Adenosine inhibits cytosolic calcium signals and chemotaxis in hepatic stellate cells

Ardeshir Z. Hashmi,1 Wyel Hakim,1 Emma A. Kruglov,1 Azuma Watanabe,1 William Watkins,2 Jonathan A. Dranoff,1 and Wajahat Z. Mehal1

1Section of Digestive Diseases, Yale University, New Haven, Connecticut; and 2Gilead Sciences Incorporated, Foster City, California

Submitted 12 May 2006; accepted in final form 6 September 2006

Hashmi AZ, Hakim W, Kruglov EA, Watanabe A, Watkins W, Dranoff JA, Mehal WZ. Adenosine inhibits cytosolic calcium signals and chemotaxis in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 292: G395–G401, 2007; doi:10.1152/ajpgi.00208.2006.—Adenosine is produced during cellular hypoxia and apoptosis, resulting in elevated tissue levels at sites of injury. Adenosine is also known to regulate a number of cellular responses to injury, but its role in hepatic stellate cell (HSC) biology and liver fibrosis is poorly understood. We tested the effect of adenosine on the cytosolic Ca2+ concentration, chemotaxis, and upregulation of activation markers in HSCs. We showed that adenosine did not induce an increase in the cytosolic Ca2+ concentration in LX-2 cells and, in addition, inhibited increases in the cytosolic Ca2+ concentration in response to ATP and PDGF. Using a Transwell system, we showed that adenosine strongly inhibited PDGF-induced HSC chemotaxis in a dose-dependent manner. This inhibition was mediated via the A2a receptor, was reversible, was reproduced by forskolin, and was blocked by the adenylyl cyclase inhibitor 2,5-dideoxyadenosine. Adenosine also upregulated the production of TGF-β and collagen I mRNA. In conclusion, adenosine reversibly inhibits Ca2+ fluxes and chemotaxis of HSCs and upregulates TGF-β and collagen I mRNA. We propose that adenosine provides a “stop” signal to HSCs when they reach sites of tissue injury with high adenosine concentrations and 2) stimulates transdifferentiation of HSCs by upregulating collagen and TGF-β production.

platelet-derived growth factor; Ca2+; fibrosis

ADENOSINE is produced extracellularly and intracellularly from the dephosphorylation of adenosine tri-, di-, and monophosphates (27). A further source of adenosine is the degradation of nucleic acids, via the uric acid pathway, during cellular injury and apoptosis (12). These sources of adenosine result in elevated levels at sites of tissue ischemia and cellular apoptosis, with concentrations increasing 100-fold from the 30- to 300-nM range present in health (16, 17). Adenosine is efficiently metabolized by deamination or phosphorylation to inosine and adenine, respectively. The tight association of adenosine levels with tissue injury provides a potential means to communicate tissue injury to adaptive cellular responses.

Hepatic stellate cells (HSCs) are central to the adaptive response of the liver to hepatocyte injury and apoptosis (2). Such injury results in the chemotaxis of HSCs to the sites of cellular damage and differentiation with upregulation of TGF-β and collagen in addition to many other changes. PDGF induces HSC chemotaxis and is thought to be important in localizing HSCs to sites of liver injury (26). PDGF is produced by a variety of cells, including bile duct epithelia, and is upregulated during liver injury (3). In this model, HSCs move via chemotaxis up a gradient of PDGF until they reach the point of maximal concentration. There, they are subsequently retained at this point and may receive additional signals such as TGF-β to promote further differentiation.

It is clear that chronic liver injury results in excess liver fibrosis, which can progress to cirrhosis. However, the relative contribution of the injury stimulus and the subsequent hepatocyte apoptosis in the development of liver fibrosis remain unknown. The recent demonstration that hepatocyte apoptosis, in the absence of an injury stimulus, can result in liver fibrosis has made the identity of signals generated from apoptotic cells that regulate liver fibrosis of great interest (24). The known increase in tissue adenosine at sites of cellular injury and apoptosis stimulated us to test whether adenosine is involved in the regulation of HSCs. We studied the ability of adenosine to regulate HSC cytosolic Ca2+ fluxes, chemotaxis, and collagen production.

In this study, we demonstrated that adenosine inhibits the increase in HSC cytosolic Ca2+ concentration induced by ATP and PDGF. This inhibition was associated with an inhibition of PDGF-induced HSC chemotaxis, and the effect was dose dependent, reversible, and likely mediated via the A2a receptor subtype. We further showed that adenosine upregulates TGF-β and collagen mRNA. This identifies a novel role for adenosine in providing a “stop” signal for HSCs when they have migrated to a site of cellular injury, with upregulation of collagen and TGF-β synthesis.

MATERIALS AND METHODS

Cell culture and reagents. LX-2 cells are immortalized human HSCs and were a gift from Dr. Scott Friedman (25). LX-2 cells were cultured in RPMI plus 5% FBS and 1% penicillin-streptomycin, and the media were changed every 3 days. Primary HSCs were cultured in medium 199, 10% FBS, 1% penicillin-streptomycin, 2% gentamycin, and fungizone at a 1:100 density, with a media change 24 h after cell isolation and then after every 3 days. Histodenz, DNase, collagenase, hematoxylin-eosin stain, forskolin, xanthine, inosine, adenine, hypoxanthine, uric acid, MRS-1523 (an A1 antagonist), 8-(p-sulfophenyl)theophylline (8-SPT; a peripheral nonselective adenosine antagonist), and 5′-(N-ethylcarboxamido)adenosine (NECA; a nonselective adenosine receptor agonist) were obtained from Sigma (St. Louis, MO). Trypan blue, trypan reagent, fungizone, trypsin-EDTA, RNAse-free water, PBS, medium 199, RPMI, DMEM, HBSS, and F-12 (HAM) were purchased from GIBCO-BRL Invitrogen (Carlsbad, CA). NaOH,

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
HCl, and glutaraldehyde were bought from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ). 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; an A2a antagonist), ZM-241385 (an A2a antagonist), and MRS-1706 (an A2b antagonist) were obtained from Tocris (Ellisville, MI). Monocyte chemotactic protein (MCP-1) and PDGF were from Pepro-Tech (Rocky Hill, NJ). Triton X-100 was from Cole-Parmer (Vernon Hills, IL). Transwell inserts were from Corning Costar (Corning, NY).

All reagents were at the highest quality grade commercially available. HEPES, bicarbonate, HCl, and glutaraldehyde were bought from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ). 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; an A2a antagonist), ZM-241385 (an A2a antagonist), and MRS-1706 (an A2b antagonist) were obtained from Tocris (Ellisville, MI). Monocyte chemotactic protein (MCP-1) and PDGF were from Pepro-Tech (Rocky Hill, NJ). Triton X-100 was from Cole-Parmer (Vernon Hills, IL). Transwell inserts were from Corning Costar (Corning, NY).

Animals and HSC isolation. Mice of the C57BL/6 strain were used for HSC isolation. All experiments and animal handling were done according to Yale University Institutional Animal Care and Use Committee guidelines. HSCs were isolated by in situ pronase-collagenase perfusion followed by density gradient centrifugation, as described previously (1). Primary cells were >95% pure. Cells were grown on standard tissue culture plastic dishes in medium 199 with 10% FCS and antibiotics. Primary cells were used at 7 days after isolation.

Semiquantitative real-time RT-PCR for expression of TGF-β and collagen I mRNA. LX-2 cells were incubated with the adenosine receptor agonist NECA at a concentration of 10 μM for 24 h or incubated in media alone. After incubation, total RNA was extracted, and cDNA was prepared using murine Moloney leukemia virus reverse transcriptase (BD Biosciences, Clontech, Palo Alto, CA). Semiquantitative real-time PCR was performed for TGF-β and collagen I using commercial primer-probe sets (Applied Biosystems) and the Applied Biosystem 7500 real-time PCR system. The expression of GAPDH was used to standardize the samples, and the results were expressed as a ratio compared with untreated LX-2 cells. Nonquantitative RT-PCR was used to determine adenosine receptor mRNA expression in LX-2 cells. Specific oligonucleotide primers were made at the Yale Keck Facility, and the thermal cycles used were based on the sequences used in a previous study (10).

Measurement of changes in cytosolic Ca2+ concentration. LX-2 cells were grown on glass coverslips, loaded with the Ca2+-sensitive fluorophore fluo-4 AM (Molecular Probes), and put into a specially designed chamber for use on a confocal microscope as previously described (13). Cells were perfused initially with HEPES buffer and then with buffer containing ATP (100 μM) or PDGF (10 ng/ml). Changes in fluo-4 fluorescence were monitored using a Zeiss confocal imaging system. Fluo-4 fluorescence was excited using a Kr/Ar laser at 488 nm; emitted fluorescence >515 nm was collected. Changes in fluorescence over time were expressed as peak fluorescence (fP) divided by initial fluorescence (f0).

Chemotaxis assay. The migration of LX-2 cells was studied using Transwell inserts equipped with 8-μm pore polycarbonate-free filters as previously described (6). Briefly, 2 × 10^4 LX-2 cells were plated per well. Cells were treated with the appropriate adenosine receptor antagonist (8-SPT or ZM-241385) 10 min before the addition of either adenosine, NECA, or forskolin. MCP-1 or PDGF was added to the lower chamber 2 h afterward. After 24 h, the lower surface of the membrane was stained using hematoxylin-eosin, 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 being expressed as average numbers of cells per high-power field. Statistical analysis in the form of a Student’s t-test was performed with P ≤ 0.05 as significant.

RESULTS

Adenosine does not induce an increase in cytosolic Ca2+ concentration. Confocal video microscopy was used to quantify changes in cytosolic Ca2+ concentrations in response to adenosine. Adenosine did not induce an increase in the cytosolic Ca2+ concentration in LX-2 cells at concentrations between 10 and 100 μM (Fig. 1, A and B).

Adenosine inhibits increases in cytosolic Ca2+ concentrations induced by ATP and PDGF. Confocal video microscopy was used to quantify changes in cytosolic Ca2+ concentration in response to ATP and PDGF. ATP induced a rapid increase in cytosolic Ca2+ concentration in LX-2 cells (Fig. 1, C and
adenosine of ATP- and PDGF-induced increases in cytosolic 
A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub>. These are widely expressed including in the 
pretreatment with adenosine. Figure 1

demonstrates that pretreatment of cells with the adenylate 
cyclase inhibitor 2,5-DDA blocked the ability of adenosine to 
reproduce the adenosine-induced inhibition of HSC chemo-
taxis as the combination of all four antagonists. This strongly 
implicates the A<sub>2a</sub> receptor subtype as being responsible for the 
hit HSC chemotaxis to PDGF as significantly antagonized by the 
A<sub>2a</sub> receptor subtype antagonist but not by the A<sub>1</sub>, A<sub>2b</sub>, and A<sub>3</sub> 
receptor-selective agents. The A<sub>2a</sub> antagonist was as effective 
at blocking adenosine-mediated inhibition of PDGF chemotaxis 
as the combination of all four antagonists. This strongly 
implies the A<sub>2a</sub> receptor subtype as being responsible for the 
hit HSC chemotaxis. The inability of A<sub>1</sub>, A<sub>2b</sub>, and A<sub>3</sub> 
receptor antagonists to block the adenosine-induced inhibition 
of PDGF chemotaxis suggests that these receptor subtypes 
have minimal roles or no role at all. Caffeine is known to be a 
pan-adenosine receptor antagonist, and we found that it was 
also (at 10 μM) able to block the adenosine-mediated inhibi-
tion of chemotaxis (data not shown, experiments repeated in 
triplicate).

Expression of adenosine receptors in HSCs. The presence of 
mRNA for adenosine receptor subtypes on LX-2 HSCs was 
assayed using RT-PCR. As shown in Fig. 5, LX-2 cells 
contained mRNA for A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> (but not A<sub>1</sub>) receptor 
subtypes. Human peripheral blood lymphocytes were used as a 
positive control, and signals for all four receptor subtypes were 
detected.

The A<sub>2a</sub> receptor effect is mediated via cAMP and is reversible. 
Signaling downstream of the A<sub>2a</sub> receptor subtype is 
mediated predominantly via G<sub>s</sub>-dependent adenylate cyclase 
activation, resulting in an increase in cAMP. An additional 
G<sub>q</sub>-independent, G<sub>12</sub>/G<sub>13</sub> pathway of Ras and ERK1/2 activation 
has also been documented (21, 22). Forskolin, an activator of 
adénylate cyclase, was used to test if elevations in cAMP could 
reproduce the adenosine-induced inhibition of HSC chemotaxis. 
As shown in Fig. 6A, forskolin also had potent inhibitory 
activity on HSC chemotaxis to PDGF. The importance of 
cAMP in the adenosine effect was further confirmed by the 
demonstration that pretreatment of cells with the adénylate 
cyclase inhibitor 2,5-DDA blocked the ability of adenosine to

---

**Fig. 2.** A: adenosine inhibited PDGF-induced chemotaxis in a dose-dependent manner. Human LX-2 HSCs (20,000 cells) were plated in the upper chamber of Transwell inserts with recombinant human PDGF-BB (10 ng/ml) as the chemoattractant in the lower chamber. Hematoxylin and eosin staining of the undersurface was done at the 24-h time point with migrating cells counted per high-power field (HPF). To test for the effect of adenosine on chemotaxis, cells were incubated with adenosine (at doses of 0.01, 0.1, 1, 10, and 100 μM) for 120 min before the addition of PDGF, and adenosine was maintained in the culture medium until the end of the 24-h study period (*P < 0.05 vs. the PDGF control by Student’s t-test). B: primary mouse HSC chemotaxis was inhibited by adenosine. Primary mouse HSCs were isolated from B6 mice and cultured on plastic. Cells at day 5 were plated (20,000 cells) in the upper chamber of Transwell inserts, with PDGF-BB (10 ng/ml) as the chemoattractant in the lower chamber. Experiments were performed as in A with the addition of adenosine 120 min before the addition of PDGF (*P < 0.05 vs. the PDGF control by Student’s t-test).
inhibit chemotaxis of HSCs in response to PDGF (Fig. 6B). Forskolin was found to completely block the ATP-induced increase in cytoplasmic Ca\textsuperscript{2+} concentration, further confirming the role of cAMP in this phenomenon (data not shown, experiments repeated in triplicate). It was of interest for us to know whether, at the cessation of cellular injury, when adenosine levels normalize, HSCs could be expected to recover the ability to undergo PDGF-mediated chemotaxis. As shown in Fig. 6C, 24 h after we replaced adenosine-containing with adenosine-free media, HSCs were again able to undergo chemotaxis in response to PDGF.

Adenosine upregulates collagen and TGF-\beta mRNA. HSCs are known to upregulate TGF-\beta (which subsequently functions in autocrine and paracrine loops) and also to upregulate a number of matrix constituents including collagen I. We tested whether adenosine upregulated the expression of TGF-\beta and collagen genes in HSCs by performing semiquantitative real-time PCR. As shown in Fig. 7, adenosine at 100 \mu M resulted in an approximately threefold increase in TGF-\beta and collagen mRNA at 24 h. At 48 h, there was a decrease in the level of collagen mRNA to basal levels, but TGF-\beta levels were maintained. The assay of the TGF-\beta concentration in the supernatant by ELISA showed a significant increase between control and adenosine-treated plates at 3 and 24 h (3-h control: 3.6 ± 0.9 ng/ml, 3-h adenosine: 12.1 ± 2.0 ng/ml, 24-h control: 4.9 ± 1.0 ng/ml, 24-h adenosine: 15.1 ± 1.1 ng/ml, means ± SE; this was significant at <0.01 for both time points). This demonstrates that at sites of high adenosine concentrations, adenosine induces HSC differentiation in addition to stopping HSC chemotaxis.

DISCUSSION

Developmental apoptosis is programmed at the level of the cell and organ, in contrast with necrosis, which is unpredictable and not programmed. Pathological apoptosis has features of...
developmental apoptosis, in that it uses much of the same cellular machinery but is not programmed at the level of the organ and is usually unpredictable. Pathological apoptosis, therefore, requires a complex adaptive response, which in the liver includes HSC migration, differentiation, and matrix remodeling (5). HSC numbers increase at the site of hepatocyte injury, and this local increase in HSCs is partly due to chemotaxis toward a gradient of a number of cytokines. For HSCs, PDGF is well characterized to induce chemotaxis and is produced by macrophages, activated HSCs, and injured bile duct segments (9). In such a model, HSCs are retained at the site of highest concentration of the chemotactic cytokine.

The role of hepatocyte apoptosis (as distinct from the injury stimulus and immune response) in the development of liver fibrosis has been of great interest. Clinical specimens from patients with hepatitis C infection have revealed a correlation between hepatocyte apoptosis, HSC activation, and increased stage of fibrosis (15). More convincing evidence that hepatocyte apoptosis is sufficient to stimulate liver fibrosis has been provided by a number of experimental models (23, 24). These studies bring to the forefront questions about the identity of the molecules that communicate hepatocyte apoptosis to the hepatic repair response. We chose to test whether adenosine might fulfill some of this function because it is produced during conditions of energy imbalance by the dephosphorylation of adenosine tri-, di-, and monophosphates and is also produced from the metabolism of purines during the degradation of nucleic acids of apoptosing cells. Adenosine is also rapidly metabolized to inosine by adenosine deaminase. The production of adenosine therefore does not require any new protein synthesis, and extracellular concentrations rise rapidly in tissue injury from the 0.1- to 0.3-μM range to >100 μM. The rapid metabolism limits the half-life to a few minutes. There is a very large body of literature on the regulation by adenosine of adaptive responses to tissue injury in a number of organs, but very little is known about its role in liver injury and repair (8).

Our finding that adenosine inhibits increases in cytosolic Ca2+ concentration induced by ATP and PDGF is novel, and, as predicted, adenosine resulted in inhibition of HSC chemotaxis by PDGF. Since adenosine levels are highest in the immediate microenvironment of cellular injury and apoptosis, this new function of adenosine adds an important component to the model of migration of HSCs. The previously described chemotaxis of HSC toward an increasing gradient of PDGF is
still valid, but our data suggest that upon arriving at a site of
cellular injury with high adenosine levels, chemotaxis will be
inhibited (11). This provides a stop signal to HSCs. A system
in which there is a start signal (chemokine/cytokine) and a
protein synthesis-independent stop signal (adenosine) has dis-
tinct advantages. First, it stops HSCs at the site where they are
most needed and keeps them there until hepatocyte apoptosis is
no longer occurring and adenosine levels drop. This is neces-
sary as PDGF and other chemokines induce chemokinesis as
well as chemotaxis; thus, even when HSCs have reached the
peak of the chemokine gradient, they will be actively moving
in the absence of a stop signal. Another advantage is that it may
allow fine localization of HSCs to the site of cellular injury. In
pathological states, chemokines are produced by many tissues
and cells including Kupffer cells and bile segments. Such
chemokine production may function to bring HSCs into a
general area (i.e., around a bile duct). The adenosine-induced
stop signal may function to localize HSCs into the exact
vicinity of cellular injury. The demonstration that the adeno-
sine stop signal is reversible suggests that after the local injury
has resolved, HSCs are able to respond to new chemokine
gradients. In addition to stopping HSCs, we demonstrated that
adenosine increases HSC differentiation by an upregulation of
TGF-β and collagen I mRNA. This is consistent with increas-
ing HSC survival and stimulating matrix remodeling once
HSCs have reached the site of tissue injury.

The important role of adenosine in liver fibrosis proposed by
us is consistent with a study (14) in experimental lesions in the
skin, where activation of the A2a receptor accelerated wound
healing. Elevated adenosine levels also result in pulmonary
fibrosis. A number of studies have correlated coffee and tea
consumption with a reduced risk of chronic liver disease. This
was recently confirmed in a prospective study (19) of a popu-
lation in the United States. Caffeine and its metabolites have a
number of properties that may be responsible for this effect.
These include antioxidant properties, inhibition of lipid per-
oxidation, and possibly an improvement of insulin sensitivity
(18). However, the only known biological function of caffeine
for which it has significant activity at concentrations achieved
during normal human consumption (peak: 70 μM) is antago-
nism of A1 and A2 adenosine receptors. Our demonstration of
a role for the A2a receptor in localizing HSCs to sites of tissue
injury suggests that the effect of caffeine in decreasing liver
fibrosis may be by A2a receptor antagonism with a consequent
interference with HSC trafficking and differentiation. This is
further supported by our demonstration of the ability of cafe-
zeine to antagonize the effect of adenosine to stop HSC
chemotaxis.

A previous study (7) on CCl4-induced liver fibrosis in rats
demonstrated a decrease in the development of fibrosis and a
more rapid resolution of fibrosis when adenosine was admin-
istered. The results of this study are very surprising because
adenosine was administered 3 times/wk and was not in any
sustained release form. Since adenosine is rapidly degraded,
this would have resulted in elevated adenosine levels for a
matter of minutes for 3 times/wk. How such transient eleva-
tions in adenosine can have such significant effects is difficul-
to explain.

The adenosine A2a receptor is Goi coupled and results in an
increase in cAMP (20). Additional downstream pathways in-
clude PKA, CREB, and p38 activation. A separate G12/13/Ras/
MEK pathway has also been identified. Adenosine has been
shown to increase cAMP levels in primary rat HSCs, and our
replication of the antichemotactic effects by forskolin demon-
strates that elevations in cAMP are sufficient for this effect.
The inability of adenosine to inhibit chemotaxis in the presence
of the adenylate cyclase inhibitor 2,5-DDA, moreover, dem-
onstrates that elevations in cAMP are required for this effect.

In summary, we demonstrated that adenosine blocks cyto-
solic elevations in Ca2+ concentration in HSCs and is a potent
stop signal for chemotaxis, and we propose that adenosine has
an important role in liver fibrosis by localizing HSCs to the site
of tissue injury and inducing HSC differentiation. This identi-
fies adenosine receptors as potential targets for modulating
HSC biology and regulating the development of liver fibrosis.

ACKNOWLEDGMENTS

We appreciate Dr. Scott Friedman’s expert advice and the use of the LX-2
cell line.

GRANTS

This work was supported by National Institute of Diabetes and Digestive
and Kidney Diseases Grants K08-DK-02965-04 and P30-DK-34989 and by a
research gift from Gilead Pharmaceuticals. J. A. Dranoff was supported by an
American Heart Association grant-in-aid.
REFERENCES