S-Adenosylmethionine and Methylthioadenosine Are Antiapoptotic in Cultured Rat Hepatocytes but Proapoptotic in Human Hepatoma Cells

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S-adenosylmethionine (AdoMet) is an essential compound in cellular transmethylation reactions and a precursor of polyamine and glutathione synthesis in the liver. In liver injury, the synthesis of AdoMet is impaired and its availability limited. AdoMet administration attenuates experimental liver damage, improves survival of alcoholic patients with cirrhosis, and prevents experimental hepatocarcinogenesis. Apoptosis contributes to different liver injuries, many of which are protected by AdoMet. The mechanism of AdoMet’s hepatoprotective and chemopreventive effects are largely unknown. The effect of AdoMet on okadaic acid (OA)-induced apoptosis was evaluated using primary cultures of rat hepatocytes and human hepatoma cell lines. AdoMet protected rat hepatocytes from OA-induced apoptosis dose dependently. It attenuated mitochondrial cytochrome c release, caspase 3 activation, and poly(ADP-ribose) polymerase cleavage. These effects were independent from AdoMet-dependent glutathione synthesis, and mimicked by 5’-methylthioadenosine (MTA), which is derived from AdoMet. Interestingly, AdoMet and MTA did not protect HuH7 cells from OA-induced apoptosis; conversely both compounds behaved as proapoptotic agents. AdoMet’s proapoptotic effect was dose dependent and observed also in HepG2 cells. In conclusion, AdoMet exerts opposing effects on apoptosis in normal versus transformed hepatocytes that could be mediated through its conversion to MTA. These effects may participate in the hepatoprotective and chemopreventive properties of this safe and well-tolerated drug. (HEPATOLOGY 2002;35:274-280.)
Materials and Methods

**Materials.** AdoMet, in the stable form of sulfate-p-toluensulfonate salt, and 5’-deoxy-5’-methyl-thioadenosine (MTA) were from Knoll Farmaceutici (Milan, Italy). D-L-Propargyglycine (PPG) was from Sigma (St. Louis, MO). OA, the caspase 3 inhibitor Ac-DEVD-CHO, and anti-actin antibody were from Calbiochem (La Jolla, CA). Cell culture reagents were from Gibco BRL (Grand Island, NY). Poly(ADP-ribose)polymerase (PARP) polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Cytochrome c monoclonal antibody was from Pharmingen (San Diego, CA). Other reagents were from Sigma.

**Isolation, Culture, and Treatment of Rat Hepatocytes and Human Liver Cancer Cells.** Hepatocytes were isolated from male Wistar rats (200-250 g) by collagenase perfusion and cultured as previously described. All animals received humane care in compliance with the National Research Council’s criteria for humane care as outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication no 86-23, revised 1985).

AdoMet or MTA (dissolved in dimethyl sulfoxide; final concentrations of dimethyl sulfoxide never exceeded 0.1%) was added to culture medium 30 minutes prior to the addition of 20 nmol/L OA. Experiments were performed in minimal essential medium containing 1% fetal bovine serum. PPG was added at a concentration of 2 mmol/L 30 minutes prior to the addition of AdoMet. Where indicated, the caspase 3 inhibitor Ac-DEVD-CHO (0.1 mmol/L) was added 3 hours before OA addition. HuH7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, glutamine, and antibiotics. They were treated with OA (125 nmol/L) in the presence or absence of AdoMet or MTA. In separate experiments, HuH7 and HepG2 cells were cultured using Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, antibiotics, and glutamine. Cells were plated at a density of 5 × 10^5 cells per 35-mm well, treated with varying concentrations of AdoMet (0 to 1 mmol/L) for 24 hours, and processed for detection of apoptotic cells using flow cytometry (see below).

**Determination of Oligonucleosomal (Histone-Associated) DNA Fragments.** The presence of soluble histone-DNA complexes was measured by the Cell Death Detection Assay (Boehringer Mannheim, Mannheim, Germany). Cell death enzyme-linked immunosorbent assays were performed according to the manufacturer’s instructions. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor, EF) was calculated as the ratio between the absorbance values of the samples obtained from treated and control cells.

**Flow Cytometry.** After treatment with AdoMet or vehicle, HuH7 and HepG2 cells (10^6) were trypsinized and collected by centrifugation at 1,000 g for 5 minutes. Cells were washed in phosphate-buffered saline, resuspended, and fixed in 70% ethanol for at least 2 hours. Next, cells were centrifuged at 200 g for 5 minutes, washed in phosphate-buffered saline, resuspended in 500 μL phosphate-buffered saline containing 100 μg RNase, and incubated for 30 minutes at 37°C on a shaker. Cellular DNA was then stained by the addition of 10 μg propidium iodide, and a total of 10,000 cells/condition were analyzed on a FACScan utilizing Cellquest software (Becton Dickinson, Franklin Lakes, NJ).

**Measurement of Intracellular GSH Levels.** Intracellular GSH levels (reduced form) were determined as previously described.

**Western Blot Analysis.** Western blot analysis was performed as previously described. PARP was analyzed in whole cell lysates and cytochrome c in cytosolic fractions obtained by differential centrifugation in 250 mmol/L sucrose buffer as described elsewhere.

**Measurement of Caspase 3 Activity.** Caspase 3 activity was measured in rat hepatocytes treated for 4 hours with OA (20 nmol/L) in the presence or absence of AdoMet or MTA, using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Palo Alto, CA). Cells (3 × 10^6) were scraped in culture medium, pelleted, and resuspended in lysis buffer and caspase 3 activity was measured following the manufacturer’s instructions.

**Statistical Analysis.** The data were analyzed using the Kruskal-Wallis test to determine differences between all independent groups. When significant differences were observed, data were compared using the Mann-Whitney U test.

Results

**AdoMet Prevents OA-Induced Apoptosis of Rat Hepatocytes in Primary Culture.** OA is a potent and specific inhibitor of protein phosphatases that induces apoptosis in several cell types, including rat hepatocytes. The antiapoptotic potential of AdoMet was evaluated in hepatocytes treated with 20 nmol/L OA for 8 hours. Apoptosis was assessed by the presence of oligonucleosomal fragments in the cytoplasm of the cells, which reflects the extent of DNA fragmentation and nuclear disruption characteristic of apoptosis. AdoMet dose dependently prevented the formation of oligonucleosomal fragments induced by OA (Fig. 1). The protective effect of AdoMet was also observed after 24 hours of treatment with OA (data not shown). These results were consistent with cell survival measured by MTT assay in hepatocytes (data not shown). The inactive structural analog norokadaone exerted no apoptotic effect indicating that, as reported, inhibition of phosphatase(s) activity by OA is responsible for the induction of cell death.

**The Antiapoptotic Action of AdoMet is GSH Independent and Mimicked by MTA.** One of the AdoMet’s mechanisms of hepatoprotection may be its ability to serve as a precursor for GSH synthesis. In this regard, AdoMet prevented tumor necrosis factor-α–induced necrotic cell death in hepatocytes from ethanol-fed rats through the restoration of depleted mitochondrial GSH levels. To evaluate whether AdoMet exerted its antiapoptotic effect on OA-induced apoptosis through GSH synthesis, we determined the intracellular levels of GSH in hepatocytes treated with OA. OA significantly increased total cellular GSH levels (from control 19.3 ± 6.2 to 44.3 ± 4.1 nmol/10^6 cells in OA-treated hepatocytes, P < .05), ruling out the possibility of GSH depletion as a mechanism for apoptosis. This increase could be attributed to induction by OA of γ-glutamylcysteine synthetase expression, the rate-limiting enzyme for GSH synthesis. The role of GSH was further analyzed in experiments using PPG, an inhibitor of
PARP is a well-characterized substrate for effector caspases, such as caspase 3. 26 Although caspase activation by several apoptotic stimuli did not result in proteolysis of PARP in hepatocytes, 27 OA was shown to induce it. 25 OA-induced PARP proteolysis was shown by the disappearance of the 116-kd intact PARP protein and the appearance of an 85-kd proteolytic fragment, both of which are recognized by the same antibody. PARP proteolysis was attenuated in cells pretreated with AdoMet and almost completely prevented by MTA (Fig. 3C).

**Effect of AdoMet and MTA on OA-Induced Apoptosis in HuH7 Cells.** AdoMet and MTA prevented the development of liver tumors in different experimental models of hepatocarcinogenesis in vivo, 8,10 and this was associated with the appearance of apoptotic bodies in hepatic nodules. 28 Because AdoMet and MTA are antiapoptotic in primary hepatocytes, we determined their effects in HuH7 cells. It is known that OA induces apoptosis in HuH7 cells, although at higher doses than that required for primary hepatocytes (up to 500 nmol/L). 20 Apoptosis in HuH7 cells

γ-cystathionase, 23 which is an effective way of blocking GSH synthesis from AdoMet. As shown in Fig. 2A, the protective action of AdoMet in OA-induced apoptosis was not affected by 2 mmol/L PPG. Under these conditions we observe effective inhibition in GSH synthesis in AdoMet-treated hepatocytes (13.25 ± 1.2 nmol/10^6 cells in controls, 2.58 ± 0.6 nmol/10^6 cells in PPG-treated cultures, 20.1 ± 2.9 nmol/10^6 in AdoMet-treated cells and 2.46 ± 0.7 nmol/10^6 cells in PPG plus AdoMet-treated cultures) and cystathionine accumulation (6.45 ± 0.77 and 10.22 ± 1.43 nmol/mg of protein in PPG-treated vs. PPG plus AdoMet-treated hepatocytes, respectively, P < .05). In the absence of PPG cystathionine was not detectable.

As mentioned above, some of the effects of AdoMet on liver damage and neoplastic transformation are mimicked by MTA. This compound is derived from AdoMet metabolism in the polyamine biosynthetic pathway, and can also arise from nonenzymatic hydrolysis of AdoMet under physiologic conditions. 24 Hence, we evaluated the effect of MTA on OA-induced apoptosis. Pretreatment of hepatocytes with MTA abolished the apoptotic effect of OA (Fig. 2B). The protective effect of MTA was dose dependent, but occurred at much lower concentrations than AdoMet.

**AdoMet and MTA Attenuate Cytochrome c Release, Caspase 3 Activation, and PARP Degradation Induced by OA.** Apoptosis induced by OA in primary rat hepatocytes is mediated by the release of cytochrome c from the mitochondria and the subsequent activation of caspase 3. 25 The effect of AdoMet and MTA on these two biochemical parameters was studied. As shown in Fig. 3A, both AdoMet and MTA effectively inhibited the release of cytochrome c induced by OA. Once released into the cytosol, cytochrome c can contribute to the activation of caspase 3. 33 Caspase 3 activity was induced about 3-fold in OA-treated cells as compared with untreated controls. When hepatocytes were pretreated with AdoMet or MTA, there was a dose-dependent inhibition of caspase 3 activity (Fig. 3B).
was measured after 24 hours of treatment with 125 nmol/L OA. In some cultures, 4 mmol/L AdoMet or 500 μmol/L MTA was added prior to OA. As shown in Fig. 4A, neither AdoMet nor MTA prevented the accumulation of cytoplasmic oligonucleosomal fragments induced by OA. Moreover, treatment with AdoMet or MTA alone resulted in a proapoptotic effect similar to that observed with OA (Fig. 4A). Neither AdoMet nor MTA potentiated the effect of OA (Fig. 4A). The effect of AdoMet and MTA on cytochrome c release to the cytosol in HuH7 cells was also studied. As shown in Fig. 4B, treatment of HuH7 cells with 4 mmol/L AdoMet or 500 μmol/L MTA for 8 hours resulted in the release of cytochrome c into the cytosolic fraction. As expected, this effect was also observed in response to OA treatment (125 nmol/L, 8 hours) (Fig. 4B), and was not potentiated when cells were simultaneously treated with either AdoMet or MTA (Fig. 4B). We next determined whether AdoMet’s proapoptotic effect was unique to HuH7 cells. In these experiments, HuH7 and HepG2 cells were treated with varying concentrations of AdoMet for 24 hours and the percent of apoptotic cells was determined by flow cytometry. As shown in Fig. 4C, AdoMet exerted a dose-dependent increase in the percent of apoptotic cells in both cell lines.

**Discussion**

There is accumulating evidence on the protective potential of AdoMet in the preservation of liver function, both in a variety of experimental models of liver damage and in human alcohol-induced liver disease. As mentioned above, cell death by apoptosis is becoming a relevant process in the development of liver injury under different pathologic conditions that are palliated by AdoMet treatment. Hence, it was interesting to directly address the effect of AdoMet on apoptosis. For this purpose we have used an experimental model of rat hepatocytes in primary culture in which apoptosis was induced by OA. OA has been previously shown to induce apoptosis in primary cultured rodent hepatocytes and hepatic-derived cell lines. As opposed to other proapoptotic stimuli, such as tumor necrosis factor-α and Fas ligand, OA-mediated apoptosis of cultured hepatic cells does not need a cotreatment with inhibitors of messenger RNA or protein synthesis. In this experimental setting we have observed that AdoMet partially prevented apoptotic cell death induced by OA in a dose-dependent fashion. AdoMet effect was accompanied by the inhibition of cytochrome c release from the mitochondria to the cytosol. This is a central event in the apoptotic pathway, and a common response to different apoptosis-inducing agents. Cytochrome c release leads to the activation of downstream effector caspases, such as caspase 3, which cleaves a number of cellular proteins facilitating DNA fragmentation and cell death. Consistent with this, AdoMet treatment partially protected against OA-induced caspase 3 activation and PARP cleavage. PARP cleavage was not observed in hepatocytes undergoing death-receptor–mediated apoptosis in response to agonists such as transforming growth factor β or Fas ligand. However, we observed that hepatocyte...
apoptosis induced by OA is accompanied by PARP proteolysis, which is consistent with other reports.25 Similar to AdoMet, prevention of these events by ursodeoxycholic acid was associated with protection from apoptosis.15,25

The biochemical mechanisms underlying the hepatoprotective effects of AdoMet are not completely known. Facilitation of methylation reactions and the restoration of depleted hepatocellular GSH levels, among other effects, have been proposed4-6,34 (reviewed in Mato et al.2). AdoMet addition to isolated hepatocytes restores intracellular AdoMet concentrations and increases GSH levels.11,35 We examined whether AdoMet protection from OA-induced apoptosis could depend on AdoMet-dependent GSH synthesis. For this purpose hepatocytes were treated with AdoMet in the presence or absence of PPG, an inhibitor of γ-cystathionase, the enzyme that converts AdoMet-derived cystathionine into the GSH precursor cysteine.1,23 Under these conditions AdoMet retained its antiapoptotic properties when cells were challenged with OA, thus ruling out the possible implication of GSH in this effect of AdoMet.

As previously mentioned, MTA is a product of AdoMet metabolism in the polyamine pathway.1 Exogenous AdoMet can also undergo nonenzymatic hydrolysis in vivo into MTA and homoserine.9,24 It has been suggested that the beneficial effects of AdoMet in liver damage could be attributed in part to its conversion to MTA.9,10 Additionally, we have recently shown that MTA mimics AdoMet’s effect on gene expression in cultured rat hepatocytes.12 Now we observe that MTA also prevents OA-induced apoptosis in cultured rat hepatocytes in a dose-dependent fashion. Inhibition of apoptosis by MTA was accompanied by the prevention of cytochrome c release from the mitochondria, caspase 3 activation, and PARP cleavage. In contrast to AdoMet, MTA does not contribute to GSH synthesis, is not a methyl donor, and inhibits methyltransferases.36 These observations further support the idea that the present antiapoptotic effects of AdoMet are GSH independent and suggest that they could be mediated in part through its conversion to MTA.

A possible mechanism for the antiapoptotic effect of AdoMet could take place at the mitochondrial level. AdoMet is transported into isolated rat liver mitochondria via a specific carrier-mediated system, thus making this organelle an intracellular target for AdoMet.57 Apoptotic signals, including OA, alter mitochondrial physiology leading to organelle swelling and the physical rupture of the outer membrane.14,25,30 These alterations contribute to the release of mitochondrial proteins, such as cytochrome c and other death-promoting proteins,30 into the cytosol. AdoMet has been shown to restore the physical properties of mitochondria in etha-
nol-fed rats,\textsuperscript{34} attenuate the increase in plasma of glutamate dehydrogenase, an enzyme exclusively located in the intermembrane space of mitochondria, and decrease the number of giant mitochondria in chronically ethanol intoxicated baboons.\textsuperscript{4} Together with our present observations, these findings suggest that AdoMet can modulate mitochondrial injury. The inhibition of OA-induced cytochrome c release by MTA also suggests that this compound could mediate part of the above-mentioned effects of AdoMet at the mitochondrial level. Interestingly, higher concentrations of AdoMet were necessary to match the effects of MTA in the different apoptosis-related events measured. This could be attributed in part to the differential intracellular availability of both compounds, AdoMet being a charged molecule and MTA a non-charged compound. Additionally, and as suggested by our present experiments, AdoMet effects may depend on the conversion of this molecule, either spontaneous or via enzymatic catalysis, to MTA, which would be bypassed by the direct addition of this compound.

While prevention of hepatocyte apoptosis under conditions of liver injury contributes to the preservation of functional liver mass and organ viability, such an effect would be undesired during the clonal expansion of malignantly initiated hepatocytes. In this respect, an imbalance between cell proliferation and apoptotic cell death seems to be important in hepatocarcinogenesis, especially during the stage of progression.\textsuperscript{39} These notions led us to test the effect of AdoMet and MTA on apoptosis induced by OA in the human hepatoma cell line HuH7. In contrast to the results obtained in primary hepatocytes, AdoMet and MTA did not protect from OA-induced apoptosis. On the contrary, both agents were able to promote the release of cytochrome c from the mitochondria into the cytoplasm and to induce apoptosis in the absence of any other stimuli, mimicking the effect of OA. AdoMet’s proapoptotic effect was also not unique to HuH7 cells, because it induced apoptosis in both HepG2 and HuH7 cells in a dose-dependent manner. Additionally, AdoMet and MTA also induced a similar proapoptotic response in the rat hepatoma cell line H4-IIE (data not shown), thus excluding the possibility that species differences are in line with our previously reported growth inhibitory properties of increased intracellular AdoMet levels on HuH7 cells.\textsuperscript{41} One of the mechanisms of the growth inhibitory effect appears to be apoptosis. How AdoMet/MTA can be antiapoptotic in primary hepatocytes but proapoptotic in liver cancer cells is unknown and is an area of our future investigation. However, this differential effect seems not to be related to the fact that primary hepatocytes are quiescent cells, whereas cell lines display enhanced proliferation, since AdoMet and MTA showed a similar antiapoptotic effect on proliferating hepatocytes treated with hepatocyte growth factor (data not shown).

Taken together, this work outlines a candidate novel role for AdoMet in the preservation of liver cell viability that could be behind the widely reported hepatoprotective actions of this molecule. Its differential effects on normal and transformed hepatocytes further strengthens the therapeutic potential of a safe and well-tolerated drug in liver disease,\textsuperscript{6} and as a chemopreventive agent in human hepatocarcinoma.

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\section*{References}