In the present study, we examined the preventive effects of calpeptin, a calpain inhibitor, on the growth of A549 NSCLC cells. Methods: We determined whether calpeptin exerted inhibitory effects on the proliferation of A549 cells. Results: Calpeptin inhibited the proliferation of A549 cells. It also prevented 1) the expression of angiopoietin (Ang)-1 and Tie-2 mRNA and 2) Ang-1-induced proliferation in A549 cells, which may be the mechanisms responsible for the preventive effects of calpeptin on A549 cell growth. Conclusions: These results suggest the clinical use of calpeptin for the treatment of NSCLC.

Keywords: Calpain, lung cancer, cell proliferation, Ang-1

Introduction

Lung cancer is a leading cause of cancer-related death worldwide. Most lung cancers are non-small cell lung cancer (NSCLC). Although several treatments such as platinum doublet chemotherapy and molecular target therapy are clinically used in the treatment of NSCLC, the overall survival of NSCLC patients with advanced stage or metastatic lesions remains very poor [1, 2]. Therefore, the development of novel treatments for NCSLC is needed.

Calpain is a calcium-dependent intracellular cysteine protease that was initially identified in 1964. The calpain family constitutes fifteen gene products in mammals. Classical calpains such as calpain-1 and calpain-2 are ubiquitously expressed, whereas others are expressed in specific tissues including skeletal muscle and the gastrointestinal tract. Calpain-1 and calpain-2 are also referred to as μ-calpain and m-calpain, respectively because they require specific calcium concentrations (μM and mM, respectively) for their activation. Calpain plays important roles in various cellular processes including cell growth, remodeling, cellular signaling, and apoptosis [3]. Previous studies reported a relationship between calpain and tumorigenesis [4] such as meningioma [5], colon cancer [6], renal cell carcinoma [7], hepatocellular carcinoma [8], cholangiocarcinoma [9], acute myeloid leukemia [10], breast cancer [11] and ovarian cancer [12]; however, the relationship between calpain and the proliferation of lung cancer cells has not yet been elucidated in detail.

Therefore, we herein determined whether calpeptin, an inhibitor of calpain-1 and calpain-2, exerted inhibitory effects on the proliferation of A549 NSCLC cells.

Materials and methods

Cell culture

A549 cells, a human NSCLC cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) (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 a final DMSO solvent concentration < 0.01% (v/v). As a control, cells

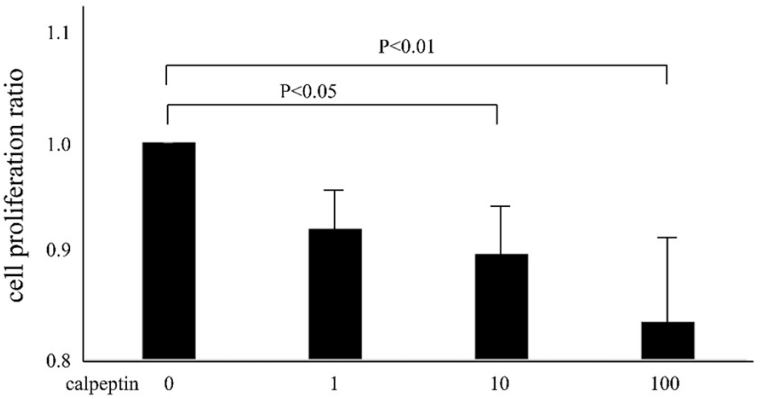

Figure 1. Inhibitory effects of calpeptin on A549 cell proliferation. A549 cells were cultured with or without calpeptin (1-100 nM) for 48 hours and cell proliferation was assayed. All results are indicated as the mean ± SD.

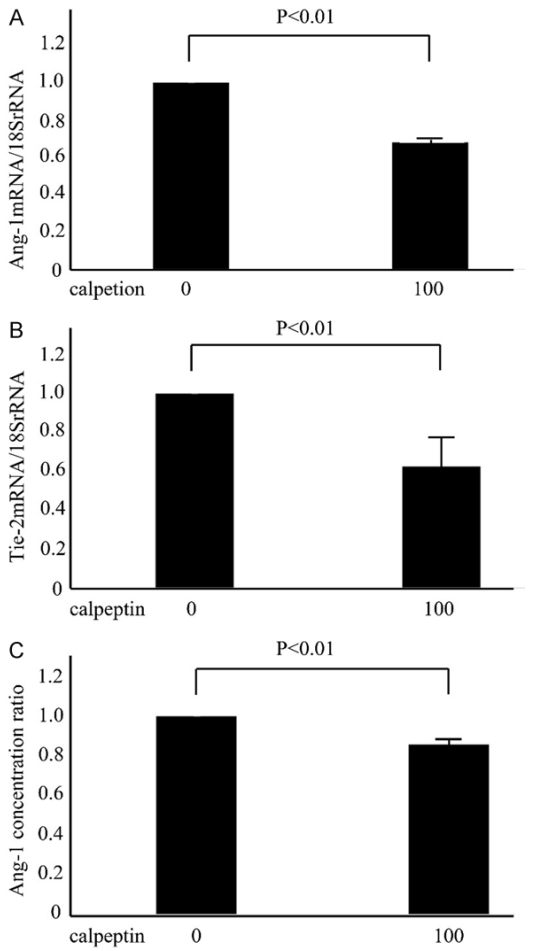

Figure 2. Inhibitory effects of calpeptin on the Ang-1/Tie-2 system. A549 cells were cultured in the presence or absence of 100 nM calpeptin for 6 hours and real-time RT-PCR was performed to determine changes in the mRNA levels of Ang-1 (A) and Tie-2 (B). The Ang-1 concentration ratio was measured after 42 hours in cells treated with 100 nM calpeptin (C). All results are indicated as the mean ± SD.

were treated with the same concentration of DMSO, and all cultures in this study contained the same final concentration of DMSO. In preliminary experiments, the final concentration of DMSO had no marked effects on A549 cells.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed as previously described [13-18]. A549 cells were cultured with or without 100 nM Cal for 6 hours. Total RNA was isolated using the RNeasy Mini kit (QIAGEN, Valencia, CA), and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed using TaqMan Gene expression products for angiopoietin (Ang)-1, Ang-2, and Tie-2 (Applied Biosystems). 18SrRNA served as an endogenous control (Applied Biosystems).

Measurement of Ang-1

Ang-1 concentrations in culture supernatants treated with or without 100 nM Cal were measured after 42 hours using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Oxford, UK).

Cell proliferation assay

A549 cells were cultured in 96-well flat-bottomed culture plates with or without 1-100 nM Cal or recombinant human Ang-1 for 48 hours, and a cell proliferation assay was performed using Cell Counting Kit-8 (Dojindo, Tokyo, Japan) as previously described [13].

Statistical analysis

Results are given as the mean ± SD of values. Statistical analyses were performed using the Bonferroni-Dunn multiple comparisons test.

Results

Inhibitory effects of calpeptin on A549 cell proliferation

We investigated the effects of calpeptin on the growth of human A549 NSCLC cells. The addi-

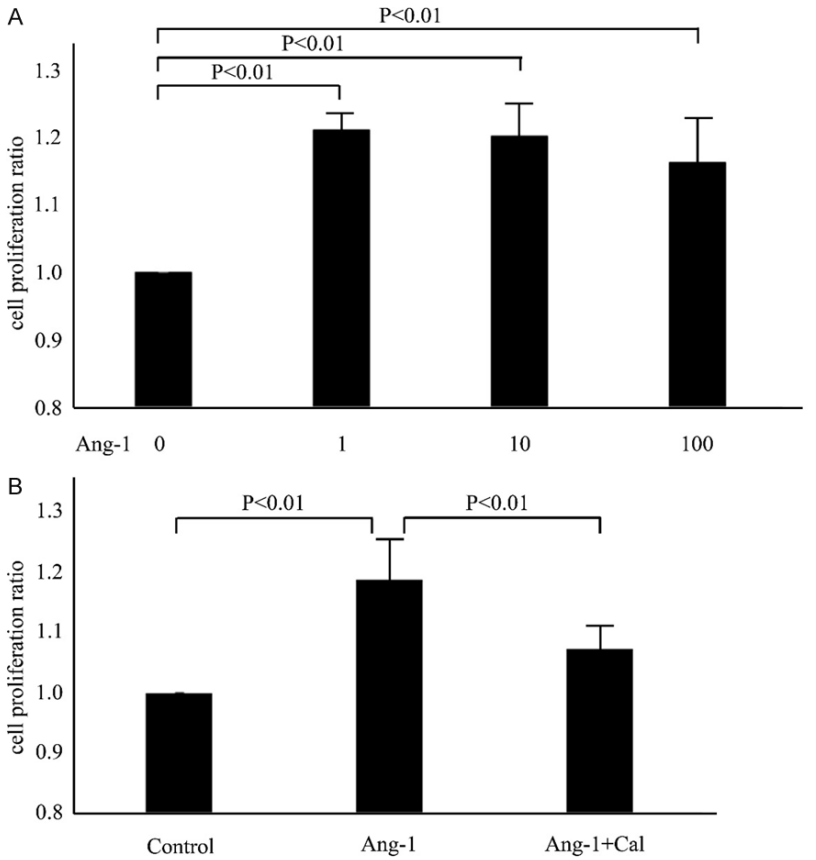

Figure 3. Effects of calpeptin on Ang-1-mediated A549 cell proliferation. A. A549 cells were cultured with or without Ang-1 (1-100 ng/ml) for 48 hours and cell proliferation was assayed. B. A549 cells were cultured in the presence of 100 ng/ml Ang-1 with or without 100 nM calpeptin for 48 hours, and cell proliferation was assayed. The results are indicated as the mean ± SD.

tion of calpeptin suppressed the proliferation of these cells in a dose-dependent manner (**Figure 1**). The maximum inhibitory effect was reached at a concentration of 100 nM calpeptin (17% decrease [P < 0.01]); lower concentrations (1 nM or 10 nM) of calpeptin exerted weaker effects than those observed at 100 nM. The final concentration of DMSO (< 0.01% (v/v)) had no marked effect on these cells (data not shown). Furthermore, calpeptin at a concentration of 100 nM had no effects on the viability of A549 cells (data not shown).

Effects of calpeptin on Ang-1 and Tie-2 expression in A549 cells

We examined the effects of calpeptin on Ang-1/Tie-2 expression in A549 cells. **Figure 2A** and **2B** show that the Ang-1 mRNA and Tie-2 mRNA/18SrRNA ratios were reduced by 100 nM calpeptin after 6 hours (P < 0.01 and P < 0.01, respectively). **Figure 2C** shows that the Ang-1 concentration ratio was decreased by 100 nM calpeptin after 42 hours (P < 0.01). However, the expression of Ang-2 mRNA was not detected in A549 cells treated with or without 100 nM calpeptin (data not shown).

Effects of Ang-1 on A549 cell proliferation

In order to clarify the involvement of Ang-1 in lung cancer cell growth, we investigated its effects on A549 cell proliferation. As shown in **Figure 3A**, the addition of Ang-1 stimulated A549 cell growth, which reached a plateau at a concentration of 100 ng/ml (1.2±0.1-fold increase [P < 0.01]).

Effects of calpeptin on Ang-1-induced A549 cell proliferation

We examined the effects of 100nM calpeptin on 100 ng/ml Ang-1-mediated cell proliferation, and found that it inhibited the Ang-1-induced proliferation of A549 cells (P < 0.05) (**Figure 3B**).

Discussion

In the present study, we investigated the relationship between calpeptin and A549 cell growth, and found that it inhibited the proliferation of these cells in a dose-dependent manner. The inhibitory effects of calpeptin on cell proliferation do not appear to be derived from its toxicity because it had no effect on the viability of A549 cells, as demonstrated by trypan blue staining (data not shown). We then attempted to elucidate the mechanism underly-

ing the suppression of A549 cell proliferation by calpeptin.

Malignant tumors require angiogenesis [19], which is associated with a poor prognosis in patients with lung cancer [20]. Ang-1 is one of the important regulators of blood vessel development. Ang-1 and Ang-2 are counteracting ligands for the endothelial receptor, tyrosine kinase Tie-2, and are also important regulators of blood vessel growth, maturation, and function. Ang-1 promotes angiogenesis, induces vascular maturation, and decreases vascular permeability, whereas Ang-2 destabilizes blood vessels, enhances vascular leaking, and antagonizes Ang-1 [21-23]. Previous studies reported a relationship between angiopoietin and lung cancer [24, 25]. In the present study, we investigated the effects of calpeptin on the expression of Ang-1 in A549 cells. We found that it suppressed the mRNA expression of Ang-1 and the Ang-1 receptor, as well as the production of Tie-2 and Ang-1 in A549 cells, and also inhibited Ang-1-dependent cell proliferation. These results suggest that calpeptin suppresses A549 cell proliferation through the "Ang-1/Tie-2 autocrine and/or paracrine mechanism" of A549 cells and Ang-1-producing surrounding cells, such as fibroblasts [15] and pericytes [22]. Therefore, the inhibitory effects of calpeptin on the expression of Ang-1 in A549 cells may play an important role in the development of NSCLC.

Lung cancer occurs in patients with idiopathic pulmonary fibrosis (IPF) (9.8-38%) [26, 27]; however, the mechanisms responsible remain obscure. Since lung cancer commonly occurs in areas of fibrosis in patients with IPF, one of the pathogenic mechanisms of lung cancer with IPF may be inflammation or fibrosis. Pulmonary fibrosis is a chronic, progressive, and irreversible lung disease that is characterized by subpleural fibrosis and honeycombing. IPF is the most common type of pulmonary fibrosis with a prevalence of 16-18 per 100,000 people, and is associated with a high incidence of death (median survival after diagnosis: 2.5-3.5 years) due to respiratory failure [28-30]. We previously reported that calpeptin prevented bleomycin-induced pulmonary fibrosis in mice [16]. It also decreased the expression of IL-6, TGF-β_1, angiopoietin-1, and collagen type Iα1 mRNA in mouse lung tissues. *In vitro* studies showed that calpeptin decreased 1) the production of IL-6, TGF-β_1, and angiopoietin-1 as well as the synthesis of collagen by lung fibroblasts, and 2) the IL-6-dependent proliferation and angiopoietin-1-dependent migration of lung fibroblasts, and these may be the mechanisms underlying the suppressive effects of calpeptin on pulmonary fibrosis. In the present study, we showed that calpeptin prevented the proliferation of A549 cells, suggesting its clinical application to the treatment of patients with NCSLC and IPF.

In summary, we herein demonstrated the preventive effects of calpeptin on the proliferation of A549 cells, for which one of the possible mechanisms was the suppression of Ang-1-induced A549 cell proliferation. Although the precise cellular mechanism underlying the suppression of A549 cell proliferation by calpeptin has not been fully elucidated and requires further study in order to aid its clinical use, our results may lead to the development of novel strategies for the treatment of NSCLC.

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Disclosure of conflict of interest

None.

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