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ORIGINAL ARTICLE

NAFAMOSTAT MESILATE (FUT-175) INHIBITS CELL GROWTH AND INVASION OF MALIGNANT PLEURAL MESOTHELIOMA CELL LINE, MSTO-211H

Takemichi Sutoh, Ikuo Fukuda, Daisuke Kimura and Takao Tsushima

Abstract Nafamostat mesilate (FUT-175), a synthetic serine protease inhibitor, has been reported to have antitumour activities toward solid tumours. The objective of this study was to characterize the biological activities of FUT-175 in a malignant mesothelioma cell line. We used MSTO-211H, a biphasic-type human malignant pleural mesothelioma cell line. The effect on cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Secretion of urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor-1 (PAI-1) was analysed by enzyme-linked immunosorbent assay. The effects on relative mRNA expression levels were measured by reverse transcription polymerase chain reaction. The effect on cell invasiveness was evaluated by cell invasion assay. FUT-175 at 10^{-5} M significantly inhibited cell growth and cell invasiveness. Cell growth reduced to 47.0 ± 2.1% compared with the control. The number of invasive cells also reduced to 16.0 ± 0.7 cells/hpf, while that of control cells was 41.4 ± 8.0 cells /hpf. U-PA and PAI-1 secreted from the cells were also reduced by FUT-175 in a dose-dependent manner. These results suggest that FUT-175 has the potential to act as a therapeutic agent against local growth and invasion, and functions by reducing PAI-1 and u-PA production of the human malignant mesothelioma. (195 words)

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Key words: nafamostat mesilate; malignant pleural mesothelioma; urokinase-type plasminogen activator; plasminogen activator inhibitor-1.

原　著

悪性胸膜中皮腫細胞株に対するメシル酸ナファモスタット(FUT-175)の細胞増殖及び細胞浸潤抑制効果の検討

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抄録　セリンプロテアーゼ阻害剤であるメシル酸ナファモスタット(FUT-175)は，臨床的には急性膵炎や播種性血管内凝固症候群に対する治療薬として用いられているが，悪性腫瘍に対する抗腫瘍活性に関しても報告がなされている．本研究では，ヒト悪性胸膜中皮腫細胞株における，FUT-175の抗腫瘍活性に関して検討した．その結果，細胞増殖能は対照群に対しFUT-175 濃度　10^{-5}M で47.0 ± 2.1%と有意に減少した($p<0.05$)．また細胞浸潤能もFUT-175 濃度　10^{-5}M で有意に減少した($p<0.05$)．さらに，ウロキナーゼ型プラスミノーゲン活性化因子(u-PA)蛋白とプラスミノーゲン活性化因子インヒビター1 (PAI-1)蛋白の分泌も同様に抑制された．これらの結果から，FUT-175 が u-PA 及び PAI-1 蛋白の分泌抑制を介し，ヒト悪性胸膜中皮腫細胞の細胞増殖能及び細胞浸潤能を抑制することが示唆された．

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キーワード：メシル酸ナファモスタット；悪性胸膜中皮腫；ウロキナーゼ型プラスミノーゲン活性化因子；プラスミノーゲン活性化因子インヒビター1．

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Introduction

Nafamostat mesilate (FUT-175) is a serine protease inhibitor widely used in Japan for acute pancreatitis, haemodialysis and disseminated intravascular coagulation. It has antitumour activities toward solid tumours[1-2]. However, its antitumour effects on malignant mesothelioma have not been studied.

Malignant mesothelioma is an aggressive neoplasm arising from human mesothelial cells and most often occurs in the pleural cavity as malignant pleural mesothelioma(MPM). It also occurs in peritoneum, pericardium and tunica vaginalis testis. MPM is considered to be associated with previous exposure to asbestos fibers. MPM has demonstrated resistance to all conventional therapy regimens, including chemotherapy, radiotherapy and surgery. The prognosis of MPM patients remains extremely poor. In Japan, 500 patients with MPM died in 1995, and this number increased to approximately 900 patients in 2003. The predicted numbers of deaths in the next 4 decades are 103,000 in Japan at predicted peak years of 2025[3-5].

Urokinase-type plasminogen activator (u-PA) protein and its inhibitor, plasminogen activator inhibitor-1 (PAI-1) are responsible for tumour invasion and cell proliferation in malignant cells. Shetty et al. reported that u-PA stimulated proliferation of human MPM cells[6]. Tumour invasion is regarded as multistep phenomena that involve proteolytic degradation of the basement membrane and extracellular matrix (ECM), altered cell adhesion and physical movement of the tumour cells. The u-PA system plays a role in the early steps of the process. MPM has the significant local invasiveness and it is related to the poor prognosis. It also has a tendency for aggressive local growth and invasion without metastases. These findings suggest that the regulation of the u-PA system has the feasibility of inhibiting tumour progression in MPM. We hypothesized that FUT-175 inhibited tumour invasion and cell proliferation by reducing u-PA production from malignant mesothelioma cells. Therefore, the objective of this study was to investigate the effect of FUT-175 on cell proliferation, cell invasion and the u-PA system in malignant mesothelioma cell line, MSTO-211H.

Materials and Methods

Cell line and cell culture

MSTO-211H, a biphasic-type human MPM cell line, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cell line was cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Grand Island, NY, USA). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagent

6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate (Nafamostat mesilate, FUT-175) was diluted with phosphate-buffered saline and stored at −20°C. The solution was diluted with culture medium. Diluted concentrations used were 10^{-5} M, 10^{-7} M and 10^{-9} M.

Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay method was carried out following the manufacturer's instruction (ATCC, Manassas, VA, USA)[7]. MSTO-211H cells were seeded at a density of 1×10^6 cells/well in 96-well plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) and incubated in complete fresh media for 24 h. The cells were then incubated for 48 h with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175. After treatment, MTT solution was added to the cells at a final concentration of 500 mg/mL and incubated for 4 h. MTT was reduced

to formazan in the living cells. The medium was then aspirated, and the formazan product was dissolved with dimethyl sulfoxide. The absorbance of formazan solution was measured using a spectrophotometer at 570 nm. Cell viability was determined by differences in absorbance and reported as a percentage of control culture conditions.

Quantification of u-PA and PAI-1 protein secretion by enzyme-linked immunosorbent assay (ELISA)

MSTO-211H cells were cultured in 60-mm cell culture dish (IWAKI, Tokyo, Japan). When the cells achieved 70% confluency, they were treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 for 48 h. U-PA and PAI-1 protein concentration in the culture supernatants were determined by ELISA using the Assaymax ELISA kit (Assaypro, St. Charles, MO, USA). The cell concentration (cells/mL) was determined using a haematocytometer, following which the protein concentration secreted from each cell (protein concentration/cell concentration) was measured.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from the cells treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 for 48 h using the RNeasy total RNA isolation kit (Qiagen, Inc, Valencia, CA, USA). cDNA synthesis and amplification by RT-PCR were carried out using the One-Step PCR kit (Qiagen, Inc, Valencia, CA, USA). The primer sequences for u-PA and PAI-1 were previously reported[8]. The polymerase chain reaction (PCR) fragments were electrophoresed on a 2% agarose gel and subsequently stained with ethidium bromide.

Cell invasion assay

The transwell cell culture chambers containing 6.4 mm diameter polycarbonate filters with 8μl pores (BD Biosciences, Bedford, MA, USA) were used for the assay. These filters were coated with Matrigel basement membrane matrix for the invasion assay. In the upper compartment, 5×10^5 cells were incubated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 for 22 h. After incubation, the non-invasive cells on the top of the filter were removed using cotton swabs. The invasive cells beneath the filters were manually counted under a microscope at × 400 magnification.

Statistical analysis

All experiments were performed at least three times and data are expressed as mean ± S.D. Student's t-test was used to determine the differences. Statistical significance was defined as p-value <0.05.

Results

Cell growth inhibition in MSTO-211H cells

MTT assay revealed that cell viability of MSTO-211H cells significantly reduced by treatment with FUT-175. The cell viability of cells treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 decreased to 78.0 ± 0.9, 81.0 ± 3.0 and 47.0 ± 2.1%, respectively, compared with control cells (Figure 1). FUT-175 at 10^{-5} M significantly reduced cell viability.

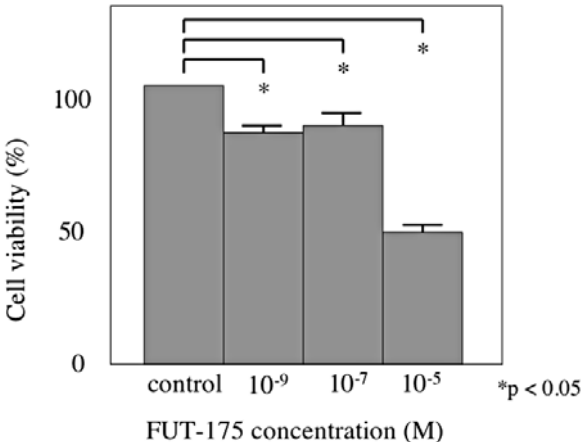

Fig. 1 Effect of FUT-175 on MSTO-211H cell proliferation. Cells were treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 for 48 h and viability was determined by MTT assay. Data represent the mean ± S.D. of triplicate experiments. FUT-175 at 10^{-5} M significantly reduced cell viability. *p < 0.05.

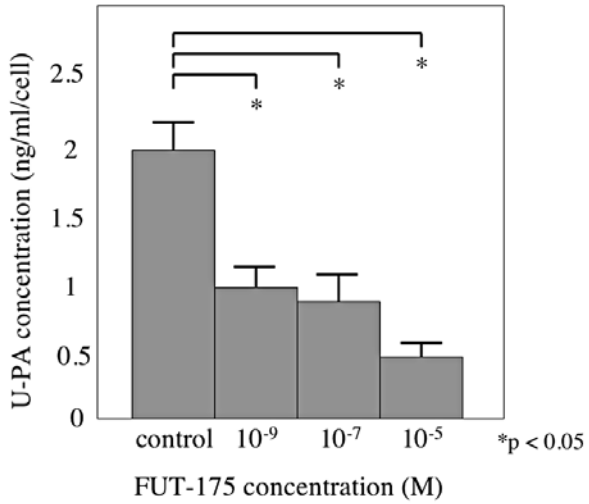

Fig. 2 Effect of FUT-175 on the production of u-PA protein. The concentration of u-PA protein in cell culture supernatant was measured by ELISA. U-PA concentration significantly reduced in a dose-dependent manner. Data represent the mean ± S.D. of triplicate experiments. *p < 0.05.

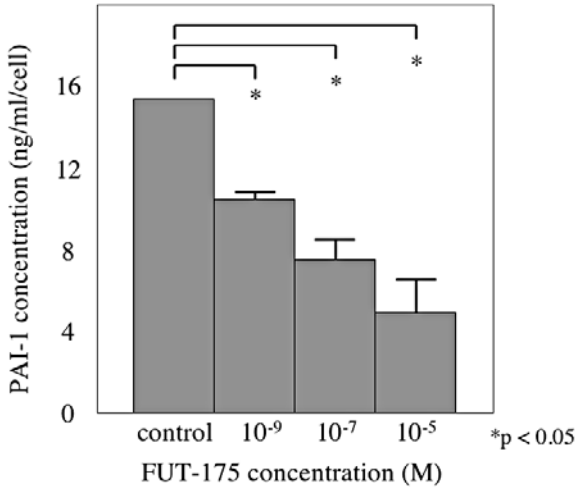

Fig. 3 Effect of FUT-175 on the production of PAI-1 protein. The concentration of PAI-1 protein in cell culture supernatant was measured by ELISA. PAI-1 concentration significantly reduced in a dose-dependent manner. Data represent the mean ± S.D. of triplicate experiments. *p < 0.05.

Inhibition of u-PA and PAI-1 protein secretion

The concentration of u-PA protein secreted from each cell treated with FUT-175 reduced in a dose-dependent manner. The u-PA concentration of untreated cells (control) was 1.69 ± 0.13 ng/mL/cell, while that of cells treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 reduced to 0.93 ± 0.05, 0.83 ± 0.04 and 0.35 ± 0.05 ng/mL/cell, respectively (Figure 2). PAI-1 protein concentration also reduced in a dose-dependent manner. PAI-1 concentration of control cells was 14.00 ± 0.15 ng/mL/cell, while that of cells treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 reduced to 10.35 ± 0.19, 7.77 ± 0.33 and 5.38 ± 0.35 ng/mL/cell, respectively (Figure 3).

Effect of FUT-175 on mRNA expression

PCR analysis results demonstrated that u-PA mRNA expression reduced in cells treated with 10^{-5} M FUT-175. However, PAI-1 mRNA expression was not affected by FUT-175 at any concentration (Figure 4).

Inhibition of tumour invasion by FUT-175 in MSTO-211H cells

Optical microscopy revealed that the number of cells beneath the filters, which represent invasive cells, decreased in cells treated with FUT-175. The number of control cells was 41.4 ± 8.0 cells/hpf, while that of cells treated with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 reduced to 21.3 ± 3.3, 17.0 ± 2.9 and 16.0 ± 0.7 cells/hpf, respectively (Figure 5).

Discussion

We found that treatment of MSTO-211H cells with FUT-175 inhibited tumour proliferation and invasion. Furthermore, we showed that FUT-175 reduced secretion of u-PA and PAI-1 protein in MSTO-211H cells.

Yamashita et al. reported that FUT-175 suppressed cell proliferation and invasion of squamous cell carcinoma[1]. Kimura et al. reported that FUT-175 inhibited liver metastasis in a CDF1 mice model with colon 26 adenocarcinoma cells[2], and Yoon et al. reported that gabexate mesilate, which was also a protease inhibitor worked similarly[9]. However, the mechanism by

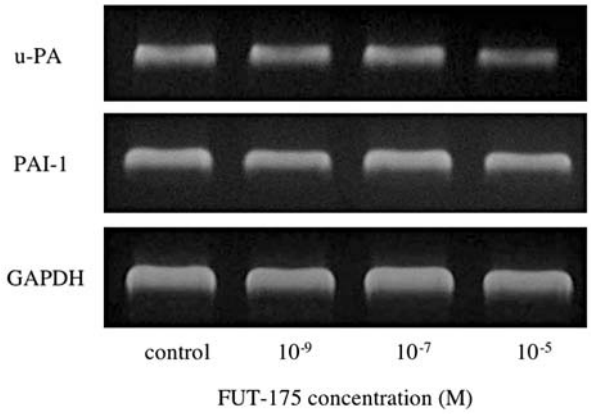

Fig. 4 Measurement of u-PA and PAI-1 expression levels in MSTO-211H cells. After treatment with 10^{-9} M, 10^{-7} M and 10^{-5} M FUT-175 for 48 h, mRNA expression was examined by RT-PCR. U-PA mRNA expression reduced at concentration of 10^{-5} M, but PAI-1 mRNA expression was not affected at any concentration. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

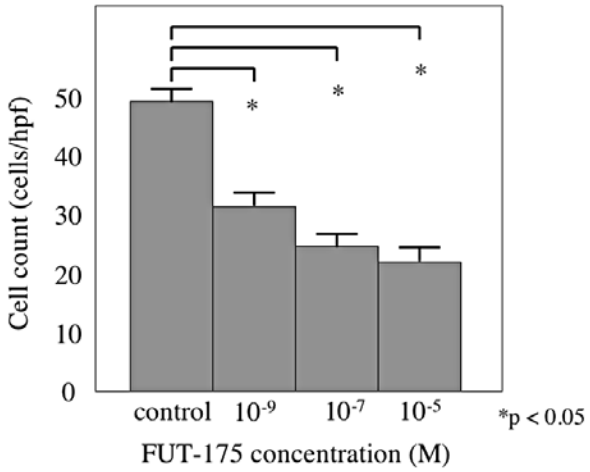

Fig. 5 The quantity of invasive cells. Cells in a high-power field (magnification × 400) were counted. Data represent the mean ± S.D. of triplicate experiments. The number of invasive cells treated with FUT-175 significantly decreased. *$p < 0.05$.

which serine protease inhibitor induced reduction of tumour proliferation and invasion is not clear. Yamashita et al. showed that the antitumour effect of FUT-175 was mediated through the down-regulation of matrix metalloprotease-2, matrix metalloprotease-9 and vascular endothelial growth factor[1]. In addition, Uwagawa reported that FUT-175 suppressed the anti-apoptotic activity of nuclear factor-kappa B in pancreatic cancer cells through induction of tumour necrosis factor receptor-mediated apoptosis[10].

The u-PA system and matrix metalloproteinases (MMPs) play a role in the early steps of tumour progression, by stimulating cell proliferation and promoting angiogenesis[11-17]. Many human cancer cells express high levels of u-PA, which may provide them a growth advantage. U-PA converts plasminogen to plasmin and pro-MMPs to MMPs, following which plasmin and MMPs enhance cell migration through the extracellular matrix degradation. Carmeliet et al. reported u-PA receptor (u-PAR) -independent activity of u-PA[14], and Koopman et al. reported the mitogenic effects of u-PA on melanoma cells[15]. The u-PA interacts with u-PAR to exert mitogenic effects on MPM cells as well as migration[16].

Our result suggests that FUT-175 may inhibit activation of plasmin and MMPs by reducing u-PA production from the malignant mesothelioma cells.

PAI-1, the primary inhibitor of u-PA, has multiple functions affecting not only cell adhesion and migration but also protease inhibition[18]. The ability of PAI-1 to modulate adhesion and migration is related to its capacity to detach cells from the extracellular matrix. PAI-1 has been shown to act as an adhesive protein, disrupting both u-PA receptor-vitronectin and integrin-vitronectin interactions[19]. Therefore, regulation of PAI-1 could be a therapeutic target through inhibition of u-PA.

FUT-175 reduced secretion of u-PA and PAI-1 protein simultaneously. During mRNA expression, however, u-PA mRNA reduced in conjunction with reduction in u-PA protein secretion, whereas PAI-1 mRNA did not change. These findings suggested that serine proteases were involved in the translation of u-PA protein, and the posttranslational processing of PAI-1 protein.

MPM has been demonstrated to be resistant to all conventional therapy regimens, including chemotherapy, radiotherapy and surgery, and the prognosis of patients remains very poor. Iyoda et al. reported that the median survival was 12.3 month and the overall survival rates of 1, 2, and 3 years after the diagnosis were 50.6%, 25.0%, and 12.7% in the Multi-institutional study in Japan[20]. The choice of chemotherapy regimens for mesothelioma patients is limited [21] and new approaches are required.

FUT-175 is clinically used in Japan, and does not have serious adverse effects. It can be used safely with other therapeutic modalities. It would be a safe and useful therapeutic agent against local growth, invasion combined with chemotherapy.

In conclusion, we found that FUT-175 reduced cell growth and cell invasiveness in a human MPM cell line, MSTO-211H. It also reduced u-PA and PAI-1 production. Therefore, our results suggest that FUT-175 may potentially be a useful therapeutic agent against local growth, invasion and metastasis of MPM by reducing PAI-1 and u-PA production.

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