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Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase

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Abstract

Extracellular adenosine significantly reduced cell viability in a dose (0.1–20 mM)- and treatment time (24–72 h)-dependent manner in GT3-TKB cells, a human gastric cancer cell line. Nuclei of cells were reactive to Hoechst 33342, a marker of apoptosis, and an anti-single-stranded DNA. Adenosine-induced GT3-TKB cell death was significantly inhibited by dipyridamole, an inhibitor of adenosine transporter, and 5′-amino-5′-deoxyadenosine, an inhibitor of adenosine kinase, but the effect was not affected by theophylline, a broad inhibitor of adenosine receptors, 8-cyclopentyltheophylline, an inhibitor of A_1 adenosine receptors or 3,7-dimethyl-1-propargylxanthine, an inhibitor of A_{2a} adenosine receptors. Adenosine had no effect on mitochondrial membrane potentials. The effect of adenosine on GT3-TKB cell death was not inhibited by a pancaspase inhibitor or inhibitors of caspase-1,-3,-4,-8, and -9. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMP-activated protein kinase (AMPK), significantly reduced GT3-TKB cell viability, but the AICAR action was not reinforced in the presence of adenosine. The results of the present study, thus, suggest that extracellular adenosine induces apoptosis in GT3-TKB cells by its uptake into cells and conversion to AMP followed by activation of AMPK, regardless of caspase activation linked to the mitochondria and the endoplasmic reticulum.

Keywords: Adenosine; GT3-TKB cells; Apoptosis; AMP; AMP-activated protein kinase

1. Introduction

Adenosine is ubiquitously present in a wide range of organs and tissues. In the central nervous systems, adenosine exhibits a variety of neuromodulatory actions, that include fine tuning in the excitatory and inhibitory neurotransmissions via the adenosine receptors, A_1, A_{2a}, A_{2b}, and A_3 receptors [1], or exerts its neuroprotective action against ischemic insult by increasing production of the energy source ATP [2].

Recent studies have shown that adenosine induces apoptosis in various cell types via receptor-mediated and non-receptor-mediated pathway [3–7]. For the extrinsic pathway, A_{2b} and A_3 adenosine receptors appear to bear apoptosis in arterial smooth muscle cells, glial cells and glomerular mesangial cells [4,6,7]. For the intrinsic pathway, extracellular adenosine seems to induce apoptosis in epithelial cancer cells originated from the breast, the colon, and the ovary or neuroblastoma cells by its uptake and conversion to AMP [3,5]. A central question, however, remains to be answered about the downstream signaling pathway. Then, we hypothesized that AMP-activated protein kinase (AMPK) might be involved in the apoptosis. AMPK, that is composed of the catalytic subunit, α subunit, and the non-catalytic subunits, β and γ subunits, is activated along an increase in intracellular AMP levels under a variety of conditions [8,9]. AMPK increases intracellular ATP levels by stimulating fatty acid oxidation

Abbreviations: AIF, apoptosis-inducing factor; AMPK, AMP-activated protein kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate-buffered saline; ssDNA, single-stranded DNA; 8-CPT, 8-cyclopentyltheophylline; DMPX, 3,7-dimethyl-1-propargylxanthine; EHNA, erythro-9 (2-hydroxy-3-nonyl)-adenosine; AMDA, 5′-amino-5′-deoxyadenosine; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ER, endoplasmic reticulum

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or cellular glucose uptake [8]. AMPK also inhibits ATP consumption by inhibiting acetyl-CoA carboxylase for fatty acid synthesis or 3-hydroxy-3-methyl-CoA reductase for cholesterol synthesis [8]. Interestingly, AMPK is shown to suppress protein synthesis in skeletal muscle cells by down-regulating mammalian target of rapamycin or to induce apoptosis in liver cells by activation of c-Jun [10,11].

The present study aimed at understanding the mechanism of adenosine-induced cell death in the human gastric cancer cell line, GT3-TKB cells. We show here that extracellular adenosine induces GT3-TKB cell apoptosis via an intrinsic pathway independent of caspase activation and that AMPK may play a significant role in the adenosine action.

2. Materials and methods

2.1. Materials

3,7-Dimethyl-1-propargylxanthine (DMPX), 5′-amino-5′-deoxyadenosine (AMDA), and Dulbecco's modified eagles medium were purchased from Sigma. 8-Cyclopentyltheophylline (8-CPT) was from Biomol Research Laboratories. Erythro-9 (2-hydroxy-3-nonyl)-adenosine (EHNA) was from Calbiochem. Dipyridamole was from ICN Biomedicals. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was from Dojindo. Theophylline and dimethylformamide were from Wako. Vyvrant™ apoptosis assay kit was from Molecular Probes. Adenosine and streptavidin biotin complex peroxidase kit were from Nacalai Tesque. DePsipher™ kit was from Trevigen. Z-VAD-FMK, Z-WEHD-FMK, Z-DEVD-FMK, Z-YVAD-FMK, Z-IETD-FMK, and Z-LEHD were from R&D Systems. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) was from Toronto Research Chemicals. GT3-TKB cells were obtained from RIKEN cell bank. An anti-single-stranded DNA (ssDNA) antibody was a gift from Dr. Toshihiro Sugiyama (Akita University School of Medicine).

2.2. Cell culture

GT3-TKB cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. MTT assay

Cell viability was evaluated by a dye staining method using MTT [12]. Cells were incubated in 100 μl of culture medium without serum containing MTT (250 μg/ml) at 37 °C for 3 h. The reaction was stopped by adding 20% (w/v) sodium dodesyl sulfate and 50% (v/v) dimethylformamide diluted with water, and the terminated reaction mixtures stood at room temperature for 24 h. Then, MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, USA).

2.4. Fluorescent microhistochemistry

After washing with cold phosphate-buffered saline (PBS), cells were incubated in 1 ml PBS containing each 1 μl of the fluorescent dyes, propidium iodide (1.0 mg/ml) and Hoechst 33342 (5.0 mg/ml) on ice for 30 min. Reactions to each dye were detected with a fluorescent photomicroscope (ECLIPSE TE300, NIKON Co., Japan) equipped with an epifluorescence device.

2.5. Immunohistochemical staining

GT3-TKB cells were fixed with 4% paraformaldehyde at room temperature for 1 h and washed twice with cold PBS. After inactivation of intracellular peroxidase activity with 0.1% H_2O_2 for 15 min, cells were reacted to a rabbit polyclonal antibody against ssDNA using a biotinylated anti-rabbit IgG antibody and a streptavidin biotin complex peroxidase kit.

2.6. Assay of mitochondrial membrane potentials

Mitochondrial membrane potentials were measured using a DePsipher™ kit. After washing with cold PBS, cells were incubated in a DePsipher™ solution at 37 °C for 20 min. Then, cells were washed with 1 ml of a reaction buffer containing a stabilizer solution. The fluorescent signals were obtained with a fluorescent photomicroscope (ECLIPSE TE300, NIKON Co., Japan) equipped with an epifluorescence device using a fluorescine long-pass filter (fluorescine and rhodamine) at an absorbance of 590 nm for red aggregations and at an absorbance of 530 nm for green aggregations.

3. Results

3.1. Extracellular adenosine-induced apoptosis

Our first attempt was to assess the effect of extracellular adenosine on GT3-TKB cell viability with an MTT assay. Adenosine significantly reduced cell viability in a dose-dependent manner at concentrations ranged from 0.1 to 20 mM and in a treatment time-dependent manner at duration ranged from 24 to 72 h (Fig. 1), suggesting that adenosine induces cell death. We subsequently examined whether the cell death is necrosis or apoptosis. In the phase-contrast microscopic study, cells were treated with adenosine (5 mM) for 48 h shrank and in spite of seeding at