Effects of butyric acid, a bacterial metabolite, on the migration of ameloblastoma mediated by laminin 332
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Abstract: Ameloblastoma is a benign tumor that develops in the jaw-bone. Occasionally, however, it may become malignant and metastasize to other tissues. Although it has been suggested that various cytokines and several adhesion factors may play a role in its malignant transformation, the details have not been elucidated. In this context, it has been reported that butyric acid produced by periodontopathic bacteria causes progression of malignant tumors occurring in the mouth via podoplanin. However, the influence of butyric acid on ameloblastoma has not been clarified. In the present study, therefore, the expression of various cytokines and adhesion factors in ameloblastoma upon stimulation with butyric acid or cytokines was investigated using real-time reverse-transcription polymerase chain reaction. Three cell lines (HAM1, HAM2 and HAM3) established from the same ameloblastoma were used in the experiments. It was found that the expression of mRNAs for epidermal growth factor (EGF) and transforming growth factor beta 1 (TGFβ1) was increased in HAM2 and HAM3, respectively, upon stimulation with butyric acid. In addition, stimulation with EGF and TGFβ1 led to an increase in the expression of laminin β-3 mRNA in the respective cell lines. These results suggest that butyric acid may be involved in ameloblastoma exacerbation through the expression of laminin 332 (LM332) via EGF and TGFβ1 produced by ameloblastoma itself.

Keywords: ameloblastoma, butyric acid, laminin 332, periodontopathic bacteria

Introduction
Ameloblastoma is a tumor of odontogenic epithelial origin, accounting for approximately 1-3% of all jaw tumors and cysts [1-3]. Although usually benign, ameloblastoma may occasionally become malignant and metastasize to other tissues, mostly the lung but also to the cervical lymph nodes, brain, bones and soft tissues [3,4]. It has been suggested that several components of the basement membrane (BM) and various cytokines may be involved in this transformation [4-7]. For example, in ameloblastoma cells, BM components such as laminin 111, laminin 332 (LM332), fibronectin, nidogen and collagen (types III, IV, V, VII and XVII) have been identified [8-10], and transforming growth factor beta 1 (TGFβ1), tumor necrosis factor alpha (TNFa), interleukin-6 (IL-6) and epidermal growth factor (EGF) have also been detected [5,7,11-13]. Previous studies have shown that LM332 is an adhesion molecule in epithelial tissues and cancer cells [14-16], and some reports have suggested that in ameloblastoma, TNFa may play a role in inducing mitogen-activated protein kinase, whereas IL-6 promotes epithelial-mesenchymal transition and EGF regulates migration and invasion [5,7,12,13,17]. Butyric acid (BA), a short-chain fatty acid, is produced by periodontopathic bacteria such as Porphyromonas gingivalis and Fusobacterium nucleatum [18], and large amounts of BA have been detected in the oral cavities of patients with periodontal disease [19,20]. In addition, recent studies have suggested an association between periodontal disease and the risk of various human malignant neoplasms, such as poorly differentiated oral squamous cell carcinoma [21,22]. Previous studies have demonstrated that podoplanin, a small mucin-like protein, mediates the progression of malignant tumors of the mouth [23,24]. It also has been reported that BA is involved in the progression of oral cancer via podoplanin [25]. Moreover, enhanced podoplanin expression has been detected in ameloblastoma [26]; however, the influences of BA on LM332 in ameloblastoma have not been clarified. The present study using three ameloblastoma cell lines—HAM1, HAM2 and HAM3—was designed to investigate the mechanism by which ameloblastoma migrates through involvement of LM332. As these cell lines have different properties [27,28] despite being established from the same ameloblastoma [29], they were considered appropriate for investigating cell-cell interaction within the same ameloblastoma, an aspect that has never been examined previously. The involvement of BA in this migration was also studied.

Materials and Methods
Cell lines and cell culture
Three immortalized human ameloblastoma cell lines—HAM1, HAM2 and HAM3—derived from the same ameloblastoma patient and established at Iwate Medical University, Morioka, Japan [29], were used in this study. These cell lines were obtained from Riken BioResource Center (Ibaraki, Japan) and grown and maintained in defined keratinocyte serum-free medium (K-SFM) (Life Technologies, Tokyo, Japan) containing a growth supplement (Life Technologies) and a penicillin-streptomycin mixed solution (Nakarai Tesque, Kyoto, Japan) at 37°C in 5% CO2. The cells were extracted from 80% confluent cultures with trypsin/ethylene diamine tetraacetic acid (0.25%/1 mM) for use in experiments.

Cell migration assay
A cell migration assay was conducted as reported previously [30,31]. Briefly, polycarbonate Transwell culture insert membranes with a pore size of 8 µm (Costar, Cambridge, MA, USA) were used for the assay. The bottom of the membrane was coated with 30 nM bovine serum albumin (BSA; control) or LM332 (BioLamina, Stockholm, Sweden) and left to set for 2 h at 37°C. After coating, the membrane was washed three times with sterile phosphate-buffered saline (PBS) and blocked with 0.1% BSA for 30 min. After washing again three times with sterile PBS, 100 µL of cell suspension (1 × 106 cells/mL in K-SFM with 0.1% growth supplement and antibiotics) was seeded into each membrane insert and set in 24-well plates. The inserts were separated into upper and lower compartments. The lower compartment contained 600 µL medium with 0.1% growth supplement and antibiotics. The cells were allowed to migrate at 37°C for 18 h, after which the membranes were fixed in 2% glutaraldehyde and stained with hematoxylin. After removing the cells from the upper side of the membrane using cotton swabs, the cells attached to the lower side were counted under
a microscope. Three experiments were performed for each cell type and the mean number of cells within four different representative fields (×400) was calculated in each. Ameloblastoma cells from 80% confluent cultures were removed with trypsin/EDTA and used in all experiments. No apparent differences in cell number or viability (as measured microscopically) were observed when the cells were resuspended in K-SFM medium with supplement and incubated on BSA- or LM332-coated surfaces for 18 h—the experimental conditions for the cell migration assay—which suggested that neither cell proliferation nor variations in cell survival contributed to the cell migration results.

Quantitative polymerase chain reaction analysis
The cells were seeded into 24-well plates at 1 × 10⁵ cells/well and incubated for 72 h at 37°C in 5% CO₂. The medium was changed to K-SFM containing a mixed solution of penicillin-streptomycin, and the cells were treated with 0.02, 0.2, 2.0 and 20.0 mM sodium butyrate (NaB), 1, 10 and 100 ng/mL EGF and 5, 10 and 50 ng/mL TGFβ1 for 6 h in accordance with previous reports [32-36]. Total RNAs were then extracted from the cells using an RNeasy mini kit (Qiagen, Copenhagen, Denmark) in accordance with the manufacturer's instructions. The cDNA was synthesized from the total RNAs using PrimeScript RT Master Mix (Takara, Bio., Kusatsu, Japan). Real-time, reverse-transcription polymerase chain reaction (RT-PCR) analysis was then conducted using the Thermal Cycler Dice Real Time System (Takara Bio) in accordance with the manufacturer's instructions. TB Green Premix Ex Taq II (Takara Bio) was used for the RT-PCR reaction. The primers for podoplanin, EGF, TGFβ1 and laminin β-3 (LMβ3) (as LM332) were purchased from Takara, and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed (sense, 5ʹ-GCACCACCAAGGCTGAGAAC-3ʹ and antisense 5ʹ-TGGTGAAGACGCCAGTGGA-3ʹ). The cDNA was amplified at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. Dissociation was performed to confirm the specificity of the primers.

Statistical analyses
The results were evaluated statistically using Student's t-test at a significance level of P < 0.05. Each sample group (stimulated with 0.02, 0.2, 2 or 20 mM NaB; 1, 10 or 100 ng/mL EGF and 5, 10 or 50 ng/mL TGFβ1) was compared only with the control group (0 mM or 0 ng/mL).

Results
LM332 promotes migration of a variety of ameloblastoma cell lines
The migration assay was used to examine the effect of LM332 on the migration of HAM1, HAM2 and HAM3 cells. The results showed that LM332 promoted the migration of the three cell lines when compared with that of the control (Fig. 1). HAM1 migration more than doubled (95 cells/field for BSA and 228 cells/field for LM332), and that of HAM3 increased by approximately 18-fold (1 cell/field for BSA and 18 cells/field for LM332) relative to the control cells. The effect of LM332 on cell migration was strongest for HAM2 (3 cells/field for BSA and 302 cells/field for LM332).

Expression of mRNAs for podoplanin and laminin β-3 after stimulation with NaB
Next the effect of NaB on expression of mRNAs for podoplanin and LMβ3 was investigated. The three cell lines were cultured in different concentrations of NaB, and real-time RT-PCR was used to monitor the levels of HAM1, HAM2 and HAM3 transcripts. In the presence of 2 or 20 mM NaB, podoplanin expression decreased significantly in all three ameloblastoma cell lines at 6 h after stimulation (Fig. 2A). In contrast, there was a weak increase in the expression of HAM2 after stimulation with 0.2 mM NaB (Fig. 2A). Levels of LMβ3 mRNA expression were not significantly increased in any of the three ameloblastoma cell lines (Fig. 2B).

Epidermal growth factor expression after stimulation with NaB
After stimulation with all concentrations of NaB, the levels of expression of EGF mRNA in HAM1 did not change significantly (Fig. 2C); however,
EGF mRNA expression in HAM2 increased significantly after stimulation with 0.02 mM NaB (Fig. 2C), and that in HAM3 increased significantly by approximately 2.2-fold after stimulation with 2 mM NaB (Fig. 2C).

Expression of transforming growth factor beta 1 mRNA after stimulation with NaB

Expression of TGFβ1 mRNA in HAM1 and HAM3 was not significantly increased after stimulation with NaB (Fig. 2D); however, the results in all three cell lines showed an increasing trend (Fig. 2D), and the mRNA expression in HAM2 increased significantly by nearly 1.2-fold after stimulation with 20 mM NaB (Fig. 2D).

Expression of laminin β-3 mRNA after stimulation with epidermal growth factor or transforming growth factor beta 1

The three ameloblastoma cell lines were stimulated with either EGF or TGFβ1 to assess their effects on LMβ3 mRNA expression. It was found that LMβ3 mRNA expression increased in all three cell lines (Figs. 3, 4). In HAM1, the expression was significantly increased after stimulation with 10 and 100 ng/mL EGF (Fig. 3A). LMβ3 mRNA expression was also significantly increased in HAM1 after stimulation with 10 or 50 ng/mL TGFβ1, in HAM2 after stimulation with 5 or 10 ng/mL TGFβ1, and in HAM3 after stimulation with 5, 10 or 50 ng/mL TGFβ1 (Fig. 4).

Discussion

The present study investigated for the first time the migration-promoting activity of LM332 on ameloblastoma cell lines and identified the effect of BA produced by periodontopathic bacteria on that activity. The results of the migration assay showed that cell migration on the LM332-coated membrane increased in all three cell lines, suggesting that LM332 is involved in ameloblastoma cell migration. On the other hand, the expression of LMβ3 mRNA did not change significantly in any of the cell lines after stimulation with NaB. Some reports have claimed that podoplanin causes progression of malignant oral tumors after BA stimulation [23-25]; therefore, real-time RT-PCR was conducted to clarify whether NaB can modulate podoplanin expression in ameloblastoma cells. NaB increased the expression of podoplanin mRNA in HAM2 and decreased it when applied at a higher concentration, suggesting that NaB did not have a strong effect on podoplanin expression in ameloblastoma cells. Thus, podoplanin might not have much influence on ameloblastoma malignancy after NaB stimulation. The effect of NaB on expression of EGF and TGFβ1 mRNAs was then studied. These two cytokines have been reported to increase LM332 expression [32,36]. Furthermore, TGFβ1 plays an important role in tumor progression through regulation of transcriptional regulators that are frequently implicated in most types of human cancers [37]. It was evident that NaB stimulation enhanced the expression of EGF mRNA in HAM2 and increased TGFβ1 mRNA expression in both HAM2 and HAM3. The effects of EGF and TGFβ1 on LMβ3 mRNA expression in ameloblastoma cell lines were then investigated, and both cytokines were found to increase the expression of LMβ3 mRNA. These results are consistent with the central role of EGF and TGFβ1 in regulation of LM332 expression, and support the findings of Da Rosa [4]. In addition, LM332, EGF and TGFβ1 were expressed by some, but not all, cells within the same ameloblastoma. The three cell lines used in this study varied in their differentiation and properties, and therefore might also exhibit different responses to cytokines. There was no significant difference in the migration of each cell line upon stimulation with NaB (data not shown), suggesting that the amount of EGF and TGFβ1 secreted by HAM2 was not sufficient to induce its migration, and that EGF secreted by HAM3 was not sufficient to induce its migration mediated by LM332. However, a combination of these cytokines might affect the migration of any of these three cell lines. It was therefore considered that the present assessment of HAM1, HAM2 and HAM3 reflected the actual...
1. Adebowo ET, Fomete B, Adekeye EO (2011) Delayed soft tissue recurrence after treatment. None.

2. Conflict of interest

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5. Additional immunohistochemical analysis of ameloblastoma tissue will be needed to elucidate the effects of Tregs on the immune system. The details of ameloblastoma differentiation revealed in the present study suggest that BA derived from oral bacteria can be a risk factor for malignancy and cell migration. Therefore, local control of BA-producing bacteria seems to be particularly important as it may indirectly cause migration of ameloblastoma cells.

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9. Conflict of interest

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