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Adenine Induces Differentiation of Rat Hepatic Stellate Cells

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Abstract

Background and Aims—Adenine is a uric acid pathway metabolite of no known function, and has recently been identified as a ligand for a rat G protein-coupled receptor. Due to the known role of other uric acid pathway metabolites in HSC biology, we tested the ability of adenine to induce HSC differentiation.

Methods—RT-PCR was performed for adenine receptor expression in T-6 and primary rat HSC. T-6 and primary rats HSC were cultured with and without adenine, and stellation examined. Next, we examined inhibition of calcium signaling using caged IP₃. To test if adenine inhibits HSC chemotaxis T-6 cells and rat HSCs were cultured with or without adenine for 24 h in a transwell assay with PDGF as the chemoattractant. cDNA was prepared from T-6 and primary HSC for quantification of collagen 1 mRNA using real-time PCR.

Results—We found that mRNA for the adenine receptor is expressed in T-6 cells and primary rat HSC. Also, adenine induces HSC stellation and adenine inhibits IP₃ mediated increase in cytosolic [Ca²⁺]_i and inhibits chemotaxis in T-6 cells and primary rat HSC. Adenine was also shown to up-regulate α -SMA and collagen 1, and this effect is lost by using specific si-RNA for the adenine receptor. Finally, adenine inhibits endothelin-1-induced gel contraction.

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Electronic supplementary material

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Conflict of interest

None of the authors have a conflict of interest.

Conclusions—The adenine receptor is present in T-6 cells and primary rats HSC. Adenine, via the adenine receptor, induces morphological change, and cytosolic calcium signaling, inhibits chemotaxis, and up-regulates collagen 1 mRNA in HSCs.

Keywords

Adenine; Stellate cells; Chemotaxis; Differentiation

Introduction

The uric acid pathway is a nucleic acid degradative pathway with increased flux after cell injury and death [1]. The pathway consists of sequential degradation of adenosine monophosphate to adenosine, adenine, hypoxanthine, xanthine and finally uric acid. This pathway is of particular interest as adenosine and uric acid are known to have a significant role in adaptation to tissue injury, including regulation of inflammation and fibrosis [1]. Adenine is immediately downstream of adenosine and was not recognized as having any signaling capacity, but a receptor for adenine was recently identified in rat neuronal tissue, and its 3D structure has been predicted [2, 3]. The adenine receptor is a member of the MAS-value gene (Mrg) receptor family, and although the highest expression is in dorsal root ganglia, expression was also found in a wide range of tissues [2]. Due to the important role of adenosine on hepatic stellate cell differentiation, and the recent identification of an adenine receptor, we hypothesized that adenine has the ability to induce HSC differentiation.

HSC have the capacity to undergo a wide range of functional changes in response to paracrine and matrix signals [4]. In this study we show that mRNA for the adenine receptor is expressed in T-6 cells and primary rats HSCs. Adenine induces actin reorganization, and inhibits IP₃ mediated increase in cytosolic [Ca²⁺]_i. Furthermore adenine inhibits chemotaxis in T-6 cells and primary rat HSC, and up-regulates α -SMA and collagen 1. This is the first study to report that HSC express the adenine receptor and undergo functional changes in response to adenine. Collectively these data suggest that adenine, along with adenosine, is an important late differentiation signal for HSC.

Methods

Cell Culture and Reagents

T-6 rat HSC cells were cultured in Waymouth MB 752/1 medium supplemented with 10 % FBS and penicillin–streptomycin (100 IU/ml and 100 mg/ml media, respectively) and used after three to six passages [5]. Primary HSCs were cultured in M199, 10 % FBS, penicillin–streptomycin (100 IU/ml and 100 mg/ml media, respectively), 2 % gentamycin, and fungizone in a 1:1,000 density, with a media change 24 h after isolation and then every 3 days. Histodenz, DNase, Pronase, Collagenase, TRIZOL reagent, Fungizone, Trypsin–EDTA, M199, Waymouth MB 752/1, DMEM, HBSS, and F-12 (HAM) were purchased from GIBCO/ Invitrogen (Grand Island, NY, USA). All reagents were of the highest quality grade commercially available.

Animals and HSC Isolation

HSC were isolated from retired breeder rats (500–700 g) by in situ pronase/collagenase perfusion followed by density gradient centrifugation, 9–11 % Nycodenz (Sigma-Aldrich, St. Louis, MO, USA). Primary cells were >95 % pure and were used at days 5–7 after isolation. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA, USA) weighing 200–250 g were used throughout this study. All experiments and animal handling were performed according to the Yale University Institutional Animal Care & Use Committee guidelines.

RT-PCR for Expression of Adenine Receptor

Complementary DNA was prepared on day 7 with primary rat HSC and amplified with primers of adenine (5'-CACC ATGGGGGAAAGCTTCACCGGTACAG-3') (5'-CGGC TC CACCTTGCTGCTTGACATC-3'), and β -actin (5'-G TGGGGCGCCCCA GGCACCA-3') (5'-CTCCTTAATGT CACGCAC GATTTC-3'). The polymerase chain reaction (PCR) was performed with 1 μ l of the complementary DNA, 1 $\times$ buffer, 1 mM MgCl₂, 200 μ M of each deoxyribonucleotide triphosphate, 2.5 U Taq polymerase, and 0.2 μ M of each adenine and β -actin specific primers.

Real-Time PCR for mRNA Expression

T-6 cells and primary rat HSCs were cultured in the presence of adenine (500 μ M). Twenty-four hours after culture, complementary DNA was prepared, and quantitative real-time PCR was performed for collagen 1 using commercial primer-probe sets (Applied Biosystems, Framingham, MA, USA) and the Applied Biosystem 7500 real-time PCR system. Expression of glyceraldehyde 3-phosphate dehydrogenase was used to standardize the samples, and the results were expressed as a ratio compared with untreated HSCs.

Chemotaxis Assay

The migration of HSC-T6 cells primary HSCs from rat was studied using transwell inserts equipped with 8- μ m pore polycarbonate-free filters as described previously [6]. For the primary HSC, two rats were used per isolation and 2×10^4 HSCs were plated per well. Cells were treated with adenine (500 μ M). PDGF or CXCL4 (Sigma Alderich St. Louis, MO, USA) was added to the lower chamber 2 h afterwards. After 24 h, the lower surface of the membrane was stained using hematoxylineosin, photographed, and analyzed. All experiments were repeated in triplicate. For each experimental group, a total of 40 high-power field images were taken. Cells per high-power field were counted, with results expressed as the average number of cells per high-power field. All experiments were performed at least in triplicate. Statistical analysis was performed using the Student *t* test; a *P* value less than 0.05 was considered significant.

Western Blotting for α -Smooth Muscle Actin

T-6 cells were cultured with and without adenine. Twenty-four hours later, the protein was extracted from each sample and Western blotting was performed for α -smooth muscle actin (α -SMA; 1: 1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β -actin (1:1,000; Santa Cruz Biotechnology) using standard techniques. Radiographs were scanned and analyzed using TL100 software from the Total Lab range (Nonlinear Dynamics, Newcastle upon Tyne, UK). Total Lab TL100 can analyze 8/16 bit TIFF format images from HDD files. Statistical analysis was performed using the Student *t* test; a *P* value less than 0.05 was considered significant.

Analysis of Intracellular Sensitivity to IP₃ via Measurement of Changes in Cytosolic Ca²⁺ Concentration

T-6 cells were cultured with or without adenine on glass coverslips for 20 min and then incubated with a solution containing 3 M caged IP₃ (Alexis Biochemicals), 5 M Fluo-4 (Molecular Probes), 19.7 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 130 mM NaCl, 5 mM KCl, 1 mM MgSO₄, and 1.25 mM CaCl₂ for 30 min. These cells were placed in a perfusion chamber on the stage of a Zeiss S10NLO microscope; IP₃ was then photoreleased using a custom-built system that couples a mercury lamp to a 1-mm quartz fiber optic cable through a high-speed shutter and filter wheel, while cells were observed using time lapse confocal microscopy.

SiRNA Transfection—Two pre-designed chemically synthesized siRNA molecules against the rat adenine receptor (Ambion, Austin, TX, USA) were tested—Silencer Select siRNA ID # s141233 and s141231, proprietary sequences. Knock-down efficiency following siRNA transfection was assessed by real-time PCR quantification of adenine receptor transcript levels, normalized to GAPDH expression, using TaqMan Gene Expression assays (Ambion, Austin, TX, USA), and controlled with scrambled siRNA (Ambion, Austin, TX, USA). When transfections were performed on cells at 70 % confluence in a 24-well plate format with 2 μ L of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and 7.5 pmol siRNA according to manufacturer's instructions, s141231 achieved a higher knockdown efficiency (89 ± 5 %) and was therefore used in subsequent experiments. Following transfection, 1.5 % FBS-containing media was added to cells for 48 h to slow growth prior to starving cells until experiments at the 96 h time point.

Statistics

For the chemotaxis experiments, 40 high-power fields were counted, and for each experimental group the average number of cells per high-power field was calculated. The Student *t* test was performed, with $P < 0.05$ considered significant. For quantitative real-time PCR, data samples were run in groups of 12 samples each, generating a ratio compared with the control sample.

Results

Adenine Induces Stellation of HSC

Adenine receptor messenger RNA (mRNA) is present in the T-6 HSC line and primary rat HSCs (Fig. 1a). Activation of HSCs is associated with changes in cell shape into a more stellate morphology [4]. HSC usually have a flat, polygonal morphology as shown in primary rat HSC and T-6 HSC cell line by phase contrast (Fig. 1b, d). Twenty-four hours after exposure to adenine, morphological change is seen in both primary rat and T-6 HSC (Fig. 1c, e). Inhibition of the adenine receptor in the T-6 HSC line by siRNA abolishes the ability of adenine to induce stellation of T-6 HSC. There was 89 % knockdown efficiency (± 5 %) in two independent experiments, each performed in triplicate. Scrambled control siRNA did not produce statistically significant reductions in AR expression.

Adenosine-induced changes on HSC and mesenchymal stem cells are known to require a PKA and Rac pathway [7, 8]. We tested the role of these molecules by using a very specific PKA inhibitor (ST-HT31 at 25 μ M) and a Rac-1 inhibitor (NSC23766 at 150 μ M). Inhibition of PKA and Rac-1 resulted in the inability of adenine to induce HSC stellation (Fig. 2). To ensure that the stellation was not due to trace contamination of adenine with adenosine we attempted to inhibit it by using the adenosine 2a receptor antagonist (ZM 241385 at a concentration of 10 μ M). As can be seen in Fig. 2 this did not result in a decrease in adenine-induced HSC stellation, further confirming the data using adenine receptor specific siRNA.

Adenine Inhibits IP₃-Mediated Ca²⁺ Signaling in HSC

To test the ability of adenine to reduce IP₃ mediated Ca⁺⁺ fluxes, T-6 cells were loaded with the Ca²⁺ flurophore Fluo-4 and cell-permeant caged IP₃ ester (C-iso IP₃, Alexis Corp.). They were imaged during uncaging of IP₃. In control cells, IP₃ uncaging results in a rapid increase in [Ca²⁺]_i, which persists for approximately 30 s (Fig. 3a). In T-6 HSC pretreated with adenine, there was a reduction in [Ca²⁺]_i elevation in response to uncaging IP₃. The summarized data show the significant differences between control group and adenine treated group (* $P < 0.05$; Fig. 3b).

Adenine Inhibits PDGF-Mediated HSC Chemotaxis

We have shown that adenosine and mammalian DNA, which are produced in areas of cellular apoptosis, inhibit HSC chemotaxis induced by PDGF [9, 10]. We tested if adenine can also inhibit PDGF or CXCL4 mediated chemotaxis using a standard transwell system. Figure 4a shows transwell chemotaxis data for T-6 HSC. PDGF induces chemotaxis of T-6 HSC, and this is significantly inhibited by pretreatment with adenine (* $P < 0.001$). In an analogous manner CXCL4 induces chemotaxis of T-6 HSC which is also inhibited by adenine (supplementary Fig 1). Primary rat HSC have a very similar response, with adenine inhibiting PDGF-mediated chemotaxis (* $P < 0.001$; Fig. 4b). To determine the specificity of this response for the adenine receptor, specific siRNA was used. As can be seen in Fig. 4d treatment with specific siRNA almost entirely abolished the ability of adenine to inhibit PDGF-induced chemotaxis.

Adenine Up-regulates α -SMA Collagen 1 in HSC

We tested if adenine enhances α -SMA protein levels in primary rat HSC by western blotting. As shown in Fig. 5a and b, adenine significantly up-regulates α -SMA levels compared with the control group (rat HSC α -SMA increased 9.48 times, SD 1.89, * $P < 0.05$).

After localization to a site of injury one of the primary functions of HSC is the deposition of matrix components. We tested up-regulation of mRNA for collagen 1 as an indicator of an increase in extracellular matrix deposition. Twenty-four hours after exposure to adenine, the levels of collagen 1 in T-6 and primary HSC were significantly greater than those of controls (T-6 cells increased 14.8 times, SD 0.23; rat HSC increased 4.6 times, SD 0.12; $P < 0.01$ for both cell types). To further test the specificity of this effect for the adenine receptor specific siRNA was used on T-6 HSC. This demonstrated that in the presence of specific siRNA adenine was unable to up-regulate collagen 1 mRNA (T-6 cells increased 11.4 times, SD 0.17; T-6 cells treated with siRNA increased 2.1 times, SD 0.11 $P < 0.01$).

Adenine Inhibits Endothelin-1 Mediated HSC Contraction

The ability of adenine to inhibit Ca^{++} fluxes made us consider that one functional consequence may be loss of the ability to induce contraction. To measure the effect of adenine on the contraction induced by endothelin-1 we used the established assay of hydrated collagen gels [11]. In Fig. 6a, a representative experiment of collagen lattice contraction after 3-h incubation was shown. Figure 6a control shows a gel exposed to control buffer only, resulting in a minimal decrease of the collagen surface area from rim of the well. Adenine caused no significant decrease in surface area of gel compared with control. Endothelin-1 (10 nM) stimulated a decrease in collagen gel surface area within 1 h. Addition of adenine to the collagen gel for 60 min followed by endothelin-1 (10 nM) resulted in significant loss of the gel contraction induced by endothelin-1.

Discussion

Adenine is one of the metabolites of nucleic acid degradation via the uric acid pathway and is generated from adenosine by purine nucleotide phosphorylase [2]. There is increased flux through this pathway, and an increase in concentration of these metabolites during tissue injury [1]. The association between tissue injury and increase in uric acid pathway metabolite concentrations makes them excellent candidates for co-coordinating adaptive responses to tissue injury. This has been very well characterized for adenosine which signals via four G protein coupled receptors, and induces a wide range of tissue adaptive responses [12]. Recently, adenine has been identified as binding with high affinity and activating a widely expressed orphan rat G protein-coupled receptor belonging to the MAS-value gene

(Mrg) receptor family [2]. We have previously shown that adenosine plays an important role in hepatic stellate cells (HSCs) differentiation and inhibition of HSC chemotaxis [9]. We therefore tested if the adenine receptor is expressed on HSC, and is involved in HSC differentiation.

We initially demonstrated expression of adenine in the human T-6 HSC line, and in primary rat HSC (Fig. 1a), which is consistent with the broad expression of this receptor [2]. One characteristic feature of HSC is their response to differentiation stimuli by a change in morphology from a flat fibroblast-like shape to a more angular, or stellate shape [13]. Such a change in morphology is seen with HSC activation by a variety of stimuli including in vitro activation on plastic, and in vivo activation with liver injury. We demonstrate that adenine induces a change in morphology from a flat fibroblast-like shape to an angular stellate shape. This is visible by phase contrast microscopy (Fig. 1b–e). siRNA to knock down was performed to confirm that this effect was specific for the adenine receptor. The use of siRNA resulted in a loss of the ability of adenine to induce a stellate morphology (Fig. 1g). These changes were quantified, and further confirmation that they were not due to contamination of samples with adenosine was obtained by using an inhibitor of the A2a adenosine receptor (Fig. 2). A very specific PKA inhibitor and a Rac-1 inhibitor were used to confirm the role of cAMP mediated PKA Rac-1 pathway downstream of the adenine receptor. These data are the first demonstration of a functional role for the adenine receptor expressed by HSC.

The increase in cytosolic $[Ca^{2+}]_i$ in response to PDGF, endothelin-1 and a variety of other messengers is mediated by cytosolic IP_3 [14]. This results in activation of IP_3 receptors on the endoplasmic reticulum (ER) and release of ER Ca^{++} into the cytosol. We have previously shown that adenosine signaling via the A2a receptor results in inhibition of the secondary messenger IP_3 mediated increase in cytosolic $[Ca^{2+}]_i$ [9]. We chose to directly increase cytosolic IP_3 and test the effect of adenine on the $[Ca^{2+}]_i$ response. Directly increasing cytosolic IP_3 has advantages over using PDGF or endothelin-1 as it removes the possibility of changes in receptor function as being interpreted as changes in Ca^{++} signaling machinery. The results from our experiments using photo-release of caged IP_3 demonstrate the ability of adenine to inhibit IP_3 -mediated increase in cytosolic Ca^{++} (Fig. 3a–b). This finding suggests that adenine receptor activation results in a decrease in IP_3R function, as has been previously reported in response to a number of membrane receptors, including cholecystokinin and angiotensin II [15, 16]. IP_3 receptors have several phosphorylation sites that can alter the response of IP_3 receptors to IP_3 , and phosphorylation of the IP_3 receptor is the most likely mechanism [17].

Activated HSCs are not present randomly within the liver parenchyma in fibrosis, but are typically found in regions of fibrosis [4]. The cellular basis of this localisation is thought to be the ability of HSC to undergo chemotaxis to a range of stimuli including platelet derived growth factor (PDGF) and endothelin-1 [18–20]. We have recently shown that adenosine and apoptotic DNA associated with cellular apoptosis can inhibit PDGF-induced chemotaxis through the A2a and TLR9 receptors, respectively [9, 10]. The ability of signals from cellular apoptosis to inhibit HSC chemotaxis is proposed to provide a stop signal to HSC at sites of cellular apoptosis, allowing HSC to localize. The ability of adenine to inhibit IP_3 mediated increase in cytosolic $[Ca^{2+}]_i$ made us speculate that it may also inhibit chemotaxis. Figure 4a and b show that adenine did inhibit PDGF-induced chemotaxis, and this was dependent on the adenine receptor as demonstrated by the ability of specific siRNA to disrupt it.

HSC have been demonstrated to acquire a wide range of functions in response to a range of stimuli. These functions include proliferation, chemotaxis, contraction, matrix remodeling

and immune regulation [4]. We propose that these are not sequentially acquired as HSC undergo activation, but rather there is also selective loss of function. For example, it is not feasible for a cell to be simultaneously undergoing cell cycle, chemotaxis and contraction. From the above data we propose that HSC, on reaching the site of tissue injury, encounter high levels of adenine molecules which provide a stop signal. One prediction of this is that upon stopping and localizing HSC will up-regulate matrix deposition. This is exactly what we found by examining α -SMA levels by western blot and collagen mRNA levels by quantitative PCR. These data are entirely consistent with data on liver slices which demonstrate the ability of the related molecule adenosine to up-regulate collagen production [21].

Finally we tested the ability of adenine to functionally impair HSC contraction using the established gel contraction system. Adenine-induced inhibition of cytosolic $[Ca^{2+}]_i$ predicts that HSC contraction will be impaired. As can be seen from Fig. 6 endothelin-1 is able to induce a significant contraction. Adenine by itself has no effect but significantly inhibits the ability of endothelin-1 to induce contraction.

We have demonstrated the presence of the newly identified adenine receptor on HSC and further demonstrated a range of functional consequences for HSC of receptor activation. Collectively these functional changes point toward the adenine receptor having a role relatively late in HSC differentiation, at a stage after initial activation, proliferation and chemotaxis. After engagement the adenine receptor inhibits Ca^{++} fluxes and chemotaxis, producing a stationary, stellate and collagen producing HSC phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HSC	Hepatic stellate cells
RT-PCR	Reverse transcriptase polymerase chain reaction
TGF-β	Transforming growth factor beta
PDGF	Platelet derived growth factor
α-SMA	Alpha-smooth muscle actin
MSU	Monosodium urate
c-AMP	Cyclic adenosine monophosphate

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Fig. 1. Adenine induces stellation of T-6 cells and primary rat HSCs. **a** mRNA for the adenine receptor is expressed in the T-6 cell line and in primary rat HSC by RT-PCR. **b** and **d** Phase-contrast images of primary rat HSC and T-6 cells in culture showing a flat cuboidal shape. **c** and **e** Phase-contrast images of primary rat HSC and T-6 cells after 24 h of exposure to adenine (500 μ M) showing stellation. **f** and **g** Use of SiRNA specific for the adenine receptor abolishes the ability of adenine to induce HSC stellation. All experiments were repeated at least five times

Fig. 2.

Adenine-induced stellation is dependent on PKA and Rac-1 and is not dependent on the adenosine A2a receptor. The degree of adenine-induced stellation was significantly reduced in the presence of PKA (ST-HT31) and rac-1 (NSC23766) inhibitors. The exposure to a adenosine A2a receptor inhibitor (ZM 241385) did not reduce the ability of adenine to induce stellation. All experiments were repeated at least four times

Fig. 3.

Adenine inhibits IP_3 -mediated increase in cytosolic $[\text{Ca}^{2+}]_i$. **a** Analysis of the fluorephore emission in response to uncaging IP_3 shows a rapid increase in cytosolic $[\text{Ca}^{2+}]_i$ over approximately 5 ms, with a gradual decline over the next 40 ms, as seen in the *darker line* from a control T-6 cell. Pretreatment of T-6 cells with adenine inhibits the increase in cytosolic $[\text{Ca}^{2+}]_i$ in response to uncaging IP_3 as seen in the *lighter line*. **b** Summation of data from control and adenine-treated T-6 cells shows an inhibition of the increase in cytosolic Ca^{2+} in response to uncaging IP_3 (* $P < 0.05$). Experiment repeated four times

Fig. 4.

Adenine inhibits PDGF mediated chemotaxis of T-6 cells and primary rat HSC. **a** Adenine inhibits PDGF mediated chemotaxis of T-6 cells across a transwell (* $P < 0.001$). **b** Adenine inhibits PDGF mediated chemotaxis of primary rat HSC (* $P < 0.001$). **c** and **d** The ability of adenine to inhibit chemotaxis is abolished by an adenine receptor specific SiRNA. The control was scrambled siRNA. All experiments were repeated at least five times

Fig. 5.

Adenine up-regulates α -SMA and collagen 1. **a** and **b** Adenine up-regulates α -SMA in primary rat HSC. **c** Adenine increases production of collagen 1 mRNA by T-6 cells and rat HSC (* $P < 0.01$, ** $P < 0.01$). **d** The ability of adenine to up-regulate collagen 1 mRNA is abolished by adenine receptor specific SiRNA (* $P < 0.01$). All experiments were repeated at least three times

Fig. 6. Adenine inhibits endothelin-1-induced gel contraction by HSC. **a** and **b** Endothelin-1 induces gel contraction as previously shown. Adenine inhibits the ability of endothelin-1 to induce HSC-induced gel contraction (** $P < 0.002$). Experiment repeated four times