Inhibition of Promotion and Persistent Nodule Growth by S-Adenosyl-L-methionine in Rat Liver Carcinogenesis: Role of Remodeling and Apoptosis

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ABSTRACT

The resistant hepatocyte model (initiation/selection) and the triphasic model (initiation/selection followed by phenobarbital, for a maximum of 16 weeks) were compared for their ability to generate enzyme-altered foci (EAF) and nodules in the liver of Wistar rats initiated by diethylnitrosamine. The effects of S-adenosyl-L-methionine (SAM) on the development of preneoplastic tissue was tested in these experimental models. In the absence of phenobarbital (PB), EAF and early nodules (EN) went through a phase of rapid growth, between 4 and 9 weeks after initiation, to a phase in which progressive decrease in number and size occurred. By the 26th week only a few remodelling EAF and nodules were found. In PB-treated rats a rapid increase in the percentage of liver occupied by EAF and EN, up to the 9th week after initiation, was followed by a period of slow growth (from the 9th to the 20th week) and then, after PB withdrawal (20th week), by a drop in the number and size of EAF and EN. However, at the 26th week actively growing nodules with a low tendency to spontaneous remodelling (persistent nodules) developed. EAF and EN showed a high DNA synthesis 5 weeks after initiation. Thereafter, progressive decline in DNA synthesis, coupled with remodeling and decrease in number of biochemical markers, was seen both in the absence and, even though to a lesser extent, in the presence of PB, indicating that preneoplastic lesions became increasingly insensitive to PB. Relatively few apoptotic bodies could be observed in EAF and EN during PB treatment. After PB withdrawal, decrease in growth potential was coupled with increase in apoptotic bodies. In contrast, in persistent nodules relatively high apoptosis occurred which partially counterbalanced high DNA synthesis. Administration of SAM for a maximum of 16 weeks, starting at the 4th week after initiation, caused a great decrease in number and size of EAF and EN, associated with inhibition of DNA synthesis, high cell death by apoptosis, high remodeling, and loss of biochemical markers, in preneoplastic lesions of both PB-treated and untreated rats. A 1-8-week SAM treatment, started after the development of persistent nodules, caused a great regression of nodular lesions, coupled with a sharp fall in DNA synthesis and increase in apoptosis. It is suggested that inhibition by SAM of the development of preneoplastic tissue is linked to a shift of the equilibrium between cell production and cell death in favor of cell death. This phenomenon and differentiation of putative preneoplastic cells to normal appearing hepatocytes could accelerate disappearance of preneoplastic lesions and makes phenotype of persistent nodules highly unstable.

INTRODUCTION

Cell proliferation plays an essential role in the initiation (1-3), promotion (4-7), and progression (8) steps of chemical carcinogenesis. The size of proliferating tissues largely depends on the equilibrium between cell growth and cell loss. Extensive remodeling (8-10) but no or very limited cell death (11) has been found in early liver lesions of rats subjected to initiation/selection treatments (RH3 model) (12). Preneoplastic lesions induced in experimental models based on prolonged administration of promoters to initiated rats exhibit very low cell death by apoptosis (13) and remodeling (14). In contrast, a relatively high necrogenic index which, however, only partially counterbalanced the high proliferative rate, has been found in PN, the development of which precedes that of hepatocarcinomas (11).

Previous work from our laboratory (15-18) has shown a great fall in hepatic SAM levels during DENA-induced liver carcinogenesis of rats subjected to the initiation/selection treatments (12) followed or not by a 16-week treatment with PB. The reconstitution of the SAM liver pool, by injection of exogenous SAM, is associated with a fall in the amount of EAF and early reversible nodules and the prevention of the development of late, PN, and hepatocellular carcinomas (16, 17). This effect is largely linked to inhibition of DNA synthesis in preneoplastic lesions (15-18). However, no data are presently available on the phenotypic changes of putative preneoplastic hepatocytes during the promotion and progression steps of liver carcinogenesis as a consequence of SAM treatment. The present study is concerned with remodeling, instability of the biochemical phenotype, and apoptosis, in early lesions (EAF and EN) as well as in late PN, in the liver of SAM-treated rats. A preliminary account of some of the results in this paper has already been given (19).

MATERIALS AND METHODS

Animals and Diets. Male Wistar rats (bred in our laboratory) and male Fischer 344 rats (purchased from Charles River; The International Standards, Calco, Como, Italy) were used. The rats (160-180 g at the beginning of the experiment) were housed, three per cage, in suspended wire-bottomed cages, in a constant temperature (22°C) and humidity (55%) environment, with a 6 a.m. to 6 p.m. photoperiod. They were placed on a standard 26% casein diet (Piccioni, Brescia, Italy) with water ad libitum. Rats received a single 150-mg/kg i.p. dose of DENA (initiation) and, 2 weeks later, were fed for 2 weeks a standard diet containing 0.03% (Wistar rats) or 0.02% 2-AAF (F344 rats), and a partial hepatectomy was performed at the midpoint of this time period (selection). Preliminary experiments showed that 150 mg/kg of DENA are necrogenic for the liver of Wistar rats. In addition, DNA synthesis of EAF was poorly affected by a 7-day feeding of 0.03% 2-AAF, which instead largely inhibited DNA synthesis in surrounding hepatocytes. At the end of 2-AAF feeding, Wistar rats were divided into 6 groups (groups 1-6 of Fig. 1), and F344 rats were divided into 2 groups (groups 7 and 8). Groups 1, 2, 7, and 8 were given a basal diet, while groups 3-6 were fed a basal diet containing 0.05% PB (triphasic model) (20), for a maximum of 16 weeks, before being placed on basal diet. All rat groups were killed as indicated in Fig. 1. Rat groups 1, 3, 5, and 7 were controls; all other groups were treated with SAM. SAM treatments were started at the end of 2-AAF feeding for Wistar rats groups 2 and 4 and was continued for a maximum of 16 weeks. They were started at...

Received 8/15/88; revised 12/7/88; accepted 12/14/88.

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This research was supported by grants from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Oncologia (Grant 87.01281.44), Associazione Italiana Ricerca sul Cancro, and Ministero Pubblica Istruzione (Program 60%).

To whom requests for reprints should be addressed.

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the 26th and 12th weeks for rat groups 6 and 8, respectively, and were continued for a maximum of 11 weeks. SAM, in the relatively stable form (21) of sulfate p-toluene sulfonate (BioResearch, Liscate, Milan, Italy), was injected i.m. as freshly prepared solutions containing 0.1 m SAM, 0.045 m NaOH, and 0.4 m lysine (final pH 6.9). Controls received the same amounts of a solution containing 0.045 m NaOH and 0.4 m lysine, brought to pH 6.9 with an equimolar mixture of sulfuric and p-toluene sulfonic acid. Thin layer chromatography analysis (15) revealed that SAM preparations were free of methionine. High performance liquid chromatography analyses (see below) showed that SAH, 5'-MTA, and other unidentified contaminants did not exceed 1.7%. All rat groups received 6 daily SAM doses of 64 µmol/kg. No differences in food intake were observed among the various groups of animals (range for all rat groups, 10.2–12.8 g/100 g/day).

When used for analytical determinations, PN were separated from surrounding liver by isolating liver cells with a collagenase method (22) and collecting nodules on a nylon filter (23). By persistent nodules (hyperplastic nodules, neoplastic nodules) we mean focal proliferating, (range for all rat groups, 10.2–12.8 g/100 g/day).

To determine LI the rats were given [3H]dThd (90 Ci/mmol; Amersham International pic., Amersham, United Kingdom) i.p., at the 26th and 12th weeks for rat groups 6 and 8, respectively, and were continued for a maximum of 11 weeks. SAM, in the relatively stable form (21) of sulfate p-toluene sulfonate (BioResearch, Liscate, Milan, Italy), was injected i.m. as freshly prepared solutions containing 0.1 m SAM, 0.045 m NaOH, and 0.4 m lysine (final pH 6.9). Controls received the same amounts of a solution containing 0.045 m NaOH and 0.4 m lysine, brought to pH 6.9 with an equimolar mixture of sulfuric and p-toluene sulfonic acid. Thin layer chromatography analysis (15) revealed that SAM preparations were free of methionine. High performance liquid chromatography analyses (see below) showed that SAH, 5'-MTA, and other unidentified contaminants did not exceed 1.7%. All rat groups received 6 daily SAM doses of 64 µmol/kg. No differences in food intake were observed among the various groups of animals (range for all rat groups, 10.2–12.8 g/100 g/day).

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Histology and Histochemistry. Small pieces of liver taken from each lobe were frozen in isopentane at −147°C, embedded in Paraplast, and then cut serially in a cryostat into 5-µm-thick sections. The sections were fixed in cold acetone and then used for histochemistry. The first section was assayed for GGT, the second for G6PD, and the third for G6Pase as described (26). Alternatively, acetone-fixed material was processed for embedding in paraffin, serially sectioned, and used for H & E staining and for GGT histochemistry. Morphometric analysis was carried out by scanning 9–10 liver sections per rat with a Leitz Diaplan microscope connected, by a telecamera, with a MOP Videoplan computerized image analyzer (Kontron Electronic, Eching, Federal Republic of Germany). Remodeling EAF and nodules were identified as areas lacking uniformity for GGT histochemical reaction and exhibiting irregular boundaries with surrounding liver and relatively low LI (8–10). To determine LI the rats were given [3H]dThd (90 Ci/mmol; Amersham International plc., Amersham, United Kingdom) i.p., at the dose of 0.5 µCi/g body weight every 6 h for 48 h before killing. The slices were fixed and processed for histochemistry and then coated with Kodak NTB2 emulsion and stored for 5 weeks in the dark at 4°C. After development the hepatocytes were counterstained with hematoxylin.

In order to evaluate phenotypic complexity of EAF and EN, the transections were examined on the monitor of the image analyzer. A sheet of veilum was placed onto the monitor surface, and the transection outlines and the EAF and EN, revealed by the histochemical test represented by each slice, were traced onto the veilum sheet, using a different color for each tracing. The superimposed images of the serial sections stained for individual markers were then digitized by an image digitizer connected with the image analyzer, equipped with a computer programmed to yield the foci number and surface area for a given phenotype or for individual foci independently of the phenotypes represented (see below). The biochemical characteristics of each focus or nodule may not quantitatively involve all the cells within the focus (27). In Fig. 4, the data under “any” represent data obtained by the markers listed regardless of whether or not other markers also scored in the same transection. The other columns of data refer to the presence of 1, 2, or 3 markers. However, when a biochemical alteration occurred within the cell population of larger foci or nodules the surface area of each cell population was considered as a fraction of the total population, carrying the same biochemical alteration, in the total slide. In contrast, in Fig. 5 the percentage of liver occupied by cells carrying the various biochemical alterations has been calculated independently of the surface area covered, in each lesion, by each biochemical marker.

Sections stained with H & E were used for counting ABs. Clear cell, eosinophilic, and mixed cell foci were identified according to published criteria (28); their correspondence with GGT-positive foci was assessed in the serial sections.

Enzyme Assay. ODC activity was determined in 30,000 × g supernatants of liver and PN homogenates by measuring the release of 14CO2 from [1-14C]ornithine as published previously (15).

Analytical Methods. High performance liquid chromatography determination of SAM, SAH, and 5'-MTA was performed as published previously (17) on HC1 extracts of liver or PN. Their quantities were determined by comparing the area of the peak in the tissue extract with that of standard solutions. Proteins were determined as published previously (29).

RESULTS

Development of Putative Preneoplastic Lesions. EAF (0.1–0.2 mm in diameter) were first observed, in Wistar rats subjected to the initiation/selection treatments, as early as 3.5 weeks after initiation (Fig. 2). Later the foci increased in number and surface area up to the 5th week and thereafter in surface area alone (not shown). At the 5th week the first nodules appeared. The percentage of liver parenchyma occupied by GGT-positive foci and EN progressively increased, reaching its maximum (22%) at the 9th week, after which EAF and EN exhibited a progressive decrease in number and surface area. By the 26th week, the percentage of liver occupied by small GGT-positive lesions (mostly EAF) decreased to about 9%. PB administration, at the end of 2-AAF feeding, greatly enhanced the development of EAF and EN, which increased in size, not in number, so that about 35% of liver was occupied by these lesions at 9 weeks after initiation. Thereafter, liver occupied by EAF and EN did not increase significantly, and a sharp decrease occurred upon withdrawal of PB. At the 26th week EAF occupied only...
13% of liver, while numerous PN were seen (see below). SAM administration caused a great decrease in the percentage of GGT-positive foci, in either the presence or the absence of PB. This decrease was seen in both the number and surface area of GGT-positive lesions.

Remodeling. The data in Fig. 3 show that in the absence of PB, remodeling, which is very low at the 5th week after starting the experiment, progressively increased thereafter. At the 20th and 26th weeks 40–50% of EAF and EN showed a nonuniform pattern and irregular outlines. PB partially inhibited remodeling of GGT-positive foci. Data relative to the 26th week, in PB-treated rats, concern only microscopic lesions, not PN, which underwent no or very low remodeling (see below). SAM highly stimulated remodeling both in PB-treated and untreated rats. Remodeling inhibition by PB and its activation by SAM were also observed in foci or EN expressing high G6PD or low or no G6Pase (data not shown).

The data in Fig. 3 also show that 5 weeks after initiation, relatively high DNA synthesis characterized uniform and nonuniform GGT-positive lesions in both PB-treated and untreated rats. However, LI was significantly lower in nonuniform than in uniform lesions. As expected, PB stimulated DNA synthesis of both uniform and nonuniform lesions. A large drop in LI occurred in uniform and nonuniform lesions 2 weeks later (7th week), followed by further relatively low decreases in the following weeks. A slower decrease occurred in PB-treated rats. However, LI remained slightly but significantly lower in nonuniform than in uniform lesions throughout the duration of the experiment, both in the absence and in the presence of PB. At the 26th week, GGT-positive cells of both PB-treated and untreated rats underwent no or very low remodeling (see below). SAM highly stimulated remodeling both in PB-treated and untreated rats. Remodeling inhibition by PB and its activation by SAM were also observed in foci or EN expressing high G6PD or low or no G6Pase (data not shown).

Levels of Phenotypic Complexity. In some experimental models, putative preneoplastic lesions exhibiting a relatively slow growth rate are generally associated with low levels of phenotypic complexity (number and identities of biochemical markers per focus) (27, 30). In Fig. 4 the percentages of putative preneoplastic tissue exhibiting individual biochemical markers or combinations of different markers have been considered as fractions of total liver cell population carrying the same biochemical alteration (see "Materials and Methods"). Between the 5th and the 26th week, the preneoplastic lesions exhibiting a high GGT, either as the only marker or associated with other markers (Columns 1 under "any"), clearly occupied larger extents of liver parenchyma, in both PB-treated PB-treated and untreated rats, than the lesions expressing the other biochemical markers (Columns 2 and 3 under "any"). After 9 weeks putative preneoplastic cells exhibiting biochemical alterations of GGT/G6PD and GGT/G6PD/G6Pase increased, with respect to the 5th week, more than those showing only one biochemical marker. This behavior was particularly evident in PB-treated rats where the cells exhibiting altered GGT/G6PD or GGT/G6PD/G6Pase occupied the largest percentage of liver parenchyma. In animals not treated with PB, about 8% of liver was occupied by EAF and EN exhibiting only one marker and about 15% by lesions exhibiting 2–3 markers. In PB-treated rats these figures were 12 and 24%, respectively. A similar behavior was observed at the 20th week (data not included in Fig. 4). However, at this time the part of the liver occupied by lesions with one or more than one marker was 10 and 12%, without PB, and 18% and 21% in its presence. At the 26th week, the tendency to a preferential development of liver cells with higher biochemical complexity levels was less evident, particularly in the rats that did not receive PB. A great decrease in putative preneoplastic cells with more than one biochemical marker occurred in SAM-treated rats, particularly at the 9th week (both with or without PB), a period of time which coincides with maximal biochemical heterogeneity of preneoplastic lesions. The same period of time was taken into consideration to evaluate if phenotypic heterogeneity could be influenced by remodeling (Fig. 5). Complexity level was lower in nonuniform than in uniform lesions, especially in the absence of PB. The disappearance of biochemical markers in nonuniform foci followed the sequence G6Pase, G6PD, GGT, so that the majority of nonuniform lesions exhibiting only one marker was GGT positive. SAM caused a large decrease in the percentage of both uniform and nonuniform lesions with three markers and an increase in those exhibiting only one marker. No significant changes or very little change occurred in the lesions showing two biochemical markers. This could reflect an equilibrium between the flux:complexity level 2 to complexity level 1,
that: complexity level 3 to complexity level 2, in SAM-treated rats.

Effect of SAM on Nodule Growth. PN appeared 22–26 weeks after initiation in Wistar rats treated up to the 20th week with PB. Very few remodeling nodules were found in those rats not treated with PB (data not shown) while numerous PN developed in F344 rats 8–12 weeks after initiation in the absence of PB. All these nodules exhibited relatively high DNA synthesis (Table 1) even in the absence (F344 rats) or after interruption (Wistar rats) of PB treatment (persistent nodules). They expressed a high GGT activity; 86% of them also exhibited a high G6PD activity, while the reaction for G6Pase was low or absent (not shown). Most of these nodules showed no evidence of remodeling for at least 8–11 weeks (Fig. 6A). When Wistar rats were subjected to SAM treatment for 1–8 weeks, starting the treatment 26 weeks after initiation, PN remodeled to various extents as could be seen from the lack of uniformity of GGT histochemistry and the irregular outlines (Fig. 6, B–D). Remodeling appeared as early as 1 week after starting SAM injection. After 8 weeks of SAM treatment, PN started showing a decrease in volume. Most of the nodular cells lost GGT activity (Fig. 6, C and D) as well as other markers (not shown), and a few PN were still recognizable microscopically by H & E staining. Similar behavior was observed in F344 rats subjected to SAM for 11 weeks (not shown).

Nodule regression was also documented by a decrease in PN number and surface area in SAM-treated rats, coupled with a large fall in DNA synthesis (Table 1). These effects were correlated in Wistar rats with the duration of SAM treatment: a great decrease occurred after SAM injection for 8 weeks, while no effects were recorded after a 1-week treatment. In F344 rats a dramatic decrease in all the parameters tested occurred after an 11-week SAM treatment. At this time PN were present only in two of five rats.

The development of EAF is coupled with a fall in liver SAM content, high ODC activity, and polyamine synthesis (15–18). SAM inhibits this synthesis and causes a reconstitution of the SAM liver pool and accumulation of 5' -MTA, an inhibitor of polyamine synthesis and growth (17, 31–33). In order to assess if similar variations occur when SAM treatment is started after development of PN, the above parameters were determined in PN various times after starting this treatment. PN of both Wistar and F344 rats showed low SAM and 5'-MTA contents, low SAM/SAH ratio, and high ODC activity (Table 2). In Wistar rats a 1-week treatment with SAM caused a 42–44% increase in SAM and 5'-MTA contents, coupled with a 13% fall in ODC activity with respect to normal liver. SAM content increased 95% in PN after an 8-week treatment with SAM. This treatment also caused a 124% rise in 5'-MTA content and a 42% fall in ODC activity. No variations in SAH content were observed during SAM treatments; thus SAM/SAH ratio varied concurrently with SAM. The effect of SAM on the above parameters could not be assessed in F344 rats due to the small amount of nodular tissue present after 11 weeks under SAM treatment (cf. Table 1). Effect of SAM on Apoptosis. The data in Table 3 indicate that relatively few ABs were present in EAF and EN, 7 weeks after initiation, in Wistar rats subjected to the initiation/selection treatments without PB. ABs further decreased in PB-treated rats. However, they increased by about 10-fold 72 h after arresting PB treatment. SAM caused a 1.5–2-fold increase in ABs in all conditions tested. Very low percentages of ABs were observed in surrounding liver (0.03–0.09%) which were not modified by SAM (not included in Table 3).

Table 4 shows that a relatively high percentage of ABs was present in PN. This was particularly evident in the liver of F344 rats which exhibit a percentage of ABs 1.6–2-fold higher than that of Wistar rats. A 1-week SAM injection caused a 1.7-fold rise in ABs in Wistar rats; however, longer treatments had an opposite effect. SAM did not modify the relative percentages of intracellular and extracellular ABs in foci and nodules (not shown). This could indicate that SAM-induced modifications of the apoptotic process do not depend on a SAM effect on the endocytic phase of this process (34).

Table 1 Effect of SAM on the development of persistent nodules

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Rat group</th>
<th>Treatment</th>
<th>Time (wk)</th>
<th>Body wt (g)</th>
<th>RLW</th>
<th>No./liver</th>
<th>Surface area (mm²)</th>
<th>LI²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>5</td>
<td>None</td>
<td>27</td>
<td>387 ± 7</td>
<td>5.98 ± 0.11</td>
<td>31 ± 7</td>
<td>5.49 ± 2.0</td>
<td>5.57 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>SAM (1)</td>
<td>27</td>
<td>386 ± 11</td>
<td>6.02 ± 0.8</td>
<td>31 ± 6</td>
<td>6.32 ± 1.4</td>
<td>6.81 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>SAM (8)</td>
<td>34</td>
<td>362 ± 16</td>
<td>6.34 ± 0.06</td>
<td>32 ± 8</td>
<td>5.91 ± 2.7</td>
<td>5.18 ± 0.44</td>
</tr>
<tr>
<td>F344</td>
<td>7</td>
<td>None</td>
<td>23</td>
<td>294 ± 8</td>
<td>5.62 ± 0.12</td>
<td>31 ± 3</td>
<td>4.43 ± 2.2</td>
<td>7.64 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>SAM (11)</td>
<td>23</td>
<td>279 ± 24</td>
<td>5.10 ± 0.05</td>
<td>4²</td>
<td>1.57²</td>
<td>0.30²</td>
</tr>
</tbody>
</table>

* Period of time after initiation. SAM treatments were started 1–11 weeks (as indicated in parentheses) before killing.

± Data are means ± SD of 5 rats for each time for groups 5, 6, and 8 and of 4 rats for group 8.

Relative liver weight in g/100 g body weight.

² t test: different from the appropriate control for P < 0.001.

Visible nodules were present only in 2 rats.
DISCUSSION

Cell Growth and Remodeling in the Triphasic Model. F344 rats, treated according to the RH model of experimental carcinogenesis, undergo rapid development of a high number of EAF and nodules (12). The same treatment was less efficacious in Wistar rats, in which putative preneoplastic tissue developed rapidly between the 5th and the 9th week after initiation, but it never occupied more than 22% of liver surface. EAF and EN showed relatively high LI and relatively low remodeling. However, LI progressively decreased and remodeling increased during the period of time considered, at the end of which EAF and EN exhibited a high degree of biochemical complexity. The slow development of EAF and EN after the 9th week was associated with a further rise in remodeling and a drop in phenotypic complexity and LI. PB, given to rats after the end of the selection period, significantly increased size and biochemical complexity of early lesions in PB-treated rats (15, 20). It cannot be excluded, however, that rise in remodeling and the irregular nodule outlines. C and D, nodules 8 weeks after starting SAM treatment. Note the absence of GGT activity in the center of the nodule showing a uniform pattern of GOT histochemistry; B, nodule 1 week after starting SAM treatment. Note that the large nodule in C is not easily recognizable by GGT histochemistry; GGT activity is present only in limited areas scattered throughout the nodule. In D, a small nodule, recognized in a serial section by H & E, is almost completely GGT negative. × 16.

Table 2 Effect of SAM on SAM, SAH, and 5′-MTA content and ODC activity of persistent nodules

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Rat group</th>
<th>Timea (wk)</th>
<th>SAMb (μg/g)</th>
<th>SAHb (μg/g)</th>
<th>SAM/SAH</th>
<th>5′-MTAc (μg/g)</th>
<th>ODC activityd (pmol CO₂/h, mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>Control</td>
<td>21.05 ± 1.10</td>
<td>10.12 ± 0.57</td>
<td>2.08 ± 0.08</td>
<td>1.02 ± 0.30</td>
<td>34.25 ± 2.12</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>9.11 ± 0.36</td>
<td>10.00 ± 0.43</td>
<td>0.93 ± 0.02</td>
<td>0.43 ± 0.02</td>
<td>103.44 ± 8.97</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>9.66 ± 0.29</td>
<td>10.16 ± 0.07</td>
<td>0.95 ± 0.03</td>
<td>0.45 ± 0.04</td>
<td>101.05 ± 11.48</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SAM (1)</td>
<td>13.19 ± 1.14</td>
<td>9.60 ± 0.14</td>
<td>1.06 ± 0.02</td>
<td>0.64 ± 0.03</td>
<td>87.56 ± 8.61</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SAM (8)</td>
<td>18.81 ± 1.29</td>
<td>9.57 ± 0.28</td>
<td>1.44 ± 0.09</td>
<td>1.01 ± 0.10</td>
<td>58.16 ± 8.36</td>
<td></td>
</tr>
<tr>
<td>F344</td>
<td>Control</td>
<td>23.44 ± 1.18</td>
<td>10.01 ± 0.33</td>
<td>2.32 ± 0.08</td>
<td>0.98 ± 0.27</td>
<td>33.23 ± 3.55</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>9.57 ± 0.37</td>
<td>10.31 ± 0.11</td>
<td>0.93 ± 0.03</td>
<td>0.41 ± 0.03</td>
<td>122.40 ± 21.15</td>
<td></td>
</tr>
</tbody>
</table>

a Period of time after initiation. SAM treatments were started 1–11 weeks (as indicated in parentheses) before killing.

b Data are means ± SD of 5 rats for control (normal rats) and for groups 5 and 6 and of 4 rats for group 7.

c t test: groups 5 and 7 versus respective control and group 6 versus group 5, different for P < 0.001.

d t test: groups 5 and 7 versus respective control and group 6 versus group 5, different for P < 0.001.

PB, given to rats after the end of the selection period, significantly increased size and biochemical complexity of early lesions in PB-treated rats (15, 20). It cannot be excluded, however, that rise in remodeling and the irregular nodule outlines. C and D, nodules 8 weeks after starting SAM treatment. Note the absence of GGT activity in the center of the nodule showing a uniform pattern of GOT histochemistry; B, nodule 1 week after starting SAM treatment. Note that the large nodule in C is not easily recognizable by GGT histochemistry; GGT activity is present only in limited areas scattered throughout the nodule. In D, a small nodule, recognized in a serial section by H & E, is almost completely GGT negative. × 16.
INHIBITION OF PROMOTION AND NODULE GROWTH BY SAM

Table 3 Effect of SAM on the incidence of apoptotic bodies in EAF and EN

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Treatment</th>
<th>Time (wk)</th>
<th>ABS/100 hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>None</td>
<td>0</td>
<td>0.61 ± 0.24</td>
</tr>
<tr>
<td>6</td>
<td>SAM</td>
<td>0</td>
<td>1.47 ± 0.15*</td>
</tr>
<tr>
<td>3</td>
<td>PB</td>
<td>0</td>
<td>0.41 ± 0.16</td>
</tr>
<tr>
<td>4</td>
<td>PB + SAM</td>
<td>0</td>
<td>0.88 ± 0.18*</td>
</tr>
<tr>
<td>3</td>
<td>PB</td>
<td>0</td>
<td>3.51 ± 0.26*</td>
</tr>
<tr>
<td>4</td>
<td>PB + SAM</td>
<td>72</td>
<td>5.42 ± 0.24</td>
</tr>
</tbody>
</table>

* Rats were killed 7 weeks after initiation or, when indicated, 72 h after the arrest of PB treatment. This treatment started 4 weeks after initiation and was continued up to the end of the 7th week. SAM treatment (6 daily doses of 64 μmol/kg) was started 4 weeks after initiation and was continued up to sacrifice.

** Period of time after the arrest of PB treatment.

† Clear cells, eosinophilic foci, and EN were identified and nuclear changes representing apoptosis and ABs were determined by scoring 5000 focal cells/liver. Data are means ± SD of 5 rats for groups 1 and 2 and of 10 rats for groups 3 and 4. ABS in surrounding liver: 0.03–0.08 for all rat groups.

‡ t test: different from the appropriate control for P < 0.002.

Table 4 Effect of SAM on the incidence of apoptotic bodies in persistent nodules

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Rat group</th>
<th>Treatment</th>
<th>Time (wk)</th>
<th>ABS/100 hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>5</td>
<td>None</td>
<td>26</td>
<td>1.73 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>SAM (1)</td>
<td>27</td>
<td>2.87 ± 0.11*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>SAM (8)</td>
<td>34</td>
<td>0.22 ± 0.00*</td>
</tr>
<tr>
<td>F344</td>
<td>7</td>
<td>None</td>
<td>12</td>
<td>2.75 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>SAM (11)</td>
<td>23</td>
<td>3.43 ± 0.45</td>
</tr>
</tbody>
</table>

* Period of time after initiation. SAM treatment was started 1–11 weeks (as indicated in parentheses) before killing. It consisted of 6 daily doses (64 μmol/kg) of SAM.

† Number of ABs was determined by scoring 3500 nodular cells/liver. Data are means ± SD of 5 rats for groups 5, 6, and 8 and 4 rats for group 7.

‡ t test: different from the appropriate control for at least P < 0.002.

Another aspect of peculiar interest is the inhibition of PN growth, associated with a sharp decrease in their histochemical uniformity and in size and number in Wistar rats exposed for 8 weeks to SAM. In F344 rats an 11-week SAM treatment led to a complete disappearance of PN. Our data, however, do not permit a comparison between the two strains for their sensitivity to SAM, due to the differences in the length of SAM treatment as well as in the developmental stages of PN at the time of this treatment. Numerous observations indicate that PN are precursors of hepatocarcinomas in the rats and maybe in humans [reviewed by Farber (24)]. Thus, inhibition of PN growth and enhancement of their regression by SAM could be important for cancer prevention, taking into account that PN are easily recognizable and have a low tendency to regress spontaneously. The SAM effect on nodule development is partially linked to an alteration of the equilibrium between cell production and cell loss in favor of cell loss by apoptosis. However, apoptosis was low in nodules, after a prolonged SAM treatment. It should be noted that phenotypic stability was lost by those nodules which underwent a drop in DNA synthesis and loss of biochemical markers. This led to the appearance of liver cells with an apparently normal phenotype, which are expected to exhibit low cell production and low cell loss. Our data do not, however, clarify if these apparently redifferentiated hepatocytes were still initiated cells.

Mechanisms of the SAM Effect. SAM is a nontoxic and nonmutagenic substance (46) which enters liver cells in vitro (47–51) and in vivo (47, 52). Indirect evidence (19) suggests SAM uptake by hepatocytes, without previous splitting to adenosine and methionine. SAM treatment causes accumulation in rat liver of 5’-MTA, a SAM catabolite which inhibits polyamine synthesis and growth (17, 18, 31–33). 5’-MTA accumulation, inhibition of ODC activity, a key enzyme for polyamine synthesis, and of DNA synthesis have also been observed in PN as a consequence of SAM treatment. Thus, growth inhibition by 5’-MTA could be envisaged as a mechanism of the SAM effect on the development of preneoplastic tissue. In PB-treated rats SAM could modulate PB effects on cell production and cell death, at least in the early stages of promotion. It should also be noted, however, that the SAM/SAH ratio is low in PN. A low SAM/SAH ratio may be associated with DNA undermethylation (53). This has indeed been observed in PN (19). When SAM treatment is started after the appearance of PN, inhibition of c-Ha-ras, c-Ki-ras, and c-myc protooncogene expression takes place, which seems to correlate better with nodular DNA methylation than with accumulation of SAM catabolites which inhibit growth (19). The role of protooncogene expression in tumor promotion and progression has not yet been clarified. However, a role of these phenomena on cell growth and differentiation has been stressed (54). The possibility that interference of a high methylaing environment with gene expression is one of the mechanisms influencing cell production and cell loss is an attractive hypothesis and is currently under study.

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INHIBITION OF PROMOTION AND NODULE GROWTH BY SAM


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Inhibition of Promotion and Persistent Nodule Growth by S-
Adenosyl-l-methionine in Rat Liver Carcinogenesis: Role of
Remodeling and Apoptosis

Renato Garcea, Lucia Daino, Rosa Pascale, et al.


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