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Association of Butyric Acid Produced by Periodontopathic Bacteria with Progression of Oral Cancer

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Abstract

Objective: The association between periodontal disease and the risk of various human cancers including oral squamous cell carcinoma (OSCC) has been suggested. Butyric acid (BA), an extracellular metabolite from periodontopathic bacteria, plays an important role in the progression of periodontal disease. Recent studies have shown that podoplanin, a transmembrane glycoprotein, is expressed in various normal as well as neoplastic tissues. In this study, the effects of BA/sodium butyrate (NaB) on podoplanin expression, cell migration and epithelial-mesenchymal transition in OSCC cell lines were examined.

Methods: Ca9-22, HSC-2, -3 and -4 cells were incubated with NaB, and then subjected to real-time RT-PCR, western blotting and scratch assay.

Results: NaB increased the expression of podoplanin in HSC-2 and -3 cells and vimentin in Ca9-22 cells. Cell migration was promoted at a low concentration of NaB especially in HSC-3 cells, whereas it was inhibited in HSC-2 and -4 cells. Scratch assay of HSC-2, -3 and -4 revealed that cell migration was markedly inhibited by addition of a siRNA specific for podoplanin.

Conclusion: BA/NaB increases podoplanin expression and cell migration in certain OSCC cell lines, suggesting that the progression of periodontal disease may promote the progression of OSCC via a podoplanin-dependent pathway.

Keywords: Butyric acid/sodium butyrate; Podoplanin; Oral squamous cell carcinoma; Epithelial-mesenchymal transition; Cell migration

Introduction

Oral squamous cell carcinoma (OSCC) is an invasive epithelial neoplasm of the oral cavity showing various degrees of squamous differentiation, and a propensity for early and extensive lymph node metastasis (Johnson et al., 2005). Although tobacco use and alcohol abuse are the dominant risk factors, human papilloma viruses (HPVs) have been found in a small proportion of OSCCs (Blot et al., 1988; Paz et al., 1997). Recent studies (Michaud et al., 2008; Tezal et al., 2009) have suggested an association between periodontal disease and the risk of various human malignant neoplasms including poorly differentiated OSCC.

Chronic periodontitis, an inflammatory form of periodontal disease, is caused by microorganisms present in the plaque biofilm that forms around the teeth (Pihlstrom et al., 2005). The formation of periodontal pockets, and progressive destruction of connective tissue and alveolar bone cause tooth loss in adults (Pihlstrom et al., 2005). The infiltration and extension of the junctional epithelium along the root caused by inflammatory stimuli from periodontopathic bacteria are essential events for the progression of chronic periodontitis (Pihlstrom et al., 2005).

It has been reported that butyric acid (BA), an extracellular metabolite from periodontopathic bacteria, induces apoptosis in T cells, B cells and inflamed gingival fibroblasts (Kurita-Ochiai et al., 2008). Furthermore, the role of BA as a histone deacetylase inhibitor and an epithelial cell migration-inducing factor has been reported (Pulukuri et al., 2007; Wilson and Gibson, 1997).

Recently, we found enhanced expression of podoplanin, a transmembrane glycoprotein, in all layers of oral sulcular and junctional epithelia in patients with chronic periodontitis, while the protein was not positive in normal gingival epithelium (Miyazaki et al., 2009). Podoplanin was originally discovered on the surface of podocytes in rats with puromycin-induced nephrosis as a 38-kDa mucoprotein linked to flattening of foot processes (Boucherot et al., 2002; Matsui et al., 1998).

Although podoplanin has been recognized to be a specific marker of lymphatic endothelial cells (Hirakawa et al., 2003), recent immunohistochemical analyses have revealed podoplanin reactivity in various normal as well as neoplastic tissues, including OSCC (Oku et al., 2008; González-Alva et al., 2009; Okamoto et al., 2009; Schacht et al., 2005). Enhanced expression of podoplanin has also been demonstrated at the invasive front of a number of human cancers including human squamous cell carcinoma (Schacht et al., 2005).

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In addition, it has been reported that podoplanin contributes to tumor metastasis through its platelet aggregation-inducing activity (Kaneko et al., 2004), and to tumor invasion by binding erzin, radixin and moesin proteins, leading to activation of RhoA and remodeling of the actin cytoskeleton of tumor cells through epithelial-mesenchymal transition (EMT) and /or collective cell migration (Martín-Villar et al., 2006; Wicki et al., 2006).

In the present study, we investigated the effects of BA/sodium butyrate (NaB) on podoplanin expression, cell migration and EMT in OSCC cell lines. BA/NaB increases podoplanin expression and cell migration in certain OSCC cell lines, suggesting the possibility that the periodontal disease promotes the progression of OSCC via a podoplanin-dependent pathway.

Materials and Methods

Cell lines and cell culture

The human gingival squamous cell carcinoma-derived cell line Ca9-22, and the human tongue squamous cell carcinoma-derived cell lines HSC-2, -3, and -4, were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Each cell line was routinely grown in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin-streptomycin (GIBCO Invitrogen, Carlsbad, CA), 10 U/ml fungizone (GIBCO Invitrogen), and 10% fetal bovine serum (GIBCO Invitrogen) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell growth inhibition test (MTT assay)

Each cell line was incubated with various concentrations of NaB (Wako, Osaka, Japan) in 96-well plates. The culture fluids were discarded after 24 h, and then 200 µl/well thiazolyl blue tetrazolium bromide (Sigma-Aldrich) solution (0.2 mg/ml) was added, and incubation was continued at 37°C for 4 h, followed by addition of 200 µl/well dimethyl sulfoxide (DMSO: Sigma-Aldrich). Absorbance values were detected with an autokinetic enzyme scaling meter at a wavelength of 540 nm.

Real-time quantitative RT-PCR

Total RNAs were extracted in accordance with the description supplied with Protein and RNA Extraction Kit for mammalian cells (PAREx; Takara, Tokyo, Japan) after each cell line had been treated with NaB, and adjusted to 0.1 µg/ml. Real-time RT-PCR was performed using a Thermal Cycler Dice Real Time System (Takara) in accordance with the standard protocol for the Dice. A One-Step SYBR PrimeScript RT-PCR Kit II (Takara) was used for the RT-PCR reaction. The primers, based on sequences for E-cadherin, vimentin, podoplanin and GAPDH, are shown in Table 1. Each PCR mixture (final reaction volume, 25 µl) contained 11.3 µl of sterile water, 12.5 µl of SYBR premix Ex Taq, 0.1 µl of forward primer (50 pmol/µl), 0.1 µl of reverse primer (50 pmol/µl), and 1·0 µl of cDNA product. PCR conditions were 95°C for 10s, followed by 40 cycles of 95°C for 5s, 60°C for 30s. Dissociation was performed according to a melting program.

Product Name	Sequences (5' → 3')		Accession Number
E-cadherin	Forward:	ggattgcaaattcctgccattc	NM_004360
	Reverse:	aacgttgtcccgggtgtca	
Vimentin	Forward:	tgagtaccggagacaggtgcag	NM_003380
	Reverse:	tagcagcttcaacggcaaagttc	
Podoplanin	Forward:	tgactccaggaaccagcgaag	AF390106
	Reverse:	gcgaatgcctgttacactgttga	
GAPDH	Forward:	gcaccgtcaaggctgagaac	NM_002046
	Reverse:	tggtgaagacgccagtgga	

Table 1: Base sequences of the primers.

Cell migration assay (scratch assay)

Cell lines were seeded in 12-well tissue culture slides at 5 x 10^5 cells/well. A scratch through the central axis of the plate was gently made using a pipette tip. The cells were then incubated with 0.3, 2.5 and 20 mM NaB. Migration of the cells into the scratch was observed and measured the distance of scratched area at 8 h.

siRNA for podoplanin (Santa Cruz biotechnology, Santa Cruz, CA) was diluted 1:2 with FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany), and mixed with RPMI1640 medium. Then 100 µl of mixture (final concentration of siRNA, 50 nM) was added to each well of a 12-well tissue culture slide.

Western blotting

Cellular proteins were extracted by using PAREx (Takara). Cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatants were recovered for determination of protein content. Aliquots were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto pure-nitrocellulose membranes (Bio-Rad, Hercules, CA). Specific proteins on the membranes were detected by incubation with a specific primary antibody against β-actin (Santa Cruz) or podoplanin (Santa Cruz) for 1 h at room temperature, followed by a species-specific secondary antibody conjugated with peroxidase and DAB/H_2O_2 solution.

Statistical analysis

Statistical significance was determined using Student's t-test. Values of $p<0.05$ were considered to be statistically significant.

Results

Effects of sodium butyrate (NaB) on cell proliferation

The Ca9-22, HSC-2, HSC-3, and HSC-4 cell lines were incubated with several concentrations of sodium butyrate (NaB) (0.3 - 20 mM) to examine the effect of NaB on cell proliferation. After 24 h of incubation, the proliferative activity of each cell line was assessed by MTT assay. In all the cell lines studied, cell proliferative activities were reduced by NaB in a dose-dependent manner (Figure 1). Cell proliferation was determined at three NaB concentration points; at 0.3 mM the proliferation of Ca9-22 cells was slightly increased, at 2.5 mM a difference in influence was found among the cell lines, and at 20 mM the proliferation of all four cell lines was predominantly suppressed.

Although DNAs isolated from each cell lines treated with NaB were electrophoresed in 2% agarose gel to investigate whether apoptosis or necrosis was occurred by NaB, any ladder or smear band were not observed same as control (data not shown).

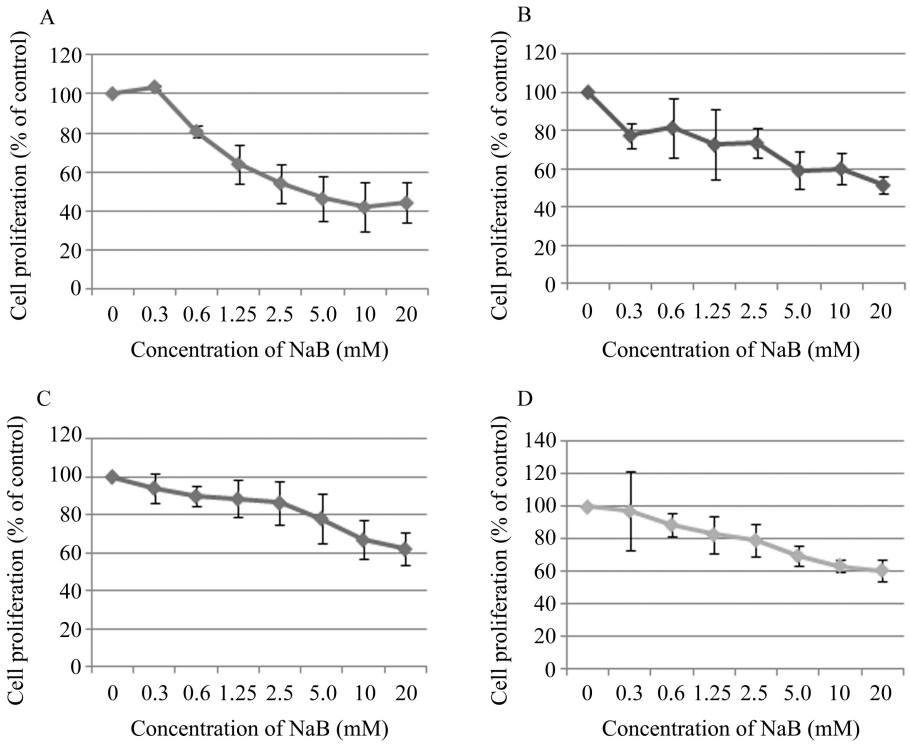

Figure 1: Effect of NaB on cell proliferation in the Ca9-22, HSC-2, -3, and -4 cell lines. Cell proliferation was assessed by MTT assay after 24 h of treatment with several concentrations of NaB. The experiment was performed at least three times for Ca9-22 (A), HSC-2 (B), -3 (C), and -4 (D). Each bar represents the mean ± SD of three separate experiments in duplicate samples.

Enhanced expression of podoplanin by NaB in HSC-2 and HSC-3 cells

After each cell line had been incubated with 0.3, 2.5 and 20 mM NaB for 4, 8, 12, and 24 h, real-time RT-PCR was performed using specific primers for podoplanin (Table 1). In HSC-2 cells, expression of podoplanin was increased after incubation with 2.5 mM NaB for 4 h (Figure 2B and Figure 2E). After 12 h incubation with 2.5 mM NaB, podoplanin expression was slightly increased in HSC-3 (Figure 2C and Figure 2E). In HSC-4 cells, expression of podoplanin was decreased by NaB in dose dependently (Figure 2D and Figure 2E). However, there were no significance effects on podoplanin expression in Ca9-22 (Figure 2A and Figure 2E).

Effect of NaB on cell migration

To examine whether NaB induces tumor cell migration by regulating the expression of podoplanin, each cell line was incubated on a plastic dish for the scratch assay. Cell migration was slightly promoted in HSC-3 cells by treatment with a low concentration of NaB, whereas it was inhibited in HSC-2 and HSC-4 cells (Figure 3B and Figure 3D). In Ca9-22 cells, NaB had no effect on cell migration (Figure 3A). siRNA for podplanin inhibited the migration of HSC-2, HSC-3, and HSC-4 cells after treatment with NaB, whereas there was no significant effect in Ca9-22 cells (Figure 3).

Western blot analysis was performed to examine whether siRNA inhibited the expression of podoplanin. Expression was inhibited in all cell lines studied, especially in HSC-3 (Figure 3E).

Effect of NaB on expression of RNA for E-cadherin and vimentin

There are two pathways that lead to progression of tumor invasion: cell migration, and EMT. Real-time RT-PCR was performed to examine the influence of NaB on the expression of E-cadherin as an epithelial marker and vimentin as a mesenchymal marker. Expression of E-cadherin was increased when Ca9-22 cells were incubated with 2.5 mM NaB for 4 and 12 h (Figure 4A), and when HSC-3 cells were incubated with 20 mM NaB for 24 h (Figure 4C). However, it was decreased in HSC-2 and HSC-4 cells (Figure 4B and Figure 4D). Vimentin expression was slightly increased when Ca9-22 cells were incubated with 0.3 mM NaB (Figure 4A) or when HSC-4 cells were incubated with 0.3 mM NaB for 12 h (Figure 4D), while there was no effect in HSC-2 cells (Figure 4B).

Discussion

Periodontal disease could be a risk factor for several systemic conditions such as a heart/vertebrate disease, diabetes, pneumonia and premature birth (Pihlstrom et al., 2005). Recent epidemiological studies (Michaud et al., 2008; Tezal et al., 2009) have suggested the association between periodontal disease and the risk of human malignant neoplasms including poorly differentiated oral squamous cell carcinoma (OSCC). Michaud et al. (2008) have described that periodontal disease might be a marker of a susceptible immune system or might directly affect cancer risk (Michaud et al., 2008).

Butyric acid (BA), an extracellular metabolite from periodontopathic bacteria, is thought to play an important role

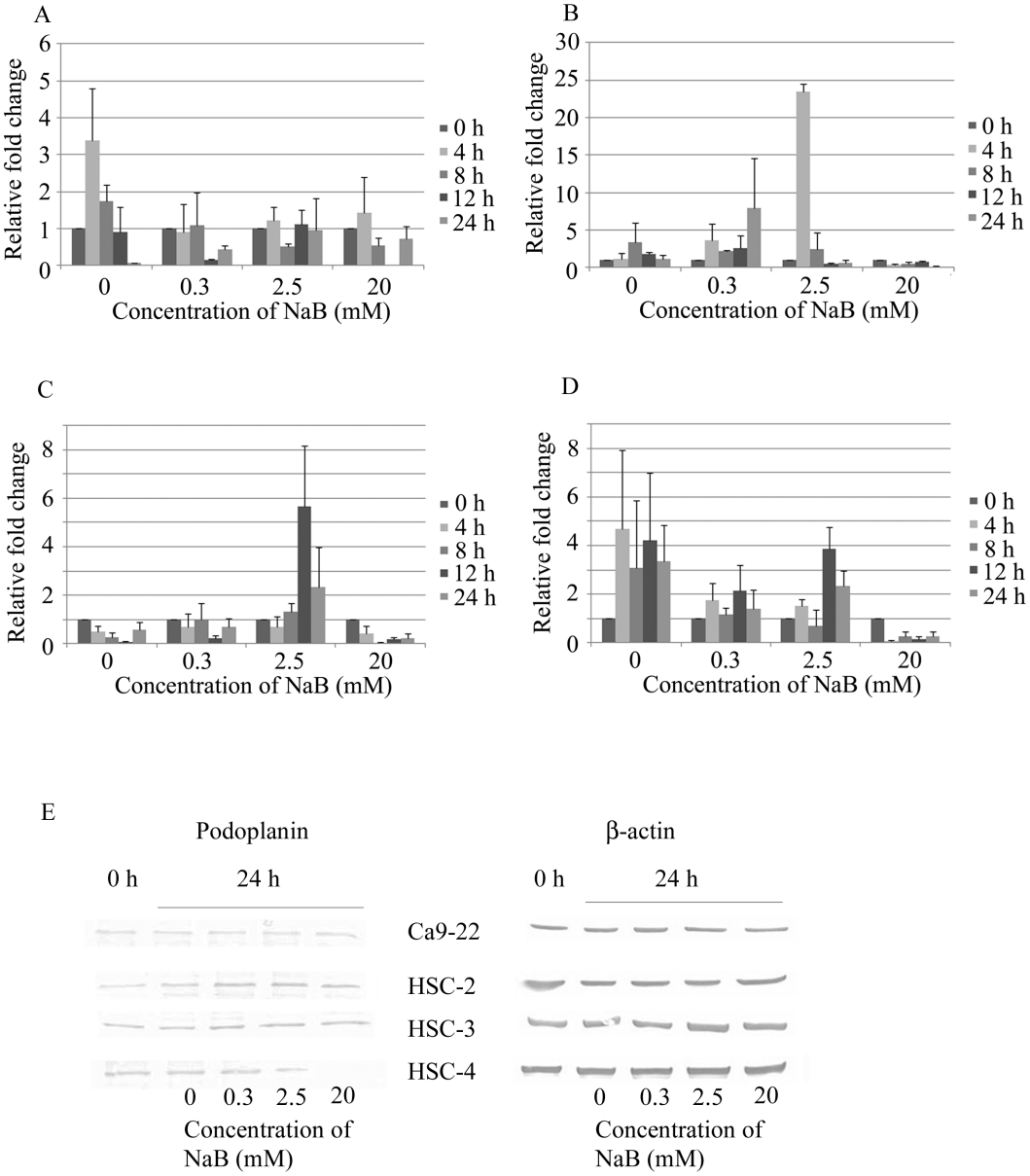

Figure 2: Effect of NaB on the expression of podoplanin. Expression of podoplanin was detected by real-time RT-PCR. Expression levels were normalized to that of the control RNA (0 mM). Total RNAs were prepared from Ca9-22 (A), HSC-2 (B), -3 (C), and -4 (D) cells treated with 0, 0.3, 2.5, and 20 mM NaB for 4, 8, 12, and 24 h. Podoplanin and β-actin expression were studied by western blot analysis after incubation of each cell line with NaB for 24 h (E). Each bar represents the mean ± SD of three separate experiments in duplicate samples.

in the progression of periodontitis through its contribution to destruction of gingival tissues and modulation of local immunity at gingival sites (Ochiai and Kurita-Ochiai, 2009). A recent study demonstrated that BA acts as a histone deacetylase inhibitor, and that periodontal disease could be as a risk factor for HIV-1 reactivation in infected individuals (Imai et al., 2009).

Furthermore, it has been reported that BA promotes the migration of normal as well as neoplastic epithelial cells (Pulukuri et al., 2007; Wilson and Gibson, 1997). The infiltration and extension of the junctional epithelium along the root caused by inflammatory stimuli from periodontopathic bacteria are essential events for the progression of chronic periodontitis (Pihlstrom et al., 2005). We previously reported that oral sulcular and junctional epithelia in severely inflamed gingiva strongly expressed podoplanin while the protein was not expressed in normal gingival epithelium (Miyazaki et al., 2009).

We have also demonstrated enhanced expression of podoplanin in limited neoplastic myoepithelial elements in pleomorphic adenoma of the salivary gland, peripheral columnar cells in ameloblastoma, and basal cells in keratocystic odontogenic tumor (Oku et al., 2008; González-Alva et al., 2009; Okamoto et al., 2009). Pleomorphic adenoma, ameloblastoma and keratocystic odontogenic tumor are characterized by benign, but also locally invasive, behavior (Eveson et al., 2005; Gardner et al., 2005; Philipsen, 2005).

In this study, we examined the effects of BA/sodium butyrate (NaB) on podoplanin expression, cell migration and EMT in 4 kinds of OSCC cell lines. The proliferative activities of HSC-2, -3 and -4 cells derived from lingual carcinomas were decreased by NaB in a dose-dependent manner. On the other hand, proliferation of Ca9-22 cells was slightly increased in the presence of a low concentration of NaB.

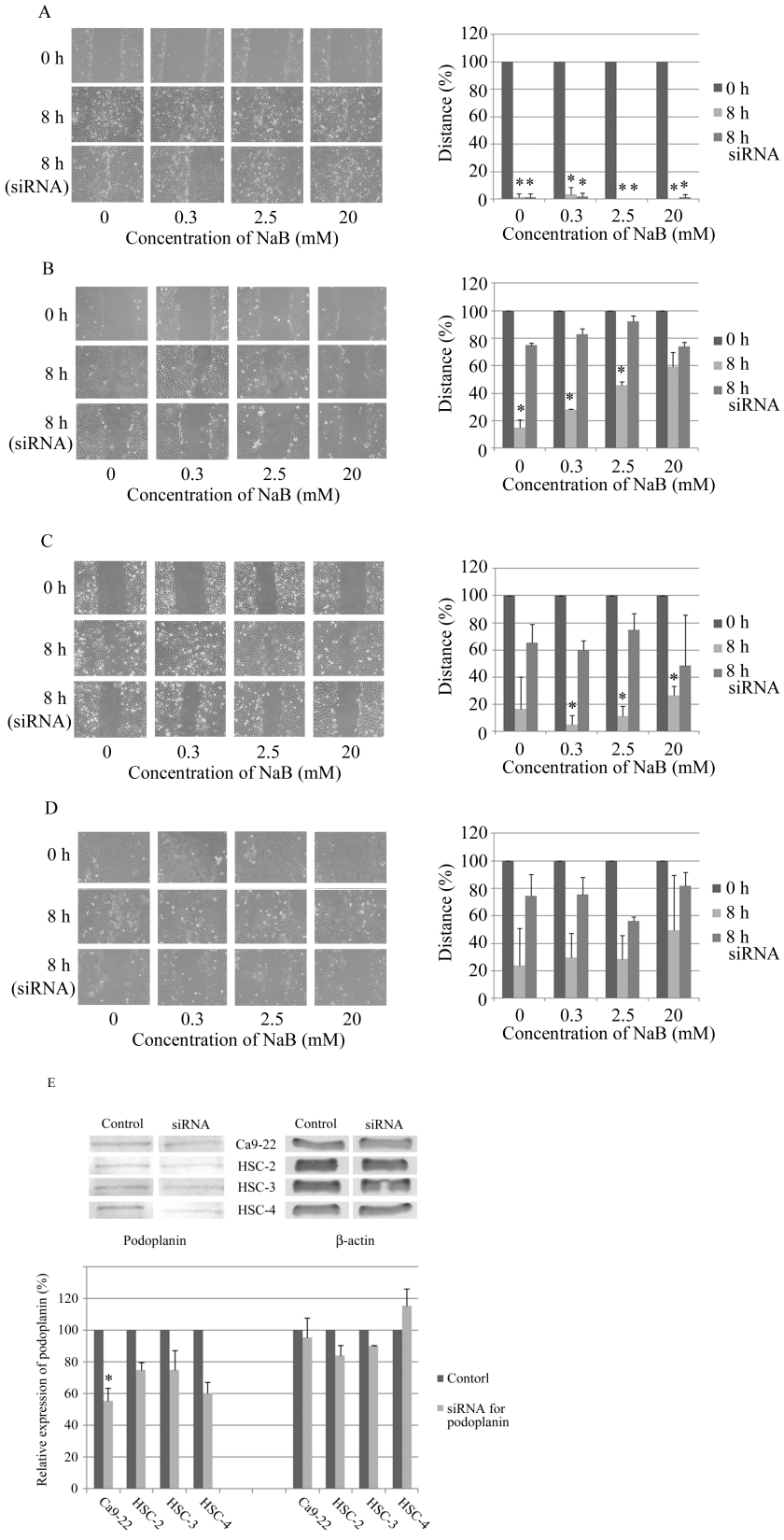

Figure 3: Effect of NaB and siRNA for podoplanin on cell migration and podoplanin expression. The scratch assay was performed to study the effect of NaB on cell migration. The Ca9-22 (A), HSC-2 (B), -3 (C), and -4 (D) cell lines were incubated into 12-well tissue culture slides with 0.3, 2.5, and 20 mM NaB with or without siRNA for podoplanin (50 nM). Pictures were taken at the beginning of the experiment (0 h), and after 8 h of incubation (8 h). Podoplanin and β-actin expression were studied by western blot analysis after incubation of each cell line with siRNA for podoplanin (50nM) for 8 h (E). *p<0.05 for NaB or siRNA treated versus control. Each bar represents the mean ± SD of two separate experiments.

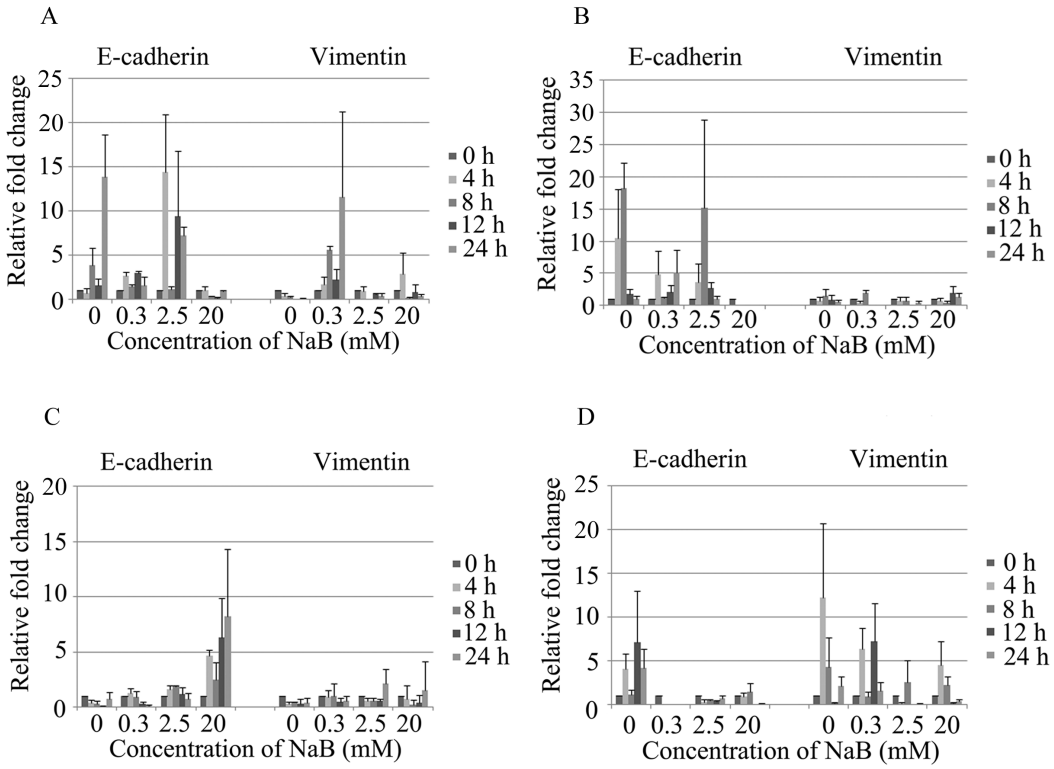

Figure 4: Effect of NaB on expression of RNA for E-cadherin and vimentin. Expression of RNA for E-cadherin and vimetin was detected by real-time RT-PCR. Expression levels were normalized to that of the control RNA (0 mM). Total RNAs were prepared from Ca9-22 (A), HSC-2 (B), -3 (C), and -4 (D) cells treated with 0, 0.3, 2.5 and 20 mM NaB for 4, 8, 12, and 24 h. Each bar represents the mean ± SD of three separate experiments in duplicate samples.

Enhanced expression of podoplanin in HSC-2 and HSC-3 cells by treatment with 2.5 mM NaB was detected by real-time RT-PCR. Furthermore, cell migration in these cell lines was accelerated at a low concentration of NaB, and was markedly suppressed by treatment with a siRNA specific for podoplanin. These results suggest that NaB/BA induces enhanced cell migration mediated by podoplanin in 2 lingual carcinoma derived cell lines. It has been reported that transcriptional factors such as c-fos and Sp1/Sp3 promote podoplanin expression, and that BA induces the expression or activation of these transcriptional factors (Durchdewald et al., 2008; Hantusch et al., 2007; Tichonicky et al., 1990).

Although NaB had no effect on podoplanin expression in Ca9-22 and HSC-4 cells, increased expression of vimentin in Ca9-22 cells and decreased expression of E-cadherin in HSC-4 cells were observed after treatment with NaB, suggesting the presence of podoplanin-independent incomplete EMT, in which the cells are partially changed towards EMT, in these cancer cells. Also, migration of HSC-4 cells was suppressed by a siRNA specific for podoplanin, suggesting podolanin dependent cell migration in this cell line.

There have been some reports that NaB can act as an anti-cancer agent because it inhibits cancer cell proliferation or induces apoptosis (Farrow et al., 2003; Wang et al., 1999). Although MTT assay had shown the decrease of cell proliferation, any ladder or smear band were not observed same as control by DNA electrophoresis. Another mechanism except apoptosis or necrosis may exist to reduce the cell proliferation, and it will be one of the future subjects to reveal it.

Podoplanin dependent progressive migration or incomplete EMT was found in the oral cancer cell lines used in the present study though NaB reduced proliferative activity. These findings suggest an association of BA produced by periodontopathic bacteria with the progression of oral cancers. Further study using normal keratinocytes is needed to examine the relevance of butyric acid produced by periodontal bacteria, and also podoplanin, in carcinoma progression and/or development.

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