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Epigenetic alteration of the purinergic type 7 receptor in salivary epithelial cells

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ABSTRACT

Purinergic receptors, particularly type 7 (P2RX7), are involved in apoptotic cell death. However, the expression and function of P2RX7 are suppressed in HSG cells. In the present study, we explored whether P2RX7 function is regulated by epigenetic alteration of the receptors in two different cell lines, HSG cells derived from human submandibular ducts, and A253 cells, originated from human submandibular carcinoma. We discovered that HSG cells expressed all subtypes of purinergic receptors, excluding P2RX7, at the mRNA level. However, treatment of the cells with 5-Aza-CdR, a DNA demethylating agent, increased the mRNA expression levels of P2RX7 in a time-dependent manner. Furthermore, 5-Aza-CdR completely rescued the calcium response induced by P2RX7 agonist BzATP, a response that was absent in untreated HSG cells. In contrast, A253 cells showed a moderate methylation pattern in the P2RX7 CpG island. Most CG pairs from the first to the 21st were methylated in untreated HSG cells, but 5-Aza-CdR-treatment partially demethylated the methylated CG pairs. We obtained similar results when investigated human tissues; the CG pairs in the P2RX7 CpG islands showed hypermethylation and hypomethylation patterns in human normal and cancer tissues, respectively. Our results suggest that the expression level and function of P2RX7 are regulated by DNA methylation in epithelial cells.

1. Introduction

DNA methylation is associated with the silencing of gene expression [1,2]. DNA methylation occurs at the 5-position of the cytosine ring in the context of CpG dinucleotides and is catalyzed by DNA methyltransferases. Epigenetic alterations target this region, thereby affecting gene expression [3]. Epigenetic alteration by DNA methylation is vital for development, and has an essential role in tumorigenesis and in the response to cancer therapy because it plays important roles in repressing the expression of tumor suppressor genes in cancer [4,5]. In general, the CpG islands in DNA often reside within gene promoters and extend downstream into transcribed regions. Recent studies have revealed that the promoters of transcriptionally activated genes are typically hypomethylated, while inactivated genes are silenced by DNA methylation of the CpG island [6–9].

5-Aza-2′-deoxycytidine (5-Aza-CdR, decitabine), an inhibitor of DNA methylation, is an S-phase-specific agent with a short *in vivo* half-life, and it rapidly reactivates the expression of genes that have undergone epigenetic silencing [10]. Whereas the covalent enzyme-DNA adduct might be responsible for cytotoxicity, particularly at high doses [11], a low dose of 5-Aza-CdR is believed to serve as a drug, inhibiting DNA methylation and even reactivating numerous genes that have been silenced by hypermethylation [12,13].

P2 receptors (P2Rs) are divided into P2Y metabotropic and P2X inotropic receptors involved in a complex signaling pathway that affects not only diverse cellular functions, such as exocrine and endocrine secretion, and inflammation, but also proliferation, differentiation, and cell death [7]. P2RX7, a member of the P2XR subfamily, is an ATP-gated plasma membrane ion channel that is ubiquitously expressed and is often overexpressed in various cancer cells such as melanoma and prostate cancer. P2RX7 is the best candidate for cancer-associated ATP effects, as it has shown an ability to confer a growth advantage to cancer cells in *in vivo* models

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[14–16]. Although the expression and activity of P2RX7 have been reported in several cancer types [6,17], the functional role of P2RX7, a subtype that seems to play a critical role in secretory epithelia, is still unknown. Particularly, the role of P2RX7 in salivary gland remains to be elucidated.

In this study, we examined whether epigenetic modification regulates the expression and function of P2RX7 in salivary epithelial cells. The results of the present study show a relationship between expression, function, and methylation status in the P2RX7 CpG island. It appears that the expression and function of P2RX7 is suppressed in normal cells by hypermethylation of the CpG island, as shown in the HSG cells.

2. Materials and methods

2.1. Reagents

5-Aza-2′-deoxycytidine (5-Aza-CdR) (Sigma Aldrich, St. Louis, MO, USA) and antibodies to P2RX7 (Abcam, Cambridge, UK) were used in the present study.

2.2. Cell culture

HSG cells derived from human submandibular ducts and A253 cells originating from human submandibular carcinoma were maintained in DMEM (Welgene, Daegu, South Korea) supplemented with 1% penicillin and streptomycin (Life Technologies, Seoul, Korea) and 10% fetal bovine serum (Welgene) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. Human tissue

Excised human salivary gland tissue was collected from the parotid gland of adult patient with malignant neoplasm. The human tissues were instantly placed in a cold (4 °C) physiological saline solution and kept for processing after surgical removal [18]. Human oral cancer tissues were used as control tissues. The usage of human tissue samples followed ethical guidelines and was approved by the Institutional Review Board of Seoul National University Dental Hospital (CRI11023G).

2.4. RT-PCR

A253 and HSG cells were collected for RNA isolation, and total RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcriptase (Invitrogen) was used with 1 μg of total RNA. The oligonucleotide primers were as follows: P2RX1: forward 5′-ggcactgcagacccatctat-3′ and reverse 5′-cccatgtcctcagcgtattt-3′; P2RX2: forward 5′-cttcctgtgcgactggatct-3′ and reverse 5′-cacactctgccccttgttg-3′; P2RX3: forward 5′-caaggattctgcccagagag-3′ and reverse 5′-agccgggtgaaggagtattt-3′; P2RX4: forward 5′-caccatgaaccagacacagg-3′ and reverse 5′-gagtacctgggcaagcagag-3′; P2RX5: forward 5′-tctgagtgccaccctcacta-3′ and reverse 5′-gatccgttccccttctgact-3′; P2RX6: forward 5′-agccacggtgtaaaaacagg-3′ and reverse 5′-aaggagtagtgaggccagca-3′; P2RX7: forward 5′-ttcctggacaaccagaggag-3′ and reverse 5′-tcctggtagagcaggaggaa-3′; and GAPDH: forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′. The amplification conditions were at 94 °C for 5 min by 35 cycles, at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. To estimate the mRNA levels of purinergic receptors, cDNA from A253 and HSG cells was amplified for 35 cycles using PCR (PTC-1148C; Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR products were subjected to electrophoresis using a Mupid®-2 plus electrophoresis system (OPTIMA, Tokyo, Japan) on 1.2% agarose gels (Sigma Aldrich), stained with 0.1 μg/ml ethidium bromide (Sigma Aldrich) and visualized directly under UV light with a bioimaging system (TS-312R; Spectroline, Westbury, NY) [18].

2.5. Quantitative real-time PCR

Total RNA was isolated from A253 and HSG cells using the Trizol reagent (Invitrogen), and 1 μg of total RNA was used to synthesize cDNA with Superscript II reverse transcriptase (Invitrogen) and oligo-(dT) primers according to the manufacturer's protocol [19]. The specific primers based on human P2RX7 cDNA sequences were as follows: forward 5′-ctcccatctcaactccctga-3′ and reverse 5′-tcctggtagagcaggaggaa-3′ and GAPDH: forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′. For quantitative real-time PCR, cDNA was amplified according to the manufacturer's SYBR PCR master mix protocol (Applied BioSystems, Foster City, CA, USA) using the ABI PRISM 7500 sequence detection system (Applied BioSystems).

2.6. Immunofluorescence confocal laser microscopy

For visualization of P2RX7, A253 and HSG cells were treated with a dimethylsulphoxide or 5-Aza-CdR vehicle on cell culture slides. The cells were rinsed three times with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature and then incubated at 4 °C overnight with anti-human P2RX7 (Abcam, Cambridge, UK) at a dilution of 1:200. After three rinses with PBS containing 1% bovine serum albumin, the cells were incubated for 1 h with an Alexa Fluor® 594 goat anti-rabbit IgG at a dilution of 1:200 The slides were then mounted by VECTASHIELD H-1200 with DAPI e (Vector Laboratories, Burlingame, CA, USA), and samples were visualized via confocal laser microscopy (Fluoview300; Olympus, Tokyo, Japan) [18].

2.7. Intracellular Ca^{2+} measurement

HSG cells were treated with fluorescent dye fura-2/AM (Molecular Probes, Eugene, OR, USA) at room temperature for 45 min. The intracellular Ca^{2+} was measured by MetaFluor® version 6.1 imaging system (Universal Imaging, West Chester, PA). In each experiment, about 10–15 cells were analyzed. The fluorescent dye fura-2/AM was recorded at excitation wavelengths of 340 and 380 nm, with an emission wavelength of 510 nm. The results are presented as 340 nm/380 nm ratios (Ca^{2+} fluorescence ratio, F340/F380) [18].

2.8. Quantification of global 5-methylcytosine content

Global 5-methylcytosine content in DNA was quantified from the genomic DNA of A253 and HSG cells using a MethylFlash Methylated DNA Quantification Colorimetric Kit (Epigentek, Farmingdale, NY, USA) [18]. The genomic DNA from A253 and HSG cells treated with a 5-Aza-CdR or dimethylsulphoxide vehicle was extracted with Qiagen Blood and Cell Culture DNA Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. Isolated DNA was used for the measurement of total DNA methylation level in the salivary gland epithelial cells.

2.9. Methylation-specific PCR and 5-Aza-CdR treatment

Bisulfite modification of genomic DNA was carried out using the EpiTect Bisulfite Kit (Qiagen). Methylation-specific PCR was achieved using the bisulfite-treated genomic DNA as the template and using specific primer sequences for the methylated or