Adenosine Inhibits Chemotaxis and Induces Hepatocyte-Specific Genes in Bone Marrow Mesenchymal Stem Cells

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Abstract
Bone marrow-derived mesenchymal stem cells (MSC) have therapeutic potential in liver injury, but the signals responsible for MSC localization to sites of injury and initiation of differentiation are not known. Adenosine concentration is increased at sites of cellular injury and inflammation, and adenosine is known to signal a variety of cellular changes. We hypothesized that local elevations in the concentration of adenosine at sites of tissue injury regulate MSC homing and differentiation. Here we demonstrate that adenosine does not induce MSC chemotaxis, but dramatically inhibits MSC chemotaxis in response to the chemoattractant hepatocyte growth factor (HGF). Inhibition of HGF-induced chemotaxis by adenosine requires the A2a receptor, and is mediated via up-regulation of the cyclic AMP/protein kinase A pathway. This results in inhibition of cytosolic calcium signaling, and down-regulation of HGF-induced Rac1. Due to the important role of Rac1 in formation of actin stress fibers, we examined the effect of adenosine on stress fiber formation and found that adenosine inhibits HGF-induced stress fiber formation. In addition, we found that adenosine induces the expression of some key endodermal and hepatocyte-specific genes in mouse and human MSC in vitro. We propose that the inhibition of MSC chemotaxis at sites of high adenosine concentration results in localization of MSC to areas of cellular injury and death in the liver. We speculate that adenosine might initiate the process of differentiation of MSC into hepatocyte-like cells.

Keywords
Cell Migration; Rac1; Protein Kinase A; Calcium; Differentiation

Introduction
Mesenchymal stem cells (MSC) are a diverse population of cells which can be isolated from multiple tissues, including bone marrow, fat, and others. Bone marrow MSC are stromal cells which support hematopoiesis during embryogenesis and in adult life. Their
mesodermal origin is reflected by their ability to differentiate into fat, cartilage and bone in vitro. In addition to their ability to differentiate into mesodermal tissues, MSC can differentiate into other cell types including hepatocyte-like cells. The ability of MSC to differentiate into multiple cell types, and the relative ease by which they can be expanded in culture makes them attractive candidates for therapy in a variety of conditions. In this context they have been tested in animal models of acute liver injury. The initial step required is localization to the site of tissue injury. After localization, MSC have been proposed to have a range of functional affects. In the liver for example there is evidence for MSC differentiating into hepatocyte like cells, as well as well as inducing stimulation of endogenous hepatocyte proliferation. In keeping with their highly plastic phenotype MSC may also differentiate into the matrix depositing hepatic myofibroblasts, but this is controversial.

There is a requirement for signals which will localize MSC to the area within the liver with hepatocyte death, and also signals which will initiate MSC differentiation. Adenosine is produced both extracellularly and intracellularly by dephosphorylation of adenosine tri-, di-, and monophosphates, and by degradation of nucleic acids via the uric acid pathway during cellular injury. These sources of adenosine result in elevated levels at sites of tissue ischemia, cellular apoptosis, and inflammation, with concentrations increasing more than 100-fold from the 30- to 300-nM range present in health. Elevated levels of adenosine are known to induce a variety of adaptive changes in response to tissue injury via four receptor subtypes A1, A2a, A2b and A3. These include matrix-remodeling, immune regulation and angiogenesis. The role of adenosine in localization of stem cells to sites of tissue injury is not known.

Our goal was to study whether adenosine induces MSC chemotaxis, determine whether adenosine regulates the response of MSC to established chemoattractants, and investigate whether adenosine has any role on differentiation of MSC. Here we demonstrate that adenosine alone does not affect MSC chemotaxis, but significantly inhibits hepatocyte growth factor induced chemotaxis. We further identify an important role for down-regulation of Rac1 in the inhibitory effect of adenosine on MSC chemotaxis. In addition to providing a chemotactic stop signal to MSC, adenosine also stimulates transcription of genes potentially associated with MSC differentiation. Based on these results, we propose that MSC reach areas of tissue injury and death due to gradients of conventional chemoattractants. However once MSC have reached these areas, adenosine provides an important stop signal, allowing them to become stationary at sites of tissue injury. Furthermore, adenosine may initiate the process of differentiation of MSC into hepatocyte-like cells at sites of liver damage.

Materials and Methods

Reagents

Forskolin (cyclic AMP analogue), MRS 1523 (A3a antagonist), 8-SPT (peripheral non-selective adenosine antagonist), adenosine, 5′-(N-ethylcarboxamido) adenosine (NECA; non-selective adenosine receptor agonist), and ionomycin were obtained from Sigma (St. Louis, MO). Trypan blue, Fungizone, Trypsin-EDTA, PBS, IMDM, MEM alpha, phenol red-free HBSS, L-Glutamine, Trizol were purchased from GIBCO/Invitrogen (Carlsbad, CA). DPCPX (A1 antagonist), ZM 241385 (A2a antagonist), and MRS 1706 (A2b antagonist) were obtained from TOCRIS (Ellisville, MI). Triton X-100 was from Cole-Parmer (Vernon Hills, IL). Eight micrometer polycarbonate transwell inserts were purchased from Corning Life Sciences (Acton, MA). ST-HT31 (Protein kinase A inhibitor) was from Promega (Madison, WI). NSC23766 (Rac1 inhibitor) and Y27632 (Rho kinase inhibitor) were from Calbiochem (Gibbstown, NJ). Fetal bovine serum was from Atlanta Biologicals.
Taqman quantitative RT-PCR assays were purchased from Applied Biosystems (Foster City, CA).

**MSC culture**

Human and mouse bone marrow MSC were provided by the Tulane Center for Gene Therapy. MSC (passage 8–15) were cultured as previously described by Peister A, et al.14. Mouse MSC media consisted of Iscove’s Modified Dulbecco Medium, supplemented with 10% fetal bovine serum, Penicillin, Streptomycin, L-Glutamine, and Amphotericin B, exchanged every 3 or 4 days. Human MSC media consisted of MEM alpha, supplemented with 16% fetal bovine serum, Penicillin, Streptomycin, L-Glutamine, and Amphotericin B. Cells were cultured in 75-cm² flasks until 80 to 90% confluence and were then used for experiments.

**Cyclic AMP Assay**

Mouse MSC were grown in 6-well plates. Serum free conditions were applied for 12 hours prior to experiments. Fresh media was added containing adenosine (10μm) or NECA (10μM) was added 20 minutes before NECA where indicated. The cyclic AMP (cAMP) levels were measured using a cAMP enzyme immunoassay system (GE Healthcare Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. After 15 minutes, cells were lysed and assayed in duplicate. The optical density was calculated against a standard curve to determine the cAMP level. Results were expressed as ratios (mean ± S.E.) relative to those of mock-transfected controls.

**Pull-down Assays for Active Rac 1**

Mouse MSC were cultured in serum free media for 12 hours, followed by HGF 50 ng/ml, with or without pre-treatment with NECA (10μM) and proteins was extracted 2h after the addition of HGF. Lysis buffer was from Upstate (Temecula, CA) consisting of 125 mm HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM MgCl2, 5 mM EDTA, 10% glycerol. Protease inhibitors aprotinin and leupeptin (10 mcg/ml each, Roche Molecular Biochemicals, Chicago, IL) were added. Following two PBS washes, lysis buffer was added, cells were scraped and incubated for 15 mins at 4°C with agitation. Rac activation was measured by affinity precipitation of cellular GTP-bound forms of Rac as previously described15. GST fused to the Rac1(p21)-binding domain of PAK (GST-PBD) bound to glutathione-coupled Sepharose beads was added and samples were incubated for 45 min at 4°C. Beads were pelleted by brief centrifugation and washed three times with lysis buffer. Beads were resuspended in Laemmli reducing buffer (Invitrogen, Carlsbad, CA) and boiled for 5 min. Supernatant and agarose pellet were mixed and 20 μl of sample was loaded on SDS-PAGE gel and blotted to a nitrocellulose membrane(Bio-Rad, Hercules, CA). Nonspecific binding was blocked with 5% milk for 1 hour and washed three times with Tris-buffered saline. Anti-Rac1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied overnight, followed by washes and appropriate secondary antibody for one hour. Chemiluminescent substrate (Pierce, Rockford, IL) was applied to the membrane for 5 mins and developed on X-ray film (Kodak, Rochester, NY).

**Transfection of MSC with constitutively active Rac 1**

cDNA encoding constitutively active Rac1 (RacQL) was a gift of Dr. V. Shah.16 Mouse MSC were grown in 12-well plate and transfected with constitutively active Rac1 plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Media was changed 3 hours after transfection and cells were kept in serum-supplemented media for an additional 36 hours. Media was then replaced with serum-free media for 12 hours prior to experiments.
Migration assay

$4 \times 10^4$ mouse MSC were plated per transwell (8 micron, Costar, Corning, NY), and MSC migration was quantified. Cells were treated with the appropriate receptor antagonist 10 min before the addition of NECA. HGF was added to the lower chamber 2 h afterward. After 24 h, the lower surface of the membrane was stained using hematoxylin, photographed, and analyzed. All experiments were repeated in triplicate. For each group, 11 high-power field images were taken. Cells per high-power field were counted. The migration index was calculated based on the ratio of cells that migrated in response to chemoattractants to cells that migrated in the absence of chemoattractants. Statistical analysis in the form of a t-test was performed using SPSS version 11.5 (Chicago, IL) with p< 0.05 considered significant.

RT-PCR

Total RNA of mouse or human MSC was extracted by Trizol, and cDNA was prepared using SuperScript III Kit (Invitrogen), or high capacity reverse transcription kit (Applied Biosystems) from 2 mcg of total RNA. Qualitative RT-PCR was used to determine adenosine receptor mRNA expression in MSC. Oligonucleotide sequences for mouse A1 and A3 receptors were used based on previously published sequences. Taqman quantitative RT-PCR (Applied Biosystems) was used to measure relative gene expression of hepatocyte-specific genes in MSC.

Analysis of Intracellular Calcium Concentration

Calcium concentration was measured with Fura2/AM (Invitrogen Molecular Probes) as calcium probe. Cells were loaded with 5 mM fura2/AM for 30 min at 37 °C. Fura2/AM-loaded MSC were stimulated with HGF (50 ng/ml). Fluorescence was monitored in ratio mode with a fluorometer (Polarstar Galaxy, BMG Lab-Technologies, Offenburg, Germany). Collected data were analyzed with Fluostar Galaxy Software (BMG Technologies). At the end of each experiment, cells were treated with 5 μM ionomycin in HBSS without phenol red. Experimental 340/380 ratios were converted to calcium concentration according to the equation previously described.

Filamentous actin staining

Mouse MSC were seeded on glass coverslips 24h before use. Following treatment/stimulation, cells were fixed with 3.7% formaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 5 min. Filamentous actin was labeled with rhodamine phalloidin (Invitrogen) for 30 min. The stained cells were imaged using con-focal microscopy (Leica TCS SP5, Leica Microsystems, Bannockburn, IL).

Results

Messenger RNA for adenosine receptor subtypes is expressed in MSC

Mouse MSC expressed mRNA for A2a and A3 receptors, but not for A1 and A2b (Fig. 1A). The expression profile of human MSC was a little different, with expression of A1, A2a, and A2b receptor mRNAs, but not for the A3 receptor (Fig. 1B).

Adenosine does not induce MSC chemotaxis

Using a transwell chamber assay with NECA in the lower chamaber, we tested whether NECA-induced MSC chemotaxis. The presence of NECA did not affect MSC chemotaxis compared to controls (Figure 1C).
Adenosine inhibits HGF-induced chemotaxis through the A2a receptor

HGF induces more than a 2-fold increase in MSC migration (p< 0.05). To test whether adenosine has an effect on HGF-induced chemotaxis, MSC were incubated with NECA (10 μM) 2 hours before the addition of HGF. Preincubation of MSC with NECA resulted in a significant decrease in HGF-induced migration index (HGF: 2.27 +/- 0.2; HGF and NECA: 1.2 +/- 0.1, P < 0.05, Figure 1C). We established that this is a receptor-mediated effect by demonstrating that it could be blocked by preadministration of the pan-adenosine receptor antagonist 8-PST (Figure 1D) (migration index, NECA + HGF: 1.45 +/- 0.13; 8PST + NECA + HGF: 2.67 +/- 0.3, P < 0.05).

Adenosine signals via four receptor subtypes: A1, A2a, A2b, and A3. Adenosine subtype-specific antagonists were used to determine the role of receptor subtypes (DPCPX (10 nM), A1; ZM241385 (1 μM), A2a; MRS-1706 (10 nM), A2b; and MRS-1523 (5 μM), A3). The ability of adenosine to inhibit MSC chemotaxis to HGF was significantly blocked by the A2a receptor subtype antagonist but not by the A1, A2b, and A3 receptor-selective antagonists (Figure 1D) (migration index, NECA + HGF: 1.45 +/- 0.13; Zm241385 + NECA + HGF: 3.6 +/- 0.3, P < 0.05). This demonstrates that the A2a receptor subtype is responsible for the inhibition of HGF-induced MSC chemotaxis.

Adenosine inhibits MSC chemotaxis through the cAMP/Protein kinase A pathway

Signaling downstream of the A2a receptor is mediated predominantly via adenylate cyclase activation, resulting in elevation in cAMP20. We found a central role for cAMP downstream of signaling of A2aR in MSC. The adenosine agonist NECA increased cAMP levels, and this effect was blocked by the A2a receptor antagonist (Figure 2A). Forskolin, which induces elevations in cAMP independent of adenosine receptor activation, mimics the inhibitory effect of NECA on HGF-induced MSC chemotaxis (Figure 2B) (migration index, HGF: 2.02 +/- 0.15; forskolin + HGF 1.08 +/- 0.05, p < 0.05). cAMP mediates downstream effects through activation of Protein kinase A (PKA) in many cells21. We tested the ability of the PKA inhibitor ST-HT31 to reverse forskolin-inhibited chemotaxis, and found that forskolin-induced inhibition of MSC migration was antagonized by the PKA inhibitor ST-HT31 (Figure 2B) (migration index, forskolin + HGF: 1.08 +/- 0.05, ST-HT31+ forskolin + HGF: 2.25 +/- 0.38, p < 0.05). We further found that the PKA inhibitor could block the effect of NECA on HGF-induced MSC chemotaxis (Figure 2C) (migration index, NECA+ HGF: 1.53 +/- 0.19, ST-HT31 + NECA + HGF: 4.2 +/- 0.69, p < 0.05). The above findings demonstrate that adenosine inhibits HGF-induced MSC chemotaxis through a cAMP/PKA-dependent pathway.

HGF increases MSC migration through the Rac1 pathway

To elucidate the intracellular signaling pathways responsible for adenosine and HGF interaction, we investigated pathways responsible for HGF-induced chemotaxis in MSC. HGF signaling via c-met is known to increase cytosolic Ca++ and lead to activation of Rac122. To determine whether Rac1 inhibition may be a mechanism by which adenosine inhibits HGF-induced chemotaxis we tested the ability of a Rac 1 inhibitor (NSC23766), and a Rho kinase inhibitor (Y27632) to inhibit HGF-induced chemotaxis. The Rho kinase inhibitor had no significant effect on HGF-induced migration (Figure 3A). However, the Rac1 inhibitor significantly blocked HGF-induced MSC migration (Figure 3A). These findings support a requirement for Rac1 in HGF-induced MSC chemotaxis (migration index, HGF: 2.0 +/- 0.2; Rac1 inhibitor + HGF: 1.2 +/- 1.2, p < 0.05).
**Adenosine inhibits MSC chemotaxis through down-regulation of Rac1**

We hypothesized that the inhibitory effect of adenosine on HGF-induced chemotaxis occurs through down-regulation of Rac1. A Rac1 pull-down assay in MSC two hours after the addition of HGF found that HGF significantly increases Rac1-GTP (e.g. active Rac1). However, in cells pre-treated with NECA, this effect of HGF was significantly inhibited (Figure 3B). Additionally, pre-treatment of cells with a PKA inhibitor (ST-HT31) before NECA, blocks the inhibitory effect of NECA on HGF, and leads to HGF-induced Rac1 activation (Figure 3B). This shows that the inhibitory effect of NECA on HGF-induced Rac1 activation, takes place through PKA pathway.

In order to confirm this effect of adenosine on Rac1 is responsible for the inhibition of MSC migration, we repeated the MSC migration assay in MSC transfected with constitutively active Rac1. We found that in MSC with constitutively active Rac1, HGF increases cell migration, but NECA could not inhibit the HGF effect (migration index, HGF: 2.23 +/- 0.39; NECA + HGF: 2.13 +/-0.32, p>0.05, Figure 3C). This demonstrates that the inhibitory effect of NECA on MSC migration takes place through down-regulation of Rac1. Rac1 is well known to be involved in actin polymerization23. We examined changes induced by HGF and adenosine on actin fibers using confocal microscopy and found that HGF increased polymerized actin fibers in MSC. In cells pre-treated with NECA, this effect of HGF was largely inhibited (Figure 4A–C). In cells pre-treated with a PKA-inhibitor before the addition of NECA, the effect of NECA on HGF was blocked and actin polymerization increased (Figure 4D). In addition to the loss of actin stress fibers NECA also induced an overall change in the cell body to a more round shape (figure 4C).

**Adenosine inhibits increases in cytosolic Ca\(^{2+}\) concentrations induced by HGF through PKA pathway**

It is known that HGF increases cytosolic free calcium concentration, and that an increase in free calcium concentration is involved in Rac1 activation22-24. We hypothesized that the inhibitory effect of adenosine on the HGF effect takes place through calcium signaling. We found that HGF leads to a large increase in intracellular calcium levels in MSC. In the cells pretreated with the adenosine receptor agonist NECA, this response was significantly attenuated (Figure 5A). The effect of NECA could be completely blocked by the pretreatment of cells with 25 micromolar ST-HT31, a selective PKA inhibitor (Figure 5A). Next, we investigated the relationship between calcium signaling and the Rac1 pathway. Ionomycin is known to intracellular calcium level, and rapidly increased intracellular calcium (Figure 5B), and Rac1 activity in MSC (Figure 5C).

**NECA Induces Endodermal, and Hepatocyte-Specific Genes**

We propose that adenosine released by damaged liver cells retains MSC at sites of tissue injury by inhibiting further migration. Next, we tested whether adenosine has any influence on hepatocyte-like differentiation of MSC in vitro. We quantitated the expression of hepatocyte-specific genes in passage 8 MSC, 4 or 8 days after the addition of NECA, and found NECA leads to nearly 20 fold induction of forkhead box A1 (Foxa 1 or HNF3-alpha) in mouse MSC (Figure 6A). Forkhead box A2 (Foxa 2 or HNF3-beta), undetectable in control mouse MSC, could be readily detected after the addition of NECA (Figure 6B). Foxa2 has been reported to have a key role on the differentiation of BM MSC into hepatocyte-like cells26. Furthermore, NECA increased the expression of Goosecoid (GSC) (Figure 6C). GSC is important for the development of mesendoderm and definitive endoderm in the mouse embryo27-28. NECA was not able to induce other endodermal, or hepatocyte-specific genes, such as Sox17, alpha-fetoprotein (AFP), albumin, epithelial gene adhesion molecule (EpCAM), tyrosine aminotransferase (TAT), in the mouse MSC (Figure 6D–H).
We found NECA induces the expression of GSC and Sox 17 in human MSC (Figure 7A–B). Both genes are critical for the development of definitive endoderm (the precursors of hepatocytes) during embryogenesis. Also, NECA induced the expression of EpCAM which is a key marker of hepatic stem cells and hepatoblasts. Furthermore, NECA induced the hepatocyte-specific genes albumin, TAT in human MSC (Figure 7 C–E). However, it did not induce the expression of AFP, Foxa1, or Foxa2 in human MSC.

Discussion

Mesenchymal stem cells (MSC) are multipotential and capable of differentiation into numerous connective tissue lineages, as well as cells of endodermal origin. This, along with ease of isolation and capacity to undergo extensive replication in vitro, have made MSC candidates for cell-based tissue engineering approaches. In an animal model of liver injury, transplanted MSC differentiated into functioning hepatocyte-like cells, and ameliorated liver injury. The mechanisms of localization to sites of injury, and differentiation into hepatocyte-like cells are not well understood.

Migration is thought to be mediated largely by soluble factors released from platelets and other cell types, sustaining chemotaxis, or movement of cells up a gradient of soluble factors. The binding of these factors to membrane receptors initiates a series of intracellular molecular events leading to the reorganization of the cytoskeleton into locomotive machinery.

Adenosine is produced from the metabolism of purines during the degradation of nucleic acids of apoptosing cells, and is rapidly metabolized by adenosine deaminase. The extracellular concentrations of adenosine rise rapidly in tissue injury from the 0.1–0.3 microM range to >100 microM. Such rapid metabolism limits the half-life to a few minutes. Since adenosine levels are highest in the immediate microenvironment of cellular injury, we were interested in examining whether adenosine has a functional affect of MSC migration, and differentiation. As can be seen in Figure 1A and B, mRNA for A2a and A2b receptors was expressed in mouse MSC and A1, A2a and A2b in human MSC. Interestingly, adenosine did not induce chemotaxis, but rather inhibited the well known chemoattractant HGF. This inhibitory affect of adenosine was receptor-mediated, and could be blocked by an antagonist specific for the A2aR. In a variety of cells, the A2aR is known to be coupled with adenylate cyclase, resulting in up-regulation of cAMP and PKA activation. We extend these findings to MSC, demonstrating an increase in cytosolic cAMP after activation by the non-hydrolysable adenosine agonist NECA. The ability of forskolin to inhibit HGF chemotaxis demonstrates that elevations in cAMP are sufficient for such inhibition. We also show a requirement for PKA using the specific PKA peptide inhibitor ST-HT31. HGF is known to increase cytosolic Ca++, and we have previously shown in hepatic stellate cells that signaling via the A2a receptor can inhibit increases in cytosolic Ca++. We therefore tested whether NECA can inhibit HGF induced increase in cytosolic Ca++. As can be seen from Figure 5A, HGF induced significant increase in cytosolic Ca++, and this was inhibited by NECA in a PKA dependent manner.

HGF has been shown to increase Rac1 activity in a Ca++ mediated manner, and we further confirm the requirement for Rac1 in HGF chemotaxis by demonstrating that a Rac1 inhibitor blocks HGF-induced chemotaxis. The inhibition of HGF-induced cytosolic Ca++ by adenosine, and the requirement for an increase in cytosolic Ca++ for Rac1 activation predicts that adenosine will inhibit HGF mediated increase in Rac1 activity. This was found to be the case, as demonstrated in Figure 3B. To definitively confirm that the inhibition of HGF induced Rac1 activation by adenosine is the mechanism of inhibition of chemotaxis we used a well tested plasmid (RacQL) expressing the constitutively active form of rac1. When MSC...
were transfected with RacQL, NECA was unable to block HGF-induced chemotaxis. Rac1 activation is known to be important in actin stress fiber formation, and to further confirm functional Rac1 inhibition in response to NECA we examined actin stress fibers in MSC. HGF increased the prominence of actin stress fibers, and as predicted from its ability to inhibit Rac1, NECA resulted in almost complete loss of actin stress fibers.

Collectively, these studies demonstrate a novel action of adenosine on MSC via the A2a receptor, resulting in inhibition of chemotaxis via a cAMP, PKA, Rac1 pathway. This has significant implications for MSC when they reach an area of cell death or inflammation with high levels of adenosine. The previously described model of chemotaxis of MSC toward an increasing gradient of HGF is still valid, but our data suggest that upon arriving at a site of cellular injury with high adenosine levels, MSC chemotaxis will be inhibited. We propose that the inhibition of chemotaxis will provide a functional stop signal, and result in localization of MSC to these sites. Such a model incorporating a stop signal in addition to the known chemotaxis signals has the advantage of localizing MSC to sites of injury, where they are most needed.

We propose a schema to describe the interaction between HGF and adenosine (Figure 8). HGF increases Rac1 activity through increasing intracellular calcium. Adenosine acts via A2a receptor and the cAMP/PKA pathway and inhibits the intracellular calcium wave induced by HGF, which in turn inhibits Rac1 activation, actin polymerization and cell migration.

In addition to inhibiting MSC chemotaxis, adenosine may also provide a differentiation signal to MSC which have stopped migrating in areas of high levels of adenosine. Adenosine receptor activation can induce the expression of several endodermal and hepatocyte-specific genes in mouse or human MSC including EpCAM, GSC, and Sox 17. These genes are critical for the development of definitive endoderm and hepatocytes during embryogenesis. These genes are up-regulated in human MSC by the effect of NECA.

We also demonstrated that NECA induces the expression of a variety of genes in MSC. In murine MSC there was up-regulation of Foxa1, Foxa2, and GSC. In human MSC there was up-regulation of GSC, Sox 17, EpCAM, albumin, and TAT. The major pathways by which MSC are thought to contribute to the hepatic response to injury are by a) stimulation of endogenous hepatocyte replication through paracrine action, b) secretion of anti-inflammatory cytokines and chemokines, c) differentiation into hepatocytes, and d) differentiation into myofibroblasts, resulting in matrix remodeling. The up-regulation of genes important in mesodermal and endodermal patterning provides support for MSC differentiation, but does not exclude paracrine effects of MSC on hepatocytes.

We have identified an important role for adenosine in the localization of MSC to sites of tissue injury, and subsequent differentiation via activation of the A2a receptor. The development of adenosine receptor agonists and antagonists is an active area of drug development, allowing for therapeutic manipulation of our findings. The full differentiation of MSC clearly requires multiple signals, and the manipulation of A2a receptor activation will form a part of this complex process. One application may be in cases of cirrhosis without on-going injury, for example with alcoholic cirrhosis where the patient has stopped drinking. By using liver specific A2a antagonists one may be able to enhance localization of MSC to the liver.

While adenosine was able to induce the expression of some important endodermal or hepatocyte-specific genes in MSC, some other important genes (e.g. AFP) could not be induced by adenosine. We propose that adenosine helps to localize MSC at sites of tissue injury and promotes differentiation of MSC; however hepatocytic differentiation in vivo is a
complex process which likely requires other factors not yet identified. In conclusion, adenosine inhibits MSC chemotaxis which may help localize MSC, and may provide differentiation signals for MSC at sites of injury.

Acknowledgments

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List of abbreviations

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<th>Abbreviation</th>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>Foxa1</td>
<td>Forkhead box A1</td>
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<td>Foxa2</td>
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<td>EpCAM</td>
<td>Epithelial gene adhesion molecule</td>
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<td>TAT</td>
<td>Tyrosine aminotransferase</td>
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References


Figure 1. NECA inhibits HGF-induced MSC chemotaxis via the A2a receptor

(A) RT-PCR. A2a, and A2b receptor mRNAs were expressed in mouse MSC, while A1 and A3 receptor mRNAs were not. (B) A1, A2a, and A2b receptor mRNAs were expressed in human MSC, while A3 receptor mRNA was not. (C) MSC migration assay. NECA (10 μM) added to the lower chamber, with only medium in the upper chamber did not result in chemotaxis. HGF (50 ng/ml) in the lower chamber with only medium in the upper chamber induced chemotaxis. To test for the effect of adenosine on chemotaxis, cells were incubated with NECA (10 μM) 2 hours before the addition of HGF. Values are migration index± SE (* P< 0.05 for control vs. HGF; † P<0.05 for HGF vs. NECA + HGF by t test). (D) The pan-adenosine receptor antagonist (8SPT) blocked the ability of NECA to inhibit HGF-induced...
chemotaxis. Furthermore, inhibition of HGF-mediated MSC chemotaxis was reversed by pre-incubation with A2a receptor antagonist (Zm 241835); whereas A1 receptor antagonist (DPCPX), A2b receptor antagonist (MRS-1706), and A3 receptor antagonist (MRS-1523) had no effect. Experiments were performed with the addition of the receptor antagonists 15 minutes before NECA. (* P<0.05 for HGF vs. NECA + HGF; †P<0.05 for NECA + HGF vs. 8SPT + NECA + HGF; ‡ P<0.05 for NECA + HGF vs. Zm241385 + NECA + HGF by t test).
Figure 2. Adenosine inhibits HGF induced chemotaxis via a cAMP and PKA mediated pathway.
(A) NECA increased cyclic AMP (cAMP) levels of MSC. This effect of NECA was blocked by Zm241385 (A2a receptor antagonist). cAMP level was measured 15 minutes after addition of NECA. A2a receptor antagonist was added 15 minutes before NECA (*p< 0.05 for control vs. NECA; †p< 0.05 for NECA vs. Zm241385 + NECA). (B) In the cells pre-treated with Forskolin (100 μM), HGF-induced chemotaxis was inhibited. In the cells pre-treated with ST-HT31-protein kinase A inhibitor- (25 μM), the effect of Forkolin was blocked (* P< 0.05 for HGF vs Forskolin + HGF; † P< 0.05 for Forskolin + HGF vs. ST-HT31 + Forskolin + HGF by t test). (C) NECA inhibited HGF-induced chemotaxis of MSC. But in the cells pre-treated with PKA inhibitor, the effect of adenosine was blocked. (* P< 0.05 for HGF vs NECA + HGF; † P< 0.05 for NECA + HGF vs. ST-HT31+ NECA + HGF by t test).
A

![Graph showing migration index](image)

B

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Rac GTP

Total GTP

*Hepatology. Author manuscript; available in PMC 2011 March 1.*
Figure 3. Adenosine inhibits HGF-induced chemotaxis via down-regulation of Rac1

(A) Rac1 inhibitor blocks MSC chemotaxis induced by HGF (* p< 0.05 HGF vs. Rac1 inhibitor + HGF). However, Rho1 inhibitor did not block HGF-induced chemotaxis in MSC.

(B) Rac1 pull down assay shows absent activity of Rac1 in control MSC. Treatment of MSC with HGF (50 ng/ml for 2h) significantly increased active Rac1. However, in cells pretreated with NECA (10 μM) 2h before the addition of HGF, the effect of HGF was significantly inhibited. Finally, pre-incubation of the cells with PKA inhibitor (25 microM) 1h before the addition of NECA blocked the effect of NECA and Rac1 activity was increased by the effect of HGF.

(C) Migration assay for MSC transfected with constitutively active Rac1. In these cells HGF increased MSC chemotaxis, but NECA did not inhibit the HGF effect.
Figure 4. Confocal microscopy after phalloidin staining demonstrating that NECA induces a loss of HGF-induced stress fibers in a PKA dependent manner
(A) Control MSC. (B) MSC treated with HGF (50 ng/ml) for 2h showing increased polymerized actin fibers. (C) MSC pre-treated with NECA (10 μM) 2h before the addition of HGF. NECA inhibited the HGF-induced increase in polymerized actin fibers, resulting in a rounded shape of the cell body. (D) MSCs pre-treated with PKA inhibitor (25 μM) for 1h, then pre-treated with NECA (100 μM) for 2h, then treated with HGF (50 ng/ml) for 2h showing increased polymerization of actin fibers. The effect of NECA was blocked by PKA inhibitor.
A

\[ [\text{Ca}^{2+}] \text{(uM)} \]

- Control
- NecA
- ST-HT31+Neca

HGF Injection

1 min
Figure 5. NECA inhibits HGF induced increase in intracellular Ca$^{2+}$

(A) In control MSCs, HGF (50 ng/ml) significantly increases intracellular Ca$^{2+}$ (full line). In MSC pre-treated with NECA 15 min before HGF infusion, the increment in intracellular Ca$^{2+}$ is inhibited (small dash line). Pretreatment with the PKA inhibitor ST-HT31 blocked the
inhibitory effect of NECA on HGF-induced upregulation of intracellular calcium (dot-dash line). (B) After the addition of Ionomycin, intracellular Ca$^{2+}$ rose immediately. (C) Rac 1 pull down assay on mouse MSC shows absence of Rac1 activity in the control MSC, and increased Rac1 activity 1 minute after the addition of ionomycin.
Figure 6. NECA up-regulates expression of hepatocyte-specific genes on mouse MSC
Mouse MSC were cultured in the presence of NECA (100 μM) and the expression of hepatocyte specific genes was quantified at day 4 and 8. (A and B) Foxa1 and Foxa2 were significantly elevated at day 4 and 8. (C): GSC was significantly elevated at day 8. (D–H) Sox17, AFP, albumin, EpCAM, and TAT were not elevated. MSC without NECA were the negative controls and normal mouse liver was the positive control (** P value < 0.01 compared to control MSC). **Abbreviation: RGE: Relative gene expression.**
Figure 7. NECA up-regulates expression of hepatocyte-specific genes on human MSC
Human MSC were cultured in the presence of NECA (100 μM) and the expression of hepatocytes specific genes was quantified at day 4 and 8. (A) GSC was significantly elevated at day 8. (B, C) Sox17 and albumin were elevated at day 8 but not day 4. (D) TAT was significantly elevated at day 4 and 8. (E) EpCAM was significantly elevated at day 8 (*: P values < 0.05 compared to control MSC; **: P value < 0.01 compared to control MSC).

Abbreviation: RGE: Relative gene expression.
Figure 8.
Schematic representation of the intracellular signaling pathways responsible for the adenosine-HGF interaction.