Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 2005–2011
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

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

Masaru Saitoh[a,b], Kaoru Nagai[a], Kazuhiko Nakagawa[b], Takehira Yamamura[b], Satoshi Yamamoto[a], Tomoyuki Nishizaki[a,*]

[a]*Department of Physiology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan*
[b]*Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan*

Received 28 November 2003; accepted 20 January 2004

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

* Corresponding author. Tel.: +81-798-45-6397; fax: +81-798-45-6649.
E-mail address: tomoyuki@hyo-med.ac.jp (T. Nishizaki).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.01.020

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

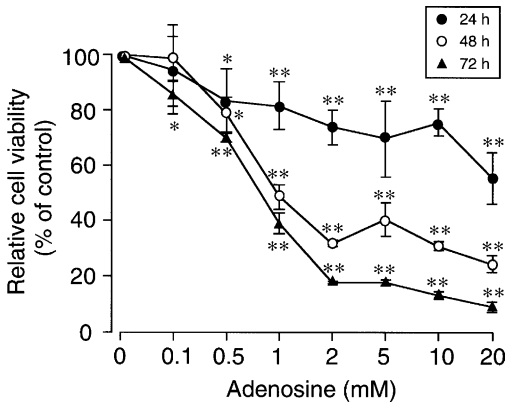

Fig. 1. Effects of extracellular adenosine on GT3-TKB cell viability. Cells were treated with adenosine at concentrations as indicated for 24–72 h, and cell viability was quantified with an MTT assay. Data represent the mean (±S.E.M.) percentage of control ($n = 4$). $^{*}P < 0.1$, $^{**}P < 0.01$, non-paired t test.

the same cell density, the number of cells markedly decreased as compared with untreated cells (Fig. 2A and D). In the fluorescent microscopic study, nuclei of cells treated with adenosine stained with propidium iodide (Fig. 2E), a marker of cell death [13], and Hoechst 33342 (Fig. 2F), a marker of apoptosis [14], while nuclei of untreated cells were not stained with each fluorescent dye (Fig. 2B and C). This suggests that extracellular adenosine reduces GT3-TKB cell viability by inducing apoptotic cell death. To obtain further evidence for this, we carried out immunohistochemical analysis using an anti-ssDNA antibody [15]. Nuclei of cells treated with adenosine were condensed and reactive to an anti-ssDNA antibody (Fig. 3). Notably, collapsed nuclei were found after treatment with much higher concentration of adenosine (10 mM) (Fig. 3), supporting the idea that extracellular adenosine induces apoptosis in GT3-TKB cells.

3.2. No implication of adenosine receptors in extracellular adenosine-induced cell death

The next question to address is as to whether adenosine-induced cell death is mediated via adenosine receptors or not. The adenosine action on GT3-TKB cell viability was not inhibited by theophylline, a broad inhibitor of adenosine receptors (Fig. 4A), 8-CPT, an inhibitor of A_1 adenosine receptors (Fig. 4B), or DMPX, a relative specific inhibitor of A_{2a} adenosine receptors (Fig. 4C). This would rule out the possibility for the implication of adenosine receptors in extracellular adenosine-induced GT3-TKB cell apoptosis.

3.3. Effects of EHNA, dipyridamole, and AMDA on extracellular adenosine-induced cell death

If the adenosine action on GT3-TKB cell death is not mediated via adenosine receptors, then adenosine should exert its action via an intrinsic pathway. Adenosine-induced cell death was not affected by EHNA [16], an

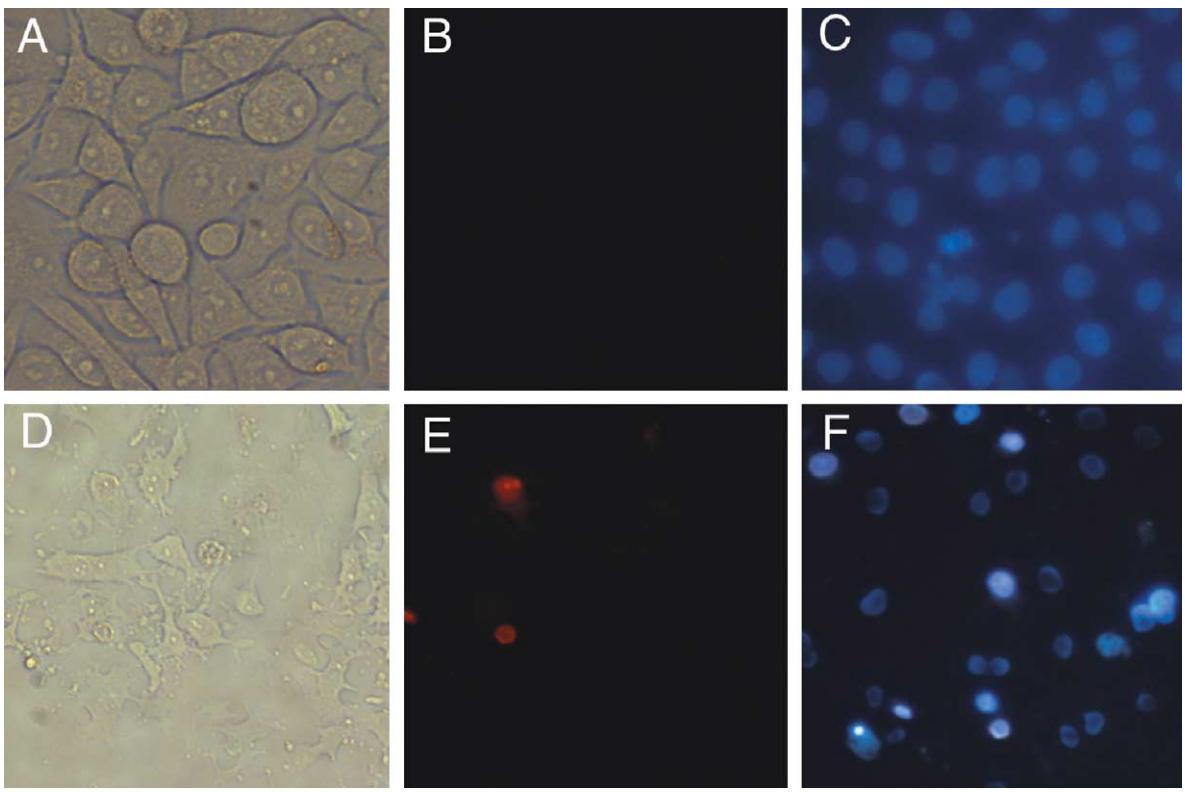

Fig. 2. Fluorescent microhistochemistry. GT3-TKB cells were incubated in the absence (A–C) and presence of adenosine (5 mM) for 48 h (D–F), and stained with propidium iodide or Hoechst 33342. (A and D) no dye; (B and E) propidium iodide; (C and F) Hoechst 33342.

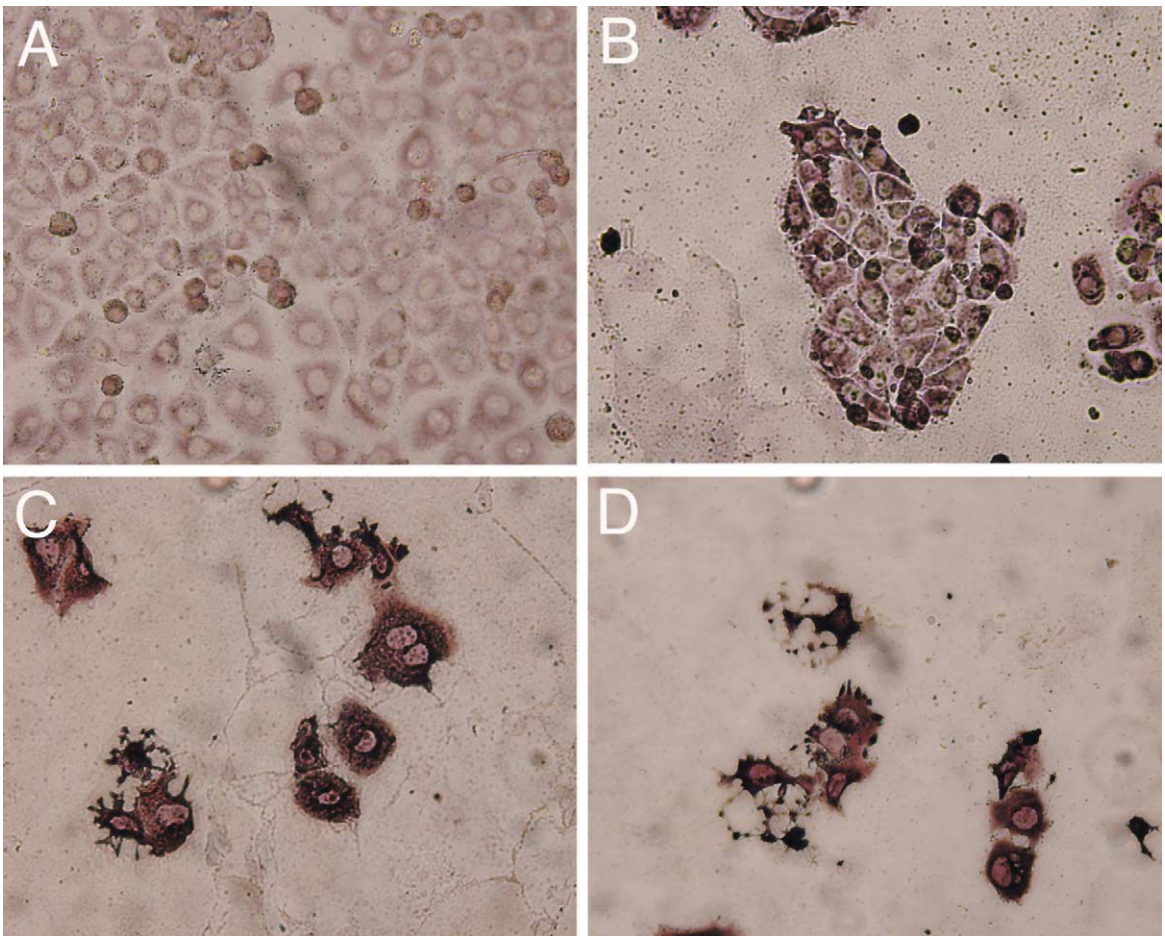

Fig. 3. Immunohistochemistry using an anti-ssDNA antibody. GT3-TKB cells were incubated in the absence (A) and presence of adenosine for 48 h (B, for 1 mM; C, for 5 mM; D, for 10 mM). Note the same magnification (200×) between them.

inhibitor of adenosine deaminase (Fig. 5A), indicating that it is not caused by the adenosine metabolites, inosine and hypoxanthine. The adenosine action, on the other hand, was significantly inhibited by dipyridamole [17], an adenosine transporter inhibitor (Fig. 5B), or AMDA [18], an adenosine kinase inhibitor (Fig. 5C). It is suggested from these results that extracellular adenosine induces apoptosis in GT3-TKB cells by adenosine uptake into cells and the ensuing AMP conversion.

3.4. Effect of extracellular adenosine on mitochondrial membrane potentials

It is well-recognized that mitochondria are the central executioners of apoptotic cell death. To ascertain whether extracellular adenosine induces GT3-TKB cell death via a mitochondrial pathway, we assayed mitochondrial membrane potentials. DePsipher^{TM}, a mitochondrial activity marker, has the properties of aggregating upon membrane polarization forming an orange-red fluorescent compound. If the potential is disturbed, the dye has no access to the transmembrane space and remain in or reverts to its green monomeric form. For both the cells untreated and treated with adenosine for 48 h, mitochondria exhibited orange-red fluorescent signals and there was no accumulation of green fluorescent signals (Fig. 6), indicating that extracellular adenosine does not perturb mitochondrial functions; in other words, adenosine-induced GT3-TKB cell death is not mediated via a mitochondrial pathway.

3.5. Effects of caspase inhibitors on extracellular adenosine-induced cell death

Adenosine-induced GT3-TKB cell death was not inhibited by Z-VAD-FMK, a pancaspase inhibitor, Z-WEHD-FMK, a caspase-1 inhibitor, Z-DEVD-FMK, a caspase-3 inhibitor, Z-YVAD-FMK, a caspase-4 inhibitor, Z-IETD-FMK, a caspase-8 inhibitor, or Z-LEHD, a caspase-9 (Fig. 7). This would imply that the adenosine action is independent of caspase, although the possibility for the implication of caspase-2,-5,-6,-7,-10,-11,-12,-13, or -14 is not ruled out.

3.6. Effect of AICAR on extracellular adenosine-induced cell death

AICAR [19], an activator of AMPK, significantly reduced GT3-TKB cell viability, reaching $45 \pm 1\%$ of

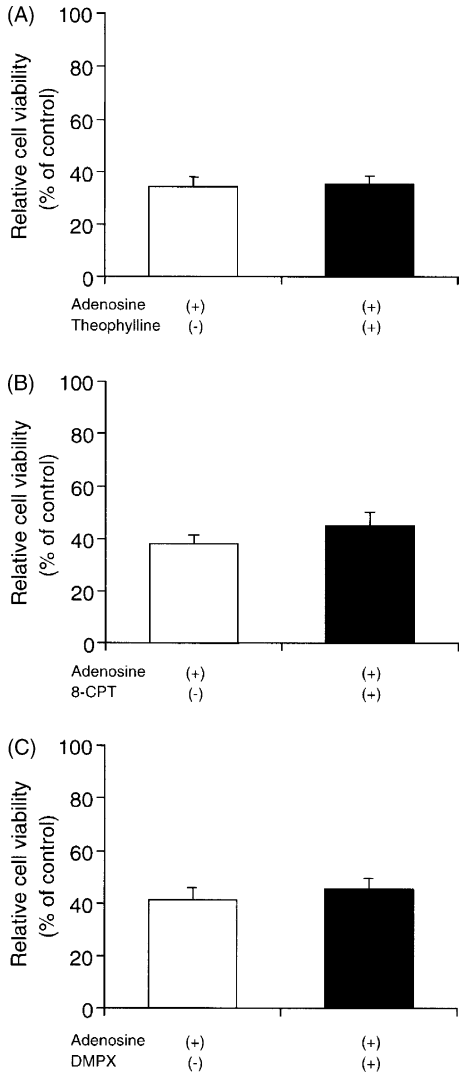

Fig. 4. Effects of theophylline, 8-CPT, and DMPX on adenosine-induced cell death. GT3-TKB cells were treated with adenosine (5 mM) in the presence and absence of theophylline (100 μM) (A), 8-CPT (10 μM) (B), or DMPX (100 μM) (C) for 48 h. Data represent the mean (±S.E.M.) percentage of control ($n = 4$).

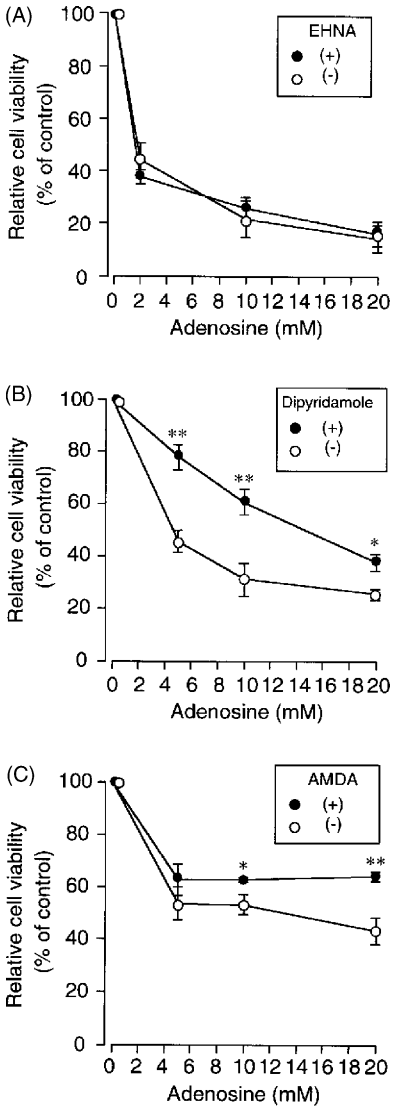

Fig. 5. Effects of EHNA, dipyridamole, and AMDA on adenosine-induced cell death. GT3-TKB cells were treated with adenosine at concentrations as indicated for 48 h in the presence and absence of EHNA (10 μM) (A), dipyridamole (100 μM) (B), or AMDA (100 μM) (C) for 48 h. Data represent the mean (±S.E.M.) percentage of control ($n = 4$). $^{*}P < 0.1$, $^{**}P < 0.01$, non-paired t test.

control (Fig. 8). A higher concentration of adenosine (5 mM) reduced the cell viability to a level similar to that achieved by AICAR alone and the AICAR action was not enhanced by adding adenosine at concentrations ranged from 1 to 5 mM (Fig. 8), suggesting a common pathway in GT3-TKB cell death between adenosine and AMPK. Overall, extracellular adenosine appears to induce apoptosis in GT3-TKB cells by its uptake, AMP conversion, and the following activation of AMPK.

4. Discussion

In the present study, extracellular adenosine significantly reduced GT3-TKB cell viability. Additionally, nuclei of cells treated with adenosine were reactive to Hoechst 33342 and an anti-ssDNA antibody. These observations indicate that extracellular adenosine induces apoptosis in GT3-TKB cells.

Apoptosis is induced by multiple mechanisms. One of the major pathways is mediated via mitochondria, and in response to apoptotic stimuli, proapoptosis-inducing factors such as cytochrome c, apoptosis-inducing factor (AIF), Smac/DAIBLO, and Omi/HtrA2, involving caspase-dependent and -independent apoptosis, were released from mitochondria [20–25]. Extracellular adenosine here did not change mitochondrial membrane potentials and the adenosine effect on the cell death was not prevented by inhibitors of caspase-3, -8, and -9, that are linked to mitochondrial apoptosis, suggesting that mitochondria are not involved in adenosine-induced GT3-TKB cell apoptosis. Several avenues of evidence have pointed to

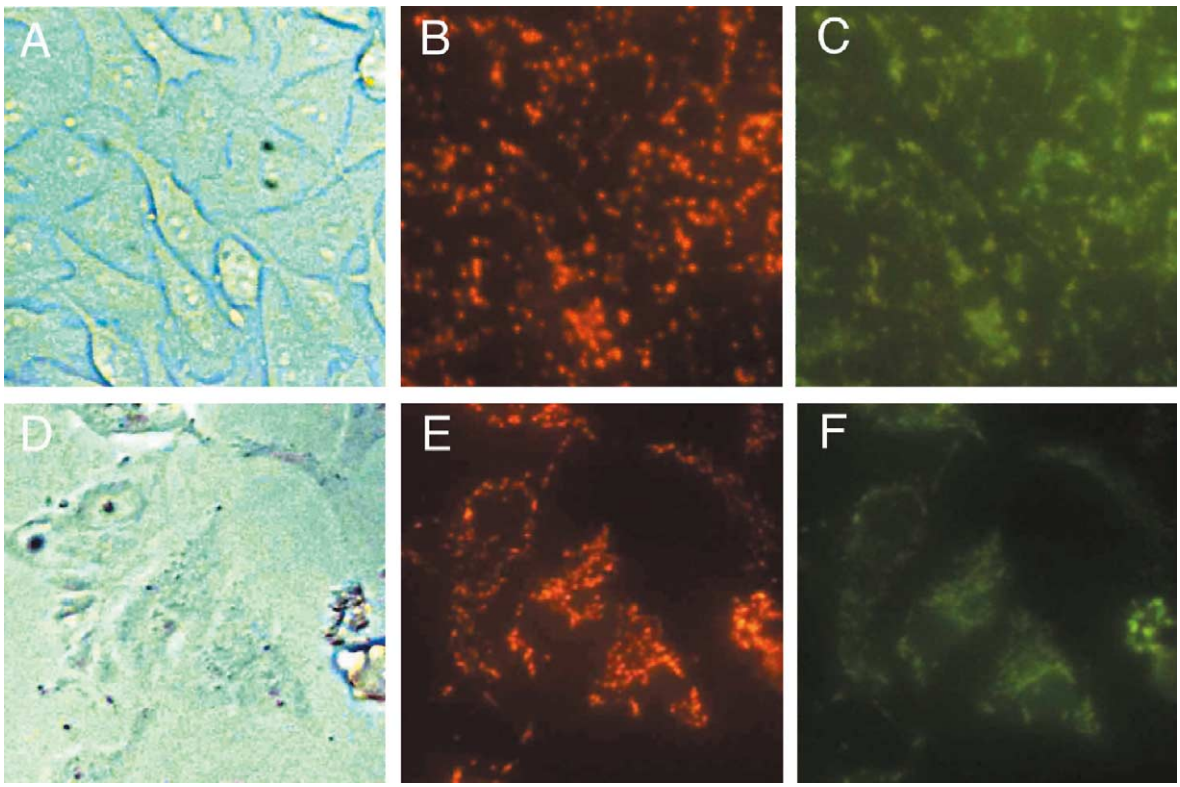

Fig. 6. Effect of extracellular adenosine on mitochondrial membrane potentials in GT3-TKB cells. Cells were incubated in the absence (A–C) and presence of adenosine (5 mM) for 48 h (D–F). (A and D) non-fluorescent image; (B and E) fluorescent image at an absorbance of 590 nm; (C and F) fluorescent image at an absorbance of 530 nm.

endoplasmic reticulum (ER) stress in apoptotic pathways [26–28]. We have found that tunicamycin, a blocker of *N*-glycosylation, or thapsigargin, an inhibitor of Ca^{2+}/ATPase pump, that causes ER stress, activates caspase-4 in the human colonic cancer cell line, Caco-2 cells (unpublished data). This accounts for the implication of caspase-4 in ER stress apoptosis. A caspase-4 inhibitor, however, failed to inhibit adenosine-induced GT3-TKB cell death, excluding the possibility for an ER pathway in the adenosine action. Taken together, it appears that extracellular adenosine induces apoptosis in GT3-TKB cells, regardless via a mitochondrial and ER pathway. The finding that adenosine-induced cell death was not inhibited by a pancaspase inhibitor also suggests that the cell death is induced in a caspase-independent manner, although the implication of caspase-2,-5,-6,-7,-10,-11,-12,-13, or -14 is not excluded.

The effect of adenosine on GT3-TKB cell death was not affected by theophylline, 8-CPT or DMPX, indicating no mediation of adenosine receptors. In contrast, it was

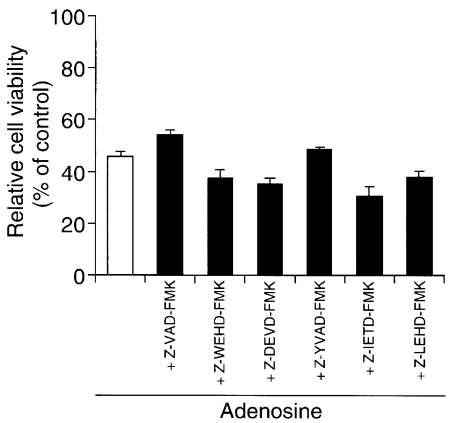

Fig. 7. Effects of caspase inhibitors on adenosine-induced cell death. GT3-TKB cells were treated with adenosine (5 mM) in the presence and absence of caspase inhibitors as indicated at a concentration of 20 μM for 48 h. Data represent the mean (±S.E.M.) percentage of control ($n = 4$).

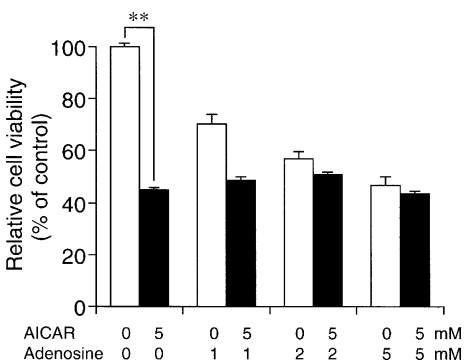

Fig. 8. Effects of AICAR on adenosine-induced cell death. GT3-TKB cells were treated with adenosine at concentrations as indicated together with and without AICAR (5 mM). Data represent the mean (±S.E.M.) percentage of control ($n = 4$). $^{**}P < 0.01$, non-paired t test.

significantly inhibited by dipyridamole or AMDA, suggesting that extracellular adenosine induces apoptosis by its uptake into cells through adenosine transporters and the ensuing conversion to AMP by adenosine kinase, leading to an increase in intracellular AMP levels. Then, one would wonder what signaling events following AMP elevation participate in the cell death. A plausible target is AMPK, since AMPK is activated along an intracellular AMP rise under conditions such as hypoxia/hypoglycemia, ischemia, exercise, and heat shock [8,9]. In support of this idea, AICAR, to activate AMPK, markedly reduced GT3-TKB cell viability. Furthermore, a higher concentration of adenosine exhibited an effect similar to AICAR- and AICAR-induced cell death was not reinforced by adding adenosine, suggesting that extracellular adenosine induces GT3-TKB cell death by a mechanism shared with the AICAR action. AMPK, thus, may be a critical factor in extracellular adenosine-induced GT3-TKB cell apoptosis. What functions downstream AMPK, however, still leaves an open question.

In conclusion, the results of the present study suggest that extracellular adenosine induces apoptosis in GT3-TKB cells via an intrinsic pathway relevant to adenosine uptake/conversion to AMP/intracellular AMP rise/AMPK activation. The results also suggest that the adenosine action is independent of caspase and that it is not mediated via a mitochondrial and ER pathway. This may represent a new mechanism underlying apoptotic cell death.

References

[1] Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 2001;24:31–55.

[2] Mori M, Nishizaki T, Okada Y. Protective effect of adenosine on the anoxic damage of hippocampal slice. Neuroscience 1992;46:301–17.

[3] Barry CP, Lind SE. Adenosine-mediated killing of cultured epithelial cancer cells. Cancer Res 2000;60:1887–94.

[4] Peyot ML, Gadeau AP, Dandré F, Belloc I, Dupuch F, Desgranges C. Extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A_{2b}-purinoceptor. Circ Res 2000;86:76–85.

[5] Schrier SM, van Tilburg EW, van der Meulen H, Ijzerman AP, Mulder GJ, Nagelkerke JF. Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells: studies on involvement of adenosine receptors and adenosine uptake. Biochem Pharmacol 2001;61:417–25.

[6] Appel E, Kazimirsky G, Ashkenazi E, Kim SG, Jacobson KA, Brodie C. Roles of BCL-2 and caspase 3 in the adenosine A3 receptor-induced apoptosis. J Mol Neurosci 2001;17:285–92.

[7] Zhao Z, Kapoian T, Shepard M, Lianos EA. Adenosine-induced apoptosis in glomerular mesangial cells. Kidney Int 2002;61:1276–85.

[8] Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 1998;67:821–55.

[9] Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen ZP, Witters LA. Dealing with energy demand: the AMP-activated protein kinase. Trends Biochem Sci 1999;24:22–5.

[10] Meisse D, Van de Casteele M, Beauloye C, Hainault I, Kefas BA, Rider MH, et al. Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. FEBS Lett 2002;526:38–42.

[11] Bolster DR, Crozier SJ, Kimball SR, Jefferson LS. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J Biol Chem 2002;277:23977–80.

[12] Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 1997;69:581–93.

[13] Walsh GM, Dewson G, Wardlaw AJ, Levi-Schaffer F, Moqbel R. A comparative study of different methods for the assessment of apoptosis and necrosis in human eosinophils. J Immunol Methods 1998;217:153–63.

[14] Chiu L, Cherwinski H, Ransom J, Dunne JF. Flow cytometric ratio analysis of the Hoechst 33342 emission spectrum: multiparametric characterization of apoptotic lymphocytes. J Immunol Methods 1996;189:157–71.

[15] Watanabe I, Toyoda M, Okuda J, Tenjo T, Tanaka K, Yamamoto T, et al. Detection of apoptotic cells in human colorectal cancer by two different in situ methods: antibody against single-stranded DNA and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) methods. Jpn J Cancer Res 1999;90:188–93.

[16] Pragnacharyulu PV, Varkhedkar V, Curtis MA, Chang IF, Abushanab E. Adenosine deaminase inhibitors: synthesis and biological evaluation of unsaturated, aromatic, and oxo derivatives of (+)-erythro-9-(2′*S*-hydroxy-3′*R*-nonyl)adenine [(+)-EHNA]. J Med Chem 2000;43:4694–700.

[17] Meester BJ, Shankley NP, Welsh NJ, Meijler FL, Black JW. Pharmacological analysis of the activity of the adenosine uptake inhibitor, dipyridamole, on the sinoatrial and atrioventricular nodes of the guinea-pig. Br J Pharmacol 1998;124:729–41.

[18] Wiesner JB, Ugarkar BG, Castellino AJ, Barankiewicz J, Dumas DP, Gruber HE, et al. Adenosine kinase inhibitors as a novel approach to anticonvulsant therapy. J Pharmacol Exp Ther 1999;289:1669–77.

[19] Kimura N, Tokunaga C, Dalal S, Richardson C, Yoshino K, Hara K, et al. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. Genes Cells 2003;8:65–79.

[20] Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 1996;86:147–57.

[21] Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999;397:441–6.

[22] Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33–42.

[23] Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102:43–53.

[24] Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 2001;8:613–21.

[25] Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem 2002;277:432–8.

[26] Chen L, Gao X. Neuronal apoptosis induced by endoplasmic reticulum stress. Neurochem Res 2002;27:891–8.

[27] Oubrahim H, Chock PB, Stadtman ER. Manganese(II) induces apoptotic cell death in NIH3T3 cells via a caspase-12-dependent pathway. J Biol Chem 2002;277:20135–8.

[28] Rao RV, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, del Rio G, et al. Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. J Biol Chem 2002;277:21836–42.