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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.

Keywords

Purinergic receptor; Salivary glands; DNA methylation; Hypomethylation; Hypermethylation; 5-Aza-CdR

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Conflict of interest

The authors declare no conflicts of interest.

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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 ionotropic 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 [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% CO₂ 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 oligo-nucleotide primers were as follows: P2RX1: forward 5'-ggcactgcagaccatctat-3' and reverse 5'-cccatgtcctcagcgtattt-3'; P2RX2: forward 5'-cttcctgtgcgactgatct-3' and reverse 5'-cacactctgcccttgttg-3'; P2RX3: forward 5'-caaggattctgccagagag-3' and reverse 5'-agccgggtgaaggagtattt-3'; P2RX4: forward 5'-caccatgaaccagacacagg-3' and reverse 5'-gagtacctgggcaagcagag-3'; P2RX5: forward 5'-tctgagtccaccctcacta-3' and reverse 5'-gatccgttccccttctgact-3'; P2RX6: forward 5'-agccacggtgtaaaacagg-3' and reverse 5'-aaggagtagtggagccagca-3'; P2RX7: forward 5'-tcctggacaaccagaggag-3' and reverse 5'-tcctgtagagcaggaggaa-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'-ctccatctcaactccctga-3' and reverse 5'-tcctgtagagcaggaggaa-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 Ca²⁺ measurement

HSG cells were treated with fluorescent dye fura-2/AM (Molecular Probes, Eugene, OR, USA) at room temperature for 45 min. The intracellular Ca²⁺ 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 (Ca²⁺ 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 unmethylated forms of the gene. A253 and HSG cells were treated with 5-Aza-CdR at a 10 µM concentration for one, two, three, or four days, after which time the media was changed every 24 h. Genomic DNA and total RNA were isolated using the QIAamp DNA Blood Mini Kit and the RNeasy Mini Kit (Qiagen) [18]. The methylation-specific primers used were as follows: M forward 5'-AGAGTTTGGTTTAGTTTCGC-3'; M reverse 5'-AAAACACGTCTCAAATTCGA-3'; U forward 5'-AGTAGAGTTTTGGTTTAGTTTTGT-3'; U reverse 5'-AAAACACATCTCAAATTCAAAA-3'.

2.10. Sequencing analysis after bisulfite treatment

In total, 2 µg of genomic DNA from HSG cells, A253 cells, and human tissues was subjected to sodium bisulfite conversion reaction, followed by use of the modified genomic DNA for PCR using a specific primer set as follows: forward 5'-tggtttgtaggaagagta-3' and reverse 5'-aaataccaacataatcacc-3'. The amplification conditions were performed as follows: 94 °C for 5 min, 35 cycles of 94 °C for 40 s, and 55 °C for 40 s, and then a final 72 °C for 1 min. The PCR products were purified with a PCR Purification Kit (Qiagen) and ligated into a pGEM-T easy vector by using the TA cloning system (Promega, Madison, WI, USA). Plasmid DNA was then isolated using a Plasmid DNA Purification Kit (iNtRON Biotechnology, Sungnam, Korea) [18]. Five independent clones were sequenced for each type of cell and tissue.

2.11. Statistical analysis

All experiments were performed in triplicate and statistical analyses were performed using Excel 2010. Statistical significance was evaluated using the Student's unpaired t-test. Asterisks indicate statistically significant *P*-values of less than 0.05 (**P* < 0.05).

3. Results

3.1. Subtypes of purinergic receptors in A253 and HSG cells

We first used RT-PCR to investigate mRNA expression levels of purinergic receptor subtypes in two different salivary gland epithelial cells, A253 and HSG cells. A253 cells were used as a positive control since all subtypes of purinergic receptors from type 1 to type 7 are expressed in the A253 cells. HSG cells also expressed all subtypes of purinergic receptors, excluding type 7, which was only barely expressed in HSG cells (Fig. 1A). Fig. 1B shows the summarized results of the RT-PCR experiments (*n* = 3, ****P* < 0.001). We next studied whether the low expression level of P2RX7 mRNA is related with DNA methylation of P2RX7 CpG islands.

3.2. Induction and localization of P2RX7 in HSG cells by 5-Aza-CdR-treatment

We next investigated whether the low expression of P2RX7 mRNA can be restored by 5-Aza-CdR treatment. Fig. 1C shows RT-PCR results over time. We incubated HSG cells with 10 µM 5-Aza-CdR. After 24 h of treatment with 5-Aza-CdR, the mRNA expression level of P2RX7 was dramatically increased for up to 4 days of incubation. Three days after culturing, the mRNA expression level of P2RX7 in HSG cells was very similar to that of the A253 cells. We also analyzed the expression level of P2RX7 mRNA using real-time PCR (*n* = 3, **P* < 0.05, ***P* < 0.01, ****P* < 0.001). The results showed a pattern similar to that seen in the results for RT-PCR (Fig. 1D).

3.3. Restoration of P2RX7 function by 5-Aza-CdR-treatment

We also examined the increase in P2RX7 protein expression by 5-Aza-CdR and further confirmed the RT-PCR results with a confocal microscope study. Although P2RX7 was strongly expressed in membranes of A253 cells (Fig. 2A), it was hardly expressed at all in HSG cells (Fig. 2B). However, after three days of treatment with 10 µM 5-Aza-CdR, the

expression level of P2RX7 in HSG cells was significantly increased in the membrane (Fig. 2C). These results demonstrate that the expression of P2RX7 is suppressed in HSG cells by hypermethylation.

We then measured intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) from fura-2-loaded cells in order to verify that the P2RX7 protein had retained its function after demethylation. Treatment with 100 μM BzATP, a P2RX7 agonist, showed little effect on the calcium response in HSG cells (Fig. 2D), although these cells responded well to 100 μM CCh, a muscarinic agonist. However, three days after treatment of HSG cells with 10 μM 5-Aza-CdR, BzATP consistently evoked a calcium response (Fig. 2E). These results suggest that 5-Aza-CdR treatment restores the P2RX7-mediated response to BzATP in HSG cells.

3.4. Global DNA methylation and hypermethylation of the P2RX7 CpG island in HSG cells

Our data shows that hypermethylation in the CpG island might have occurred in the P2RX7 protein in HSG cells. Therefore, we next studied the level of the global DNA methylation marker, 5-methylcytosine (5-mC), in HSG cells before and after treatment with 5-Aza-CdR. In A253 cells, which were used as a control, 5-mC content was significantly higher than in HSG cells (Fig. 3A). The treatment of HSG cells with 5-Aza-CdR reduced the 5-mC content in proportion to the incubation time. Two days after treatment with 5-Aza-CdR, the 5-mC content was dramatically reduced compared to untreated HSG cells ($n = 3$, $*P < 0.05$, $**P < 0.01$). These results indicate that the global DNA of the A253 cells is hypermethylated compared to HSG cells, while the expression and function of P2RX7 are specifically hypermethylated in HSG cells. Next, the methylation pattern of the P2RX7 CpG island was studied using methylation-specific PCR (MSP) in A253, HSG, and 5-Aza-CdR-treated HSG cells. Three days of 5-Aza-CdR treatment strongly induced a non-methylation-specific band, which was not detected in the untreated HSG cells (Fig. 3B). This result demonstrates that the expression and function of P2RX7 is partly regulated by the methylation pattern of the P2RX7 CpG island.

3.5. Bisulfite sequencing of the P2RX7 CpG island before and after 5-Aza-CdR treatment

Finally, we used bisulfite sequencing to investigate the methylation status within the P2RX7 CpG island in HSG cells before and after treatment with 5-Aza-CdR, as well as in human tissues. We found 21 CG pairs in the P2RX7 gene from positions -61 to +480 relative to the start codon of exon 1, a region downstream of the active promoter (Fig. 4A). In the A253 cells used as a positive control, the CG pairs showed a hypomethylation pattern compared with the untreated HSG cells, and the CG pairs in untreated HSG cells were heavily methylated from the first to 21st CGs, as we expected (Fig. 4B). However, in 5-Aza-CdR-treated HSG cells, a portion of CG pairs that were methylated in untreated HSG cells were demethylated (Fig. 4C). We next examined the methylation status of the P2RX7 CpG islands in cancer and normal human tissues (Fig. 4D). Interestingly, the results were very similar to the results seen in A253 and HSG cells. We found that the CG pairs in the P2RX7 CpG island were hypomethylated in human cancer tissue and hypermethylated in normal tissue compared to each other. These findings suggest that the P2RX7 CpG island is hypermethylated in HSG cells and in normal human tissue compared with A253 cells and human cancer tissues, which showed a hypo-methylated pattern.

4. Discussion

Epigenetics concerns heritable changes in gene expression that do not involve changes to the underlying DNA sequence [20–23]. The abnormal gene expression caused by epigenetic change is a regular and natural occurrence, but can also be influenced by several other factors such as age, disease, and the environment. Epigenetic studies on processes, including but not limited to histone modification, non-coding RNA-associated gene silencing, and impaired DNA methylation leading to aberrant hypermethylation or hypomethylation of gene CpG islands, are continuously uncovering the role of epigenetics in cancer initiation and progression [24].

Here, we applied an epigenetic approach using a comparison of A253 cells to the HSG cell line [25], well-established as an *in vitro* model for salivary gland secretion. Although HSG cells possess muscarinic receptor subtypes, they lack mRNA and functional expression of P2RX7 compared with A253 cells, which originated from human submandibular carcinoma. Thus, HSG cells do not respond to BzATP, which increases $[Ca^{2+}]_i$. P2RX7 belongs to the family of P_2 -purinergic receptors for ATP, but P2RX7 receptors respond more readily to BzATP than to ATP. Activation of purinergic receptor subtypes increases the intracellular concentration of Ca^{2+} , a second messenger with a key role in the regulation of salivary gland secretion [26]. It has been established that P2RX7 plays a crucial role in the secretory mechanism of fluid by increasing $[Ca^{2+}]_i$. However, the expression level and function of P2RX7 in salivary gland cells, such as A253 and HSG cells, are still poorly understood. Thus, we hypothesized that the hypomethylation and/or hypermethylation of the P2RX7 gene are responsible for the overexpression and suppression of P2RX7 function in salivary gland cells (A253 and HSG).

In our study, a portion of the 21 methylated CG sites were demethylated by 5-Aza-CdR-treatment, which resulted in the rescue of P2RX7 function, as determined by the HSG cells' ability to react to BzATP with a calcium response. Several previous studies support our results. Expression of the P2RX7 receptor is controlled by the hypermethylation of the CpG island's CG sites located within a 547-nt region downstream of the active promoter [27]. It is of note that demethylation occurring at even a few sites among the total 21 methylated CG sites increases the activity of the P2RX7 gene, leading to the increased expression of the P2RX7 protein. It is not certain, however, whether a particular CG site needs to be demethylated for gene activation or if demethylation of any of the CG sites leads to the restoration of P2RX7 function.

To the best of our knowledge, this study is the first to report on epigenetic regulation of the P2RX7 gene expression in both salivary gland cells and salivary gland carcinoma cells. This study shows the effects of 5-Aza-CdR on the suppressed expression and function of P2RX7 in salivary gland cells (HSG), as well as the hypomethylation pattern of P2RX7 genes, and its relation to salivary gland carcinoma cells (A253) and human cancer tissue.

In summary, our study showed that the function of P2RX7 is suppressed in normal cells by DNA hypermethylation of the CpG island. Demethylation by 5-Aza-CdR in the CpG island of P2RX7 restores not only the expression but also the function of this protein, as evidenced

by the increase in $[Ca^{2+}]_i$ in response to the stimulation of treated cells with BzATP, a specific P2RX7 agonist. In contrast, both a salivary gland cancer cell line and human cancer tissue showed hypomethylation patterns in the CpG island of P2RX7. Therefore, we conclude that P2RX7 function is suppressed by hypermethylation of the P2RX7 CpG island in normal salivary cell lines and in normal human tissues in order to maintain the integrity and function of the salivary glands.

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Fig. 1. mRNA expression of purinergic receptor subtypes and induction of P2RX7 in A253 and HSG cells. (A) mRNA expression of purinergic receptors in A253 and HSG cells. A253 cells were used as a positive control. (B) Summarized results of RT-PCR experiments. The thickness of each band was measured using a densitogram. Vertical bars in the graphs represent the mean $\pm$ standard deviation (SD) of mRNA level calibrated to the amount of GAPDH mRNA as determined using RT-PCR. Experiments were repeated three times with essentially identical results ($n = 3$, *** $P < 0.001$). Expression of P2RX7 mRNA in A253 and HSG cells treated with 5-Aza-CdR (10 μ M) for 24, 48, 72, or 96 h, measured using RT-PCR (C) and real-time PCR (D). All experiments were performed in triplicate ($n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig. 2.

Restoration of P2RX7 protein and function by 5-Aza-CdR-treatment. Immunostaining was used to detect the subcellular localization of P2RX7 in A253 and HSG cells before and after 5-Aza-CdR-treatment. (A) Expression of P2RX7 in the membranes of A253 cells. (B) Expression of P2RX7 in HSG cells before 5-Aza-CdR-treatment. (C) Expression of P2RX7 in HSG cells after 5-Aza-CdR-treatment. (Original magnification, $\times 600$) (D) Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) in fura-2-loaded cells. $[Ca^{2+}]_i$ response induced by BzATP, a P2RX7 agonist, was used to determine the function of P2RX7 in untreated and treated HSG cells. A representative $[Ca^{2+}]_i$ response to 100 μ M BzATP and 10 μ M CCh in HSG cells. (E) A representative $[Ca^{2+}]_i$ response to 100 μ M BzATP in HSG cells treated with 5-Aza-CdR.

Fig. 3. Methylation levels in total DNA and the P2RX7 CpG island by 5-Aza-CdR-treatment. (A) Global DNA hypomethylation by 5-Aza-CdR treatment in HSG cells. The total DNA was extracted from control A253 and HSG cells before and after 5-Aza-CdR treatment, and quantitative determination of 5-methylcytosine content was performed. (B) In the hypermethylated HSG cells, hypomethylation was induced by 5-Aza-CdR. Methylation-specific PCR was accomplished with modified DNA samples by sodium bisulfite from 5-Aza-CdR-treated or untreated P2RX7 cells. M indicates PCR products of the methylated P2RX7 CpG island. UM indicates PCR products of the unmethylated P2RX7 CpG island. 5-Aza-CdR treatment induced a non-methylation-specific band in the PCR results from treated HSG cells. All experiments were performed in triplicate (* $P < 0.05$, ** $P < 0.01$).

Fig. 4.

Bisulfite sequencing of the P2RX7 CpG island before and after 5-Aza-CdR-treatment. (A) The CpG island of the P2RX7 (GenBank no. Y12851.1) was analyzed. This sequence spans bp between positions -61 to +480 relative to the start codon, including 21 CGs in the exon 1 coding region, which is downstream of the active promoter. The start codon (ATG) in exon 1 is indicated. The CG sites within the sequence of the P2RX7 CpG island are indicated in bold and by underlining. TIS, transcription initiation site. (B) Bisulfite sequencing of the P2RX7 CpG island in A253 cells was used as a positive control. (C) Bisulfite sequencing of the P2RX7 CpG island in HSG cells. DNA was from HSG cells either before or after 5-Aza-CdR treatment. (D) Bisulfite sequencing of the P2RX7 CpG island in human normal tissue and cancer tissue. Symbols: ○, unmethylated cytosines; ●, methylated cytosines.