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Adenosine A_3 Receptor Suppresses Prostate Cancer Metastasis by Inhibiting NADPH Oxidase Activity[1,2]

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Abstract

Prostate cancer is the most commonly diagnosed and second most lethal malignancy in men, due mainly to a lack of effective treatment for the metastatic disease. A number of recent studies have shown that activation of the purine nucleoside receptor, adenosine A_3 receptor (A_3AR), attenuates proliferation of melanoma, colon, and prostate cancer cells. In the present study, we determined whether activation of the A_3AR reduces the ability of prostate cancer cells to migrate *in vitro* and metastasize *in vivo*. Using severe combined immunodeficient mice, we show that proliferation and metastasis of AT6.1 rat prostate cancer cells were decreased by the administration of A_3AR agonist N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide. *In vitro* studies show that activation of A_3AR decreased high basal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity present in these cells, along with the expression of Rac1 and $p47^{phox}$ subunits of this enzyme. Inhibition of NADPH oxidase activity by the dominant-negative RacN17 or short interfering (si)RNA against $p47^{phox}$ reduced both the generation of reactive oxygen species and the invasion of these cells on Matrigel. In addition, we show that membrane association of $p47^{phox}$ and activation of NADPH oxidase is dependent on the activity of the extracellular signal–regulated kinase (ERK)1/2 mitogen-activated protein kinase pathway. We also provide evidence that A_3AR inhibits ERK1/2 activity in prostate cancer cells through inhibition of adenylyl cyclase and protein kinase A. We conclude that activation of the A_3AR in prostate cancer cells reduces protein kinase A–mediated stimulation of ERK1/2, leading to reduced NADPH oxidase activity and cancer cell invasiveness.

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Introduction

In the United States, the number of new cases and deaths due to prostate cancer were estimated at 186,320 and 28,660, respectively, for the year 2008 [1]. The major cause of deaths is due to the complications resulting from cancer metastases to distant organs in the body. The 5-year survival rate for the localized disease is close to 90% to 100%, but it is just approximately 32% for the metastatic prostate cancer [1], reflecting the lack of effective treatment for the metastatic disease, associated with androgen insensitivity.

Cancer cell metastasis requires the cell to acquire an early motile phenotype followed by transformation to an adhesive phenotype to facilitate interaction with the extracellular matrix proteins. This allows for dispersion of the malignant cells through the blood stream or lymph vessels to other parts of the body [2]. The Rho family of small GTP binding proteins, namely, Rac1 and Cdc42, play crucial roles in cancer cell metastasis [3]. Aberrant activation of these proteins promotes

Abbreviations: A_3AR, adenosine A_3 receptor; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; DPI, dibenziodolium chloride; ERK1/2, extracellular signal–regulated kinase 1/2; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; MRS1523, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; PD098059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; ROS, reactive oxygen species; siRNA, short interfering RNA; SQ22536, 9-(tetrahydro-2-furanyl)-adenine

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[2]This article refers to supplementary materials, which are designated by Figures W1 to W6 and are available online at www.neoplasia.com.

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invasion of various cancers [4–6] through regulation of actin cytoskeletal structures [7].

In addition to its role in cell motility, Rac1 is also an essential component of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, a major source of superoxide generation in the cell. NADPH oxidase was initially thought to be expressed mainly in phagocytes where it contributes to killing of ingested microorganisms through the generation of superoxide [8]. However, NADPH oxidase subunits are also expressed in other tissues [9]. Full enzyme activity is conferred by four oxidase subunits, namely, $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ [10]. Other proteins associated with activation of the holoenzyme include $p40^{phox}$, Rap1A, and Rac1, whose activation promotes NADPH oxidase activity and generation of reactive oxygen species (ROS) [10].

Whereas it is known that ROS increase oncogenic transformation by damaging DNA and increasing mutation rates, recent evidence demonstrates that ROS activate cell signaling pathways to promote proliferation and metastasis [11]. ROS act as signaling molecules of growth factor receptors to activate mitogen-activated protein (MAP) kinases such as p38, extracellular signal–regulated kinase (ERK), and c-Jun N-terminal kinase [12]. These signaling pathways culminate in the regulation of cell cycle proteins, thereby influencing the proliferation of cancer cells. In the context of growth factor signaling, NADPH oxidase plays a key role in generating ROS [10].

Adenosine-based drugs represent a novel class of compounds that show antitumor effects against a number of different cancers. Four major classes of adenosine receptors (ARs), namely, the A_1, the A_{2A}, A_{2B}, and A_3AR [13], have been identified. Adenosine and different AR agonists have been shown to affect the tumorigenicity of different cancer types. A_1AR agonists inhibit the proliferation of various cancer cell lines including epidermoid cancer cells [14] and human leukemia cells [15], whereas activation of the A_{2B}AR increased tumor growth [16]. Recent studies have shown an antitumorigenic action of A_3AR agonists against melanoma [17], colon [18], and prostate cancers [19]. The mechanism underlying this action involves inhibition of cyclic AMP production, leading to reduced protein kinase A (PKA) and glycogen synthase kinase-3β activity, destabilization of β-catenin and suppression of cyclin D1 and *c-myc*, cell cycle arrest, and/or apoptosis [20,21]. A_3AR agonists were also shown to increase migration of human colon cancer cells [22]. However, this observation was obtained in *in vitro* hypoxic culture conditions. Induction of the A_3AR occurs in several types of cancer [21,23], when compared with normal adjacent tissue, suggesting that these receptors could serve as potential molecular markers of these cancers.

In this study, we show that the A_3AR agonist, IB-MECA, inhibits *in vivo* tumor growth and metastasis of prostate cancer in mice, in addition to inhibition of *in vitro* cell proliferation and invasion of prostate cancer cells. This antitumor action involves suppression of high ROS generation through the NADPH oxidase system, which is maintained through high ERK1/2 activity in these cells. We show that A_3AR suppresses ERK1/2 and NADPH oxidase activity by inhibiting a cyclic AMP/PKA pathway in these cells.

Materials and Methods

Materials

N^6-(3-Iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA), 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), dibenziodolium chloride (DPI), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD098059), 9-(tetrahydro-2-furanyl)-adenine (SQ22536), *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), and bisindolylmaleimide I hydrochloride (BIM) were obtained from Sigma Chemical, Co (St Louis, MO). Antibodies against adenosine A_3 receptor (A_3AR), phospho-ERK1/2, ERK1/2, $p47^{phox}$ Rac1, and Na^+-K^+ ATPase were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Antibodies against β-actin and against Bax and p53 were bought from Sigma Chemical, Co, and Cell Signaling Technology (Danvers, MA), respectively. Cell culture media RPMI 1640 and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA), whereas fetal bovine serum was from Atlanta Biologicals, Inc (Lawrenceville, GA). All other reagents and supplies were of highest available grade and were purchased from standard sources.

Cell Cultures

The rat prostatic carcinoma cell line AT6.1, and human prostate cancer cell lines ALVA and PC3-MM were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 25 μg/ml streptomycin. Cells were grown at 37°C in the presence of 5% CO_2 and 95% ambient air. The medium was changed every second day, and the cells were passaged twice a week. All the experiments were performed using confluent monolayers.

Immunocytochemistry

AT6.1, PC3-MM, or ALVA cells were cultured on glass coverslips. Drug treatments were performed for 24 hours, and cells were fixed with 4% paraformaldehyde. Cells were processed and imaged for A_3AR and Rac1 as described previously [24].

2′,7′-Dichlorodihydrofluorescein Diacetate Assay

After 12 hours of serum deprivation AT6.1, PC3-MM, or ALVA cells were loaded with 5 μg/ml of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; EMD Biosciences, San Diego, CA) in 2 ml of 1× PBS. After incubation at 37°C for 20 minutes, cells were washed with 1× PBS. First, baseline images were recorded by a confocal microscope (Olympus America, Inc, Melville, NY) at 488 nm, and then the image was captured after the addition of 1 μM IB-MECA. For other treatment groups, cells were pretreated with either vehicle or A_3AR inhibitor MRS 1523 (1 μM) or NADPH oxidase inhibitors DPI (10 μM) or AEBSF (100 μM) for 30 minutes before adding the dye. Quantitation of the fluorescence intensity was performed by using FluoView software (Olympus America, Inc, Melville, NY).

Preparation of Plasma Membrane and Cytosolic Extracts

Cytosolic and membrane extracts from whole cell lysates were isolated as described previously [24]. To verify the purity of the membrane isolates, we probed for the membrane-specific protein Na^+-K^+ ATPase. High Na^+-K^+ ATPase levels were obtained in the membrane fraction by Western blot analysis compared with the cytosol (data not shown).

Western Blot Analysis

Total cell lysates were used to quantitate activation/inhibition of ERK1/2 and expression of Rac1, whereas membrane preparations were made to study the expression of A_3AR. Assays were performed essentially as described previously [24]. Blots were visualized by