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Original contribution

Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells

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Summary Extracellular adenosine regulates a wide variety of physiological processes by interacting with 4 adenosine receptor subtypes: A1, A2A, A2B, and A3. However, little is known of their pathophysiological roles in human cancers. In this study, we examined the expression pattern of adenosine receptors in various colorectal tissues and human colon carcinoma cell lines and investigated the biologic functions regarding colon carcinogenesis. Using reverse transcriptase polymerase chain reaction and Western blotting, we found that adenosine receptor A2B (ADORA2B) was consistently up-regulated in colorectal carcinoma tissues and colon cancer cell lines compared with normal colorectal mucosa. In immunohistochemistry, we observed diffuse immunopositivity of ADORA2B in *67% of colorectal adenocarcinomas (39/58)*, *17% of tubular adenomas (5/30)*, and 0% of normal colon glands (0/62). During a hypoxic state, there was also a significant induction of ADORA2B expression in the messenger RNA level at 8 hours of incubation and in the protein level at 24 hours of incubation in colon carcinoma cell lines. To examine the function of ADORA2B, we applied an ADORA2B-selective antagonist (MRS1754) to the colon carcinoma cells, which significantly inhibited cell growth in a dose-dependent manner as demonstrated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay. In conclusions, ADORA2B was overexpressed in colorectal carcinomas grown under a hypoxic state, presumably promoting cancer cell growth. Our data suggest that this adenosine receptor is a potential therapeutic target for colorectal cancer.

1. Introduction

Adenosine regulates a wide variety of physiological processes by interacting with adenosine receptors. Adenosine receptors are G protein-coupled purinergic receptors, generally regarded as 4 subtypes referred to as adenosine receptor A1 (ADORA1), A2A (ADORA2A), A2B (ADORA2B), and A3 (ADORA3). Each adenosine receptor is encoded by a separate gene with different functions and tissue distribution [1]. ADORA1 together with ADORA2A play roles in the heart, regulating myocardial oxygen consumption and coronary blood flow. Both ADORA1 and ADORA2A also have important roles in the brain, regulating the release of neurotransmitters such as dopamine and glutamine [2-5].

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Furthermore, ADORA2A has antiinflammatory effects throughout the body [6]. ADORA2B and ADORA3 are mainly peripherally located and are involved in processes such as inflammation and immune response.

Pharmacological tools such as radioligands, selective agonists, and selective antagonists of adenosine receptors have revealed detailed signal transduction of each adenosine receptor subtype. Activation of ADORA1 and ADORA3 decreases cyclic adenosine monophosphate (cAMP) concentration and raises intracellular Ca^{2+} levels through a pathway involving phospholipase C activation [7,8]. On the other hand, the ADORA2A and ADORA2B subtypes are associated with stimulatory G-proteins, and activation of these 2 receptor subtypes causes activation of adenylate cyclase and phospholipase C [9]. These findings imply that extracellular adenosine, as a ligand of adenosine receptors, has different biologic effects depending on the expression and the distribution of adenosine receptor subtypes.

More recently, the adenosine-adenosine receptor pathway has been shown to modulate cell proliferation and differentiation, and apoptosis of tumor cells [10-12]. Reports indicate that adenosine accumulates in solid tumors, and a high concentration of adenosine stimulates tumor growth and tumor angiogenesis [11,13]. Also, cell surface CD73, which produces extracellular adenosine via ecto-5′-nucleotidase activity, is increased in human cancer cells [14]. Furthermore, some studies showed pro- or antimitogenic effects of ADORA1, ADORA2A, and ADORA3 subtypes [15].

Previous investigations demonstrated an association between ADORA1 and carcinogenesis; expression of this receptor was demonstrated in the human leukemia Jurkat and human melanoma A375 cell lines [16-18]. Researchers found ADORA2A on cell membranes of different human tumor cell lines including neuroblastoma and malignant melanoma [17-19]. Adenosine was found to exert its effects on proliferation and cell death mainly through the ADORA3 subtype, which is present in different cell types including astroglial, leukemia, and melanoma cells [17,18,20,21].

There is growing evidence that the adenosine-adenosine receptor pathway may provide promising therapeutic targets in a wide range of conditions such as cerebral and cardiac ischemia, nervous system disorders including dementia and Parkinson disease, and immune and inflammatory disorders [22]. At present, however, few therapeutic targets in the fight against human cancers are available from the "adenosinergic system."

To investigate the pathophysiological roles of adenosine receptors in human colorectal cancers, we evaluated the expression profiles of all 4 adenosine receptor subtypes in various colorectal tissues by reverse transcriptase polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry. Then, we examined the expression profile of adenosine receptors in human colon carcinoma cell lines under normoxic or hypoxic conditions. Moreover, we studied the effect of selective antagonism against adenosine receptors on cell proliferation and on cell invasion of the colorectal cancers.

2. Materials and methods

2.1. Case selection

We studied *88 surgical specimens*, including *tubular adenomas (30 cases)* and *tubular adenocarcinomas (58 cases)*, of the colorectum from routine surgical pathology files at University of Yamanashi Hospital, Yamanashi, Japan. Hematoxylin-eosin–stained slides of all cases were reviewed, and the diagnosis was made on the basis of the World Health Organization Classification [23]. Samples of colon mucosa, obtained 8 to 10 cm distant from the colon carcinoma were used as normal colon mucosa (62 cases). The institutional ethics board of the University of Yamanashi approved the protocols.

2.2. Cell lines and cell culture

Human colorectal adenocarcinoma-derived cell lines, DLD1, SW480, HCT-15, LOVO, and COLO205 cells (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan), were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, streptomycin sulfate (100 μg/mL), and penicillin (100 U/mL). Cells were cultured in a standard humidified incubator at 37°C in a 5% carbon dioxide atmosphere.

The hypoxic condition (1% O_2) was prepared by using an oxygen absorber–CO_2 generator (Mitsubishi Gas Chemical, Tokyo, Japan) in a humidified cell culture incubator according to the manufacturer's protocol.

For ADORA2B inhibition, we used MRS1754 (Sigma, St Louis, MO), a *p*-cyanoanilide xanthine derivative, which is a selective antagonist for the ADORA2B subtype having very low affinity for ADORA1, ADORA2A, and ADORA3 subtypes of humans and rats.

2.3. RNA extraction and RT-PCR

Total RNA was isolated from frozen human colon tissues and cultured cells using TRIzol (Invitrogen). Complementary DNA (cDNA) was generated using the TaqMan RT reagent kit (Applied Biosystems, Branchburg, NJ). Specific PCR primers targeted for *ADORA1*, *ADORA2A*, *ADORA2B*, *ADORA3*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, as an internal control) were designed as listed in Table 1. We performed amplification using HotStarTaq DNA polymerase kit (Qiagen, Tokyo, Japan). PCR conditions were as follows: (1) 95°C for 15 minutes; (2) 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; (3) 72°C for 10 minutes; and (4) 4°C hold. Negative controls