Original Paper

Adenosine Deaminase Inhibitor EHNA Exhibits a Potent Anticancer Effect Against Malignant Pleural Mesothelioma

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Key Words

Adenosine deaminase inhibitor • EHNA • Malignant pleural mesothelioma • Apoptosis

Abstract

Background/Aims: Malignant pleural mesothelioma (MPM) is an aggressive malignant tumor and an effective therapy has been little provided as yet. The present study investigated the possibility for the adenosine deaminase (ADA) inhibitor EHNA as a target of MPM treatment.

Methods: MTT assay, TUNEL staining, monitoring of intracellular adenosine concentrations, and Western blotting were carried out in cultured human MPM cell lines without and with knocking-down ADA. The *in vivo* effect of EHNA was assessed in mice inoculated with NCI-H2052 MPM cells. **Results:** EHNA induced apoptosis of human MPM cell lines in a concentration (0.01-1 mM)- and treatment time (24-48 h)-dependent manner, but such effect was not obtained with another ADA inhibitor pentostatin. EHNA increased intracellular adenosine concentrations in a treatment time (3-9 h)-dependent manner. EHNA-induced apoptosis of MPM cells was mimicked by knocking-down ADA, and the effect was neutralized by the adenosine kinase inhibitor ABT-702. EHNA clearly suppressed tumor growth in mice inoculated with NCI-H2052 MPM cells. **Conclusion:** The results of the present study show that EHNA induces apoptosis of MPM cells by increasing intracellular adenosine concentrations, to convert to AMP, and effectively prevents MPM cell proliferation. This suggests that EHNA may be useful for treatment of the tragic neoplasm MPM.

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Introduction

Malignant pleural mesothelioma (MPM), a highly aggressive neoplasm, has been increasing in incidence globally [1]. Previous exposure to asbestos fibers essentially contributes to the pathogenesis of MPM, which is characterized by insidious growth and chemotherapy resistance with poor prognosis. In spite of extensive and intensive challenges, MPM therapy has been still limited to marginally effective chemotherapy and morbid surgery.

Accumulating evidence has pointed to adenosine-induced apoptosis in a variety of cancer cells, largely as mediated through intrinsic and/or extrinsic pathways. For the intrinsic pathway, extracellular adenosine is taken into cells by adenosine transporters and converted to AMP by adenosine kinase to activate AMP-activated protein kinase (AMPK), responsible for apoptosis in GT3-TKB human lung cancer cells and HuH-7 human hepatoma cells [2, 3]. Intracellularly transported adenosine also induces apoptosis in a caspase-dependent or -independent manner in HuH-7, HepG2 human hepatocellular carcinoma, MCF-7 human breast cancer, or human MPM cells [3-8]. For the extrinsic pathway, adenosine receptors, which include A_1 , A_{2a} , A_{2b} , and A_3 receptors, mediate apoptosis. Adenosine induces apoptosis in CW2 human colonic cancer cells and RCR-1 rat astrocytoma cells via A_1 adenosine receptor [9, 10]. Adenosine induces apoptosis in Caco-2 human colonic cancer and HepG2 cells via A_{2a} adenosine receptor [11, 12]. A_3 adenosine receptor mediates apoptosis in human lung cancer cells, hepatocellular carcinoma cells, thyroid cancer cells, breast cancer cells, renal cancer cells, bladder cancer cells, and MPM cells by the diverse independent mechanisms [13-20].

Adenosine is broken down into inosine by adenosine deaminase (ADA). ADA inhibition should raise adenosine concentrations by preventing adenosine breakdown. Then, we postulated that the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) could exert its antitumor action against a variety of cancers. We show here that EHNA has the potential to induce apoptosis of MPM cells and suppress tumor growth in mice inoculated with NCI-H2052 cells.

Materials and Methods

Cell culture

Human MPM cell lines such as NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.003% L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml). Human lung cancer cell lines A549, SBC-3, and Lu-65 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). A549 and SBC-3 cells were grown in minimum essential medium (MEM) containing 0.1 mM non-essential amino acids and Lu-65 cells in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (final concentration, 100 U/ml) and streptomycin (final concentration, 0.1 mg/ml). Human hepatic cancer cell lines HepG2 and HuH-7 cells, obtained from RIKEN cell bank (Ibaraki, Japan), were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (final concentration, 100 U/ml) and streptomycin (final concentration, 0.1 mg/ml). All the cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Assay of cell viability

Cell viability was evaluated by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed to detect *in situ* DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized cells were

reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37 °C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510, Carl Zeiss Co., Ltd., Oberkochen, Germany).

Construction and transfection of siRNA

The siRNA to silence human ADA-targeted gene (*ADA* siRNA) and the negative control siRNA (*NC* siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The *ADA* siRNA or the *NC* siRNA was reverse-transfected into cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Western blotting

Cells transfected with the *ADA* siRNA or the *NC* siRNA were lysed in lysis buffer [150 mM NaCl, 20 mM EDTA, 0.5% (v/v) Nonidet P-40 and 50 mM Tris, pH 7.4] containing 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and then centrifuged at 800 *g* for 5 min at 4 °C. The supernatant was used as total cell lysate.

For Western blotting, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against ADA (Santa Cruz Biotechnology) or β -actin (Sigma, St Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific).

Assay of extra- and intra-cellular adenosine

Nucleosides were extracted from cells or extracellular solutions by adding 2% trichloroacetic acid. After centrifugation at 5,000 *g* for 5 min at 4 °C, the supernatants were loaded onto a reversed phase high-performance liquid chromatography (HPLC)(LC-10ATvp; Shimadzu Co., Kyoto, Japan). An adenosine peak was detected at an absorbance of 260 nm (SPD-10Avp UV-VIS detector, Shimadzu Co.), and adenosine was quantified.

Evaluation of tumor growth in mice inoculated with NCI-H2052 cells

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Nude KSN/Slc mice (male, 7 w), weighting the average of 26 g, were obtained from Japan SLC, Inc. (Shizuoka, Japan). NCI-H2052 cells (1×10^7 cells) suspended in 200 μ l of culture medium with 50% (v/v) matrigel (BD Biosciences, San Jose, CA, USA) was subcutaneously inoculated in the right flank of mice under pentobarbital general anesthesia. EHNA (0.3 mg/kg, corresponding to 1 μ mol/kg in 100 μ l of PBS) or 100 μ l of PBS was intraperitoneally injected to mice three times a week. The longer (L) and shorter length (S) of inoculated tumors was measured using calipers and tumor volume (V) was calculated according to the following equation: $V = L \times S^2 \times 0.5$.

Statistical analysis

Statistical analysis was carried out using unpaired *t*-test and Dunnett's test.

Results

EHNA induces apoptosis of cancer cells

For the MPM cell lines NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells, EHNA reduced cell viability in a concentration (0.01-1 mM)- and treatment time (24-48 h)-dependent manner, reaching below 20% of basal levels at 48-h treatment at 1 mM (Fig. 1A-D). In the TUNEL staining, EHNA (1 mM) significantly increased the number of TUNEL-

Fig. 1. EHNA-induced apoptosis of MPM cells. MTT assay was carried out in NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D), untreated and treated with EHNA at concentrations as indicated for 24–48 h. In the graphs, each point represents the mean ($\pm$ SEM) percentage of control (MTT intensities for cells untreated with EHNA) ($n=4$ independent experiments). TUNEL staining was carried out in NCI-H28 (E), NCI-H2052 (F), NCI-H2452 (G), and MSTO-211H cells (H), untreated (Cont) and treated with EHNA (1 mM) for 24 h. DIC, differential interference contrast. Bars, 100 μ m. TUNEL-positive cells were counted in the area (0.4 mm $\times$ 0.4 mm) selected at random. In the graphs, each column represents the mean ($\pm$ SEM) TUNEL-positive cell percentage of whole cells ($n=4$ independent experiments). P values, unpaired t -test.

positive cells as compared with that for untreated control cells for all the investigated MPM cell lines (Fig. 1E–H), indicating that EHNA induces apoptosis of MPM cells.

Likewise, EHNA reduced cell viability for the human lung cancer cell lines A549, SBC-3, and Lu-65 cells and the human hepatic cancer cell lines HepG2 and HuH-7 cells in a concentration (0.01–1 mM)- and treatment time (24–48 h)-dependent manner (Fig. 2A–C,G,H) and definitely increased TUNEL-positive cells for these cell lines (Fig. 2D–F,I,J). Taken together, these results indicate that EHNA has the potential to induce apoptosis of a variety of cancer cells.

Surprisingly, another ADA inhibitor pentostatin had no/little effect on cell viability for all the investigated MPM cell lines (Fig. 3A–D).

EHNA increases intracellular adenosine concentrations in MPM cells

We next examined whether EHNA actually increases adenosine concentrations in MPM cells. Expectedly, EHNA (0.3 mM) increased intracellular adenosine concentrations in a treatment time (3–9 h)-dependent manner for all the investigated MPM cell lines, while extracellular adenosine concentrations were not increased or conversely decreased (Fig. 4A–D).

Apoptosis of MPM cells is induced by knocking-down ADA

To ascertain whether EHNA-induced apoptosis of MPM cells is due to ADA inhibition, the ADA siRNA was constructed and transfected into cells. Expression of ADA protein for

Fig. 2. EHNA-induced apoptosis of human lung and hepatic cancer cells. MTT assay was carried out in A549 (A), SBC-3 (B), Lu-65 (C), HepG2 (G), and HuH-7 cells (H), untreated and treated with EHNA at concentrations as indicated for 24–48 h. In the graphs, each point represents the mean ($\pm$ SEM) percentage of control (MTT intensities for cells untreated with EHNA) ($n=4$ independent experiments). TUNEL staining was carried out in A549 (D), SBC-3 (E), Lu-65 (F), HepG2 (I), and HuH-7 cells (J), untreated (Cont) and treated with EHNA (1 mM) for 24 h. DIC, differential interference contrast. Bars, 100 μ m. TUNEL-positive cells were counted in the area (0.4 mm $\times$ 0.4 mm) selected at random. In the graphs, each column represents the mean ($\pm$ SEM) TUNEL-positive cell percentage of whole cells ($n=4$ independent experiments). P values, unpaired t -test.

cells transfected with the *ADA* siRNA was clearly decreased (Fig. 5A-D), confirming *ADA* knock-down. Cell viability for NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells was significantly reduced by knocking-down *ADA*, and the effect was neutralized by the adenosine kinase inhibitor 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl) pyrido[2,3-d] pyrimidine (ABT-702) (Fig. 6A-D). Moreover, a significant increase in TUNEL-positive cells was found for all the investigated MPM cell lines by knocking-down *ADA* (Fig. 6E-H). Collectively, these results suggest that EHNA increases intracellular adenosine concentrations by inhibiting *ADA* and that in turn, adenosine kinase-catalyzed conversion to AMP triggers apoptosis of MPM cells.

Fig. 3. Effect of pentostatin on MPM cell viability. MTT assay was carried out in NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D), untreated and treated with pentostatin at concentrations as indicated for 24-48 h. In the graphs, each point represents the mean ($\pm$ SEM) percentage of control (MTT intensities for cells untreated with pentostatin)(n=4 independent experiments).

Fig. 4. Effects of EHNA on extra- and intra-cellular adenosine concentrations. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with EHNA (0.3 mM) for 3-9 h, and then extra- and intra-cellular adenosine were quantified by HPLC. In the graphs, each point represents the mean ($\pm$ SEM) adenosine concentration (μ M for extracellular adenosine, nmol/mg protein for intracellular adenosine)(n=4 independent experiments).

Fig. 5. ADA knock-down. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were transfected with the NC siRNA or the ADA siRNA followed by Western blotting 48 h after transfection. Signal intensities for ADA

protein were normalized by those for β -actin. In the graphs, each column represents the mean ($\pm$ SEM) ADA protein intensity (n=4 independent experiments). P values, unpaired t -test.

Fig. 6. MPM cell apoptosis is induced by knocking-down ADA. MTT assay was carried out in NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) transfected with the NC siRNA (NC) or the ADA siRNA (ADA KD) 48 h after transfection in the presence and absence of ABT-702 (ABT) (10 nM). In the graphs, each column represents the mean ($\pm$ SEM) percentage of control (MTT intensities for cells transfected with the NC siRNA at 48 h after transfection) ($n=4$ independent experiments). P values, Dunnett's test. TUNEL staining was carried out in NCI-H28 (E), NCI-H2052 (F), NCI-H2452 (G), and MSTO-211H cells (H) transfected with the NC siRNA (NC) or the ADA siRNA (ADA KD) 48 h after transfection. DIC, differential interference contrast. Bars, 100 μ m. TUNEL-positive cells were counted in the area (0.4 mm $\times$ 0.4 mm) selected at random. In the graphs, each column represents the mean ($\pm$ SEM) TUNEL-positive cell percentage of whole cells ($n=4$ independent experiments). P values, unpaired t -test.

Fig. 7. EHNA-induced suppression of NCI-H2052 MPM cell proliferation. NCI-H2052 cells were subcutaneously inoculated in the flank of mice, and PBS or EHNA (0.3 mg/kg) was intraperitoneally injected three times a week. Time-course change of tumor volume (the mean $\pm$ SEM) ($n=6$ independent mice). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared with that for PBS-injected control mice, unpaired t -test.

Ehna suppresses NCI-H2052 tumor growth

We finally examined the effect of EHNA on tumor growth in mice inoculated with NCI-H2052 cells. Intraperitoneal injection with EHNA at a dose of 0.3 mg/kg drastically reduced NCI-H2052 cell growth as compared with that for PBS-injected control mice (Fig. 7). This indicates that EHNA exhibits a potent anticancer activity against MPM.

Discussion

A growing body of evidence has shown that high concentrations of extracellular adenosine is capable of inducing apoptosis in a variety of cancer cells mainly through intrinsic and/or extrinsic pathways [1-20]. ADA, an enzyme involved in purine metabolism, catalyzes the hydrolytic deamination of adenosine and 2'-deoxyadenosine to their corresponding hypoxanthine derivatives, inosine and 2'-deoxy-inosine. ADA inhibition, therefore, results in the accumulation of adenosine, 2'-deoxyadenosine, and 2'-deoxyATP in cells [21, 22]. Indeed, the ADA inhibitor EHNA increased intracellular adenosine concentrations in MPM cells. EHNA induced apoptosis in all the investigated MPM cell lines, human lung cancer cell lines, and human hepatic cancer cell lines. This suggests that an increase in intracellular adenosine concentrations due to ADA inhibition is essential for EHNA-induced apoptosis of MPM cells. In support of this idea, apoptosis of MPM cells was induced by knocking-down ADA. Notably, MPM cell death due to ADA knock-down was prevented by the adenosine kinase inhibitor ABT-702. This implies that AMP, produced by adenosine kinase-catalyzed phosphorylation of adenosine, following an increase in intracellular adenosine concentrations due to ADA inhibition is a key factor for EHNA-induced apoptosis of MPM cells. EHNA clearly suppressed tumor growth in mice inoculated with NCI-H2052 cells. Overall, these results indicate that EHNA could be a beneficial drug for treatment of MPM.

The most paradoxical result in the present study is that another ADA inhibitor pentostatin, whereas it increases intracellular adenosine concentrations, had no/little effect on cell viability for all the investigated MPM cell lines. This raises the possibility that EHNA-induced apoptosis of MPM cells may not be induced simply by raising intracellular adenosine levels due to ADA inhibition and that the unknown additional mechanism may participate in the EHNA action. Pentostatin in combination of 2'-deoxyadenosine is shown to activate caspase-3 and induce apoptosis of human monocytoid leukemia cells [23]. Pentostatin, alternatively, has a striking cytotoxic effect on different types of T-cell lymphomas, including T-prolymphocytic leukemia, large granular lymphocyte leukemia, and hepatosplenic gd⁺ T-cell lymphoma [24]. EHNA, on the other hand, acts as an inhibitor of cyclic nucleotide phosphodiesterase 2 (PDE2) as well as ADA [25]. Interestingly, PDE inhibitors could still induce cell death in a variety of cancer cells [26-28]. EHNA, accordingly, might exhibit an anticancer effect against MPM by inhibiting both ADA and PDE2. To answer this question, we are currently probing the target molecules responsible for EHNA-induced apoptosis of MPM cells.

Accumulating evidence has pointed that higher concentrations of adenosine induce apoptosis of a wide variety of cancer cells via intrinsic and extrinsic pathways [2-10]. This raises the possibility that adenosine is available for effective chemotherapy of cancers. Adenosine is an endogenous substance, and therefore, in use for chemotherapy less side effects are expected. From these points of view, EHNA, which induces MPM cell apoptosis by increasing endogenous adenosine concentrations, could be developed as a promising drug for MPM therapy. We are currently preparing for clinical challenge of EHNA.

In conclusion, the results of the present study clearly demonstrate that EHNA induces apoptosis of MPM cells and suppresses tumor growth in mice inoculated with NCI-H2052 cells. This may provide a new strategy for treatment of MPM.

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