Inhibition of hyaluronan synthesis suppresses tumorigenicity of osteosarcoma cells, LM-8, in vitro and in vivo via depletion of hyaluronan-rich pericellular matrix of the cells

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INTRODUCTION

Hyaluronan (HA) has been shown to regulate malignant tumor growth, invasion and metastasis [1], moreover its function and correlation with the clinical outcome of patients with malignant tumors has been demonstrated [2]. However, few reports have analyzed the role of HA for osteosarcoma.

Several studies have shown that 4-methylumbelliferone (MU), an inhibitor of HA synthesis, had anti-cancer effects via inhibition of HA synthesis in malignant tumor cells such as melanoma and pancreatic cancer in vitro and in vivo [3, 4]. We hypothesized that MU had anti-tumor effects in highly metastatic osteosarcoma cells, LM8, which have HA-rich pericellular matrix, possibly via perturbation of the matrix formation.

In this study, effects of MU on tumorigenicity of LM8 cells were investigated in vitro and in vivo.

MATERIALS AND METHODS

Cell culture: Highly metastatic osteosarcoma cells, LM8, were maintained at 37°C in an atmosphere with 5% CO2 with DMEM, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were cultured in monolayer with or without MU (0.1mM – 1mM) dissolved in DMEM, and subjected to the following assays. In Vitro Cell Proliferation Assay: Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay 24, 48 and 72 hours after treatment with or without MU (0.1mM, 0.2mM, 0.4mM, 1.0mM). Cell Motility and Invasion Assay: Chemotactic motility of LM8 cells was investigated using 12-well cell culture chambers containing inserts with 12 µm pores after 24 hours of treatment with or without MU (1.0mM). Invasion of LM8 cells was evaluated in the same chambers that also contained 100µg/ml Matrigel on the upper surface of the inserts. Migrating and invading cells on the lower surface of the membrane were counted under the light microscopy. Particle Exclusion Assay: Following the treatment of LM8 cells for 72 hours with or without MU (1.0mM), the medium was replaced with a suspension of horse erythrocytes in PBS. The pericellular matrix was visualized and photographed. The proportion of pericellular matrix area to cell areas was calculated. TUNEL Staining: Following the 24 hours treatment of LM8 cells with or without MU (1.0mM), the positive cells in TUNEL staining was counted. Real-Time RT-PCR: Total RNA was isolated from LM8 cells after the treatment with or without MU (1.0mM) for 24 hours. Expression levels of Has1, Has3, CD44, and GAPDH mRNAs were assessed by real time PCR method. They were normalized by the expression level of GAPDH mRNA. HA staining: HA accumulation was visualized using the biotinylated Hyaluronic Acid Binding Protein. In Vivo Experiments: Three weeks after implantation of LM8 cells into the dorsal flank of 5-week old C3H/He mice, daily administration of MU (10mg) was performed to the mice intraperitoneally. After 14 days of consecutive administration, mice were sacrificed, and the weight of the primary grafted sites and the number of lung metastases were evaluated.

Statistical Analysis: Bonferroni-Dunn post-hoc test and Mann-Whitney U test were used to assess differences between means. All analyses were performed using SPSS 17.0 for Windows software.

RESULTS

In Vitro Experiments: The cell proliferation was inhibited by MU in dose dependent manner (p<0.001). The motility and the invasion were inhibited by 1.0mM MU administration (Figure 1A and 1B). The pericellular coat formation in the MU treated cells was markedly inhibited compared with that in the control cells (Figure 2A, 2B), and the morphometric analysis revealed that functional pericellular matrix area significantly decreased with treatment of MU compared with control (Figure 2E). The more apoptotic cells were observed in the TUNEL assay with treatment of MU, but there was no statistical difference between two groups. The results of the real time RT-PCR exhibited the higher expression level of Has1 and Has3, and the lower expression level of CD44 with treatment of MU, but the difference was not statistically significant. Has2 was not detected in LM8 cells. The positivity of HABP staining of the cells was suppressed with treatment of MU compared with that of DMSO control cells (Figure 2C, 2D).

DISCUSSION

This study shows the inhibitory effects of MU on highly metastatic osteosarcoma cells in the various phases of tumorigenicity, possibly via suppression of HA synthesis. Upregulation of Has1 and Has3 mRNA might result from the suppression of HA synthesis by MU. Given that positive feedback of Has genes expression may recover the reduced HA synthesis after administration of MU, continuous application of MU might be required for clinical setting.

Previous studies have reported the anti-tumor effects of the genetic manipulation of HAS [5]. However, application of genetic alteration will be limited due to the predicted complications. Although HA oligosaccharides have anti-tumor effects on osteosarcoma cells [6], application of oligos to osteosarcoma is methodologically difficult. Limitation of this study is that we could not clarify the possibility of direct anti-tumor effect of MU on osteosarcoma cells, not mediated by HA inhibition.

Since MU has been now clinically used in the biliary tract disease in Japan, we can readily apply MU to patients with osteosarcoma clinically, compared with the methods of genetic manipulation or with HA oligos application. MU can be a novel therapeutic agent for patients with osteosarcoma with inhibitory effects on multi-process of tumorigenicity.

REFERENCE

1. Toole BP, Nat Rev Cancer 4; 528-539, 2004
2. Anttila MA et al, Cancer Res 60; 150-155, 2000