Chemoprevention of Rat Liver Carcinogenesis by S-Adenosyl-L-methionine: A Long-Term Study

Rosa M. Pascale, Vincenzo Marras, Maria M. Simile, Lucia Daino, Giampaola Pinna, Simona Bennati, Monica Carta, Maria A. Seddaiu, Giovanni Massarelli, and Francesco Feo

Institutes of General Pathology [R. M. P., M. M. S., L. D., G. P., S. B., M. C., M. A. S., F. F.], and Morbid Anatomy [V. M., G. M.], University of Sassari, 07100 Sassari, Italy

ABSTRACT

Previous work has shown a consistent fall in S-adenosyl-L-methionine (SAM) in the liver of diethylnitrosamine-initiated rats, during the development of preneoplastic lesions, in persistent nodules (PNs), and hepatocellular carcinomas. The injection of SAM into rats causes the reconstitution of the SAM pool, coupled with growth restraint, remodeling, and apoptosis of preneoplastic cells, and inhibits the development of PNs and hepatocellular carcinomas. To evaluate if SAM treatment causes a long-term prevention of preneoplastic and neoplastic liver lesions or merely causes a delay in their development, we evaluated the effect of a relatively short SAM treatment on the development of preneoplastic and neoplastic lesions in a long-term study. Male Wistar rats were subjected to initiation with diethylnitrosamine, followed by selection and then by the administration of phenobarbital for 16 weeks. After selection, the rats were given i.m. injections of a purified SAM preparation (384 μmol/kg/day) for 24 weeks. In SAM-treated rats, a decrease in the incidence of PNs was found 6, 14, and 24–28 months after initiation. At the end of SAM treatment the number of PNs per rat liver, nodule diameter, and labeling and mitotic indices of nodular cells decreased considerably in control rats. Nodule diameter started to increase rapidly again only 8 months after arresting SAM treatment, when complete recovery of DNA synthesis in nodular cells occurred. The majority of nodules present in the liver 6–28 months after initiation belonged to the clear and acidophilic cell types, with lower percentages of mixed cell and basophilic cell types. A decrease in basophilic nodules occurred in SAM-treated rats. Fourteen and 24–28 months after initiation hepato-cellular carcinoma incidence was 11 of 12 and 10 of 10 in control rats, respectively, and only 1 of 12 and 3 of 11 in SAM-treated rats. At the 24th–28th month all control rats had tumors identified as 2 poorly differentiated carcinomas, 6 trabecular carcinomas, or 3 adenocarcinomas, while only 2 relatively small trabecular carcinomas and 1 small glandular tumor developed in SAM-treated rats. In 3 of 11 SAM-treated rats, but in none of the control rats, leukemic infiltration of liver occurred 14–24 months after initiation. Leukemic infiltration of the spleen occurred in 5 and 3 control and SAM-treated rats, respectively. These results show that a 6-month treatment with SAM causes a great loss in the ability of initiated preneoplastic cells to evolve to cancer for a period of time (28 months) roughly corresponding to two-thirds of the rat life span. Prenecoplastic and neoplastic lesions which escape from the SAM chemopreventive effect undergo a relatively slow progression to more malignant lesions. Our results envisage the possibility of preventing human liver tumors in populations at risk.

INTRODUCTION

Previous work has shown a consistent fall in the major lipotropic compound, SAM,3 in the liver of DENA-initiated rats during the development of preneoplastic lesions (1–3), as well as in PNs and HCCs (3, 4). The injection of a highly purified and relatively stable SAM preparation caused the reconstitution of the SAM pool, coupled with the inhibition of growth and the rise in remodeling of putative preneoplastic lesions (3, 5). A rise in cell death by apoptosis also occurred in preneoplastic cells, and it was suggested that it contributes to the decrease in number and size of preneoplastic lesions in SAM-treated rats (5, 6). SAM treatment, started during the development of early preneoplastic lesions (enzyme-altered foci), greatly inhibited the development of enzyme-altered foci, PNs, and HCCs (3). In these experiments SAM was injected continuously from the fourth week after initiation, up to killing, 6 and 14 months after initiation. Thus, even though these observations suggest a strong chemopreventive effect of SAM for hepatic carcinogenesis, they do not clarify if the blocking of the evolution of initiated cells to cancer, in SAM-treated rats, is maintained for a long period of time after arresting SAM administration. Alternatively, initiated cells may survive to SAM, remaining in a silent state, and preneoplastic and neoplastic lesions may develop after arresting SAM administration. Clearly, the discrimination between these two possibilities may largely influence the strategy for the use of SAM as a chemopreventive agent for liver tumors in humans (6), taking into account that this compound is already currently used as a cytoprotective agent against acute and chronic toxic liver injury in humans (7). In this study we performed long-term experiments to evaluate the effect of a relatively short SAM treatment on the development of PNs and HCCs between 6 and 28 months after initiation of hepatocarcinogenesis in Wistar rats subjected to initiation/selection/PB treatments (5). Evidence is provided showing, in the majority of rats, a long-term decrease in the incidence, number, and size of preneoplastic and neoplastic lesions in SAM-treated rats.

MATERIALS AND METHODS

Male Wistar rats (bred in our laboratory, 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 (60%) 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% N-acetylaminoferurol. A partial hepatectomy was performed at the midpoint of this time period (selection (8)). After selection the rats were fed a standard diet containing 0.05% PB for a maximum of 16 weeks (5) after which they were given a standard diet. The rats were randomly divided into two groups. Group 1 was control and group 2 was SAM-treated. SAM treatment (6 daily i.m. doses of 64 μmol/kg each) was started at the end of selection and was continued up to the 24th week. SAM p-toluensulfonate (BioResearch S.P.A., Liscate, Milano, Italy) was injected i.m. as a freshly prepared solution in 0.045 M NaOH and 0.4 M lysine (pH 6.5). Controls received the solvent brought to pH 6.5 with an equimolar mixture of sulfuric acid and PTS. As shown in Fig. 1, high pressure liquid chromatography analysis (9) showed the presence of very low amounts of S-adenosylhomocysteine,
CHEMOPREVENTION OF LIVER TUMORS BY SAM

5'-methylthioadenosine, and other unidentified contaminants. The SAM peak corresponded to 98.2% of the theoretical SAM content (corrected for PTS content) of the injected preparation. Thin layer chromatography analysis (9) revealed that SAM preparations were free of methionine or other contaminants detectable with ninhydrin. The rats were killed 6, 14, and 24–28 months after initiation. Twenty-four-28 months after initiation, all of the rats of group 1 and 7 rats of group 2 were moribund and were killed. The remaining rats of groups 2 were killed at the 28th month.

After animals were killed the liver were excised and the liver surface was examined carefully; then the livers were rapidly sectioned into approximately 4-mm slices. All visible nodules were counted with the aid of a magnifying glass, and maximum and minimum diameters were measured. Benign lesions (PNs) were distinguished from carcinoma nodules on the basis of the published criteria (10–13). By persistent nodules we mean focal proliferating hepatocytes visible to the naked eye, larger than a liver lobule, with a diameter of at least 0.5 mm, compressing surrounding parenchyma (13). Small pieces of liver were taken from each lobe (3 from the central lobe, and 2 from the other 2 lobes) as well as from each macroscopic liver lesion, and from each pulmonary lobe and spleen, fixed with buffered formaldehyde (pH 7), and then processed for embedding in paraffin/Paraplast. They were then sectioned into 5-µm-thick sections and used for hematoxylin and eosin staining. GGT histochemistry was performed according to the method of Ruthenberg et al. (14) by using section from pieces of liver fixed with cold acetone. At least 27 sections were examined per liver, representing the superficial, medium, and profound layers of each piece of liver or visible liver lesion. Foci, nodules, and carcinomas were recognized microscopically and classified according to the published criteria (10–13). By persistent nodules we mean focal proliferating hepatocytes visible to the naked eye, larger than a liver lobule, with a diameter of at least 0.5 mm, compressing surrounding parenchyma (15). Morphometric analysis of small preneoplastic foci was carried out by scanning the sections with a Leitz Diaplan microscope connected by a telecamera with a MOP Videoplan image analyzer (Kontron Electronik, Eching, Germany). To determine the LI of GGT-positive nodules, the rats received, 1 week before killing, an i.p. implant of one osmotic minipump (model 2ML1, Alza Scientific Marketing, London) containing 1.2 mCi of [3H]deoxythymidine (90 Ci/mmol; Amersham International plc, Amersham, United Kingdom) in 2 ml (release, 11 µl/h). The slices, fixed and processed for GGT histochemistry, were coated with Kodak NTB2 emulsion and stored for 5 weeks in the dark at 4°C. After the development the slices were counterstained with hematoxylin. LI was determined by counting 4000–6000 GGT-positive hepatocytes/liver. MI was calculated by counting the mitoses in 8000 GGT-positive hepatocytes/liver.

Statistical analysis was performed by Student’s t test or by χ² test. The Yates correction for small groups was applied to the χ² test (16).

RESULTS

Toxicity. One hundred ten rats were subjected to initiation/selection/BB treatments. Two rats died during the 2nd week of AAF feeding. An additional 39 rats died in the following 2 weeks: 20 of them were treated with PTS; and 19 were treated with SAM. Finally 4 rats died between the 5th and the 24th weeks; 2 of them were treated with SAM. Thus, at the end of SAM injection (6th month) the effective number of rats was 32 and 33 for PTS-treated and SAM-treated groups, respectively.

Table 1 Effect of SAM on the incidence of neoplastic nodules and carcinomas in the rats subjected to the initiation/promotion/PB treatments

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Treatment</th>
<th>6 mob</th>
<th>14 moa</th>
<th>24–28 moa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PN</td>
<td>CA</td>
<td>PN</td>
<td>CA</td>
</tr>
<tr>
<td>1</td>
<td>PTS</td>
<td>10/10 (100)</td>
<td>0/10</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>2</td>
<td>SAM*</td>
<td>6/10 (60)</td>
<td>0/10</td>
<td>6/12 (50)</td>
</tr>
</tbody>
</table>

* Number of rats with macroscopic and/or microscopic evidence of PNs and/or carcinomas (CA), 6, 14, and 24–28 months after initiation/number of surviving rats. Numbers in parentheses, percentages of PN- or CA-bearing rats.

* χ² test (Yates corrected); SAM versus PTS: 6 months, PN, χ² = 6.25, P < 0.01; 12 months, PN, χ² = 1.04, P < 0.01, CA, χ² = 14.85, P < 0.0001; 28 months, PN and CA, χ² < 4.98, P < 0.025.

* SAM treatment was started 4 weeks after initiation and was continued up to the 24th week.
group, in which an adenocarcinoma was seen (not shown). Twenty-four-28 months after initiation nodules and tumors, belonging to the poorly differentiated, trabecular, and adenocarcinoma histological types (see below), were visible in all rats not treated with SAM. In the SAM group there occurred a great fall in the incidence of nodules and carcinomas, which were present in about 45 and 27% of rats, respectively. All differences between control and SAM groups, with respect to the incidence of nodules and carcinomas, 24–28 months after initiation, were significant.

Liver Nodule Development. In order to follow the development of PNs, the number of nodules per liver and their average diameter were determined 6–28 months after initiation (Fig. 2). PN number per rat underwent a sharp decrease between 6 and 28 months after initiation in control rats. In SAM-treated rats PN number was 33% lower than in PTS-treated rats 6 months after initiation. Thereafter it underwent a slight further decrease, but 24–28 months after initiation, the number of PNs was 36% higher in SAM-treated than in untreated rats. Nodule diameter underwent a progressive increase in control rats, while in SAM-treated rats increase in diameter was seen only between the 14th and 28th months. Moreover, the diameter of PNs was 23–43% lower in SAM-treated than in untreated rats throughout the experimental period.

The effect of SAM on PN growth was also determined by measuring the mitotic and labeling indices (Table 2). LI and MI were high in GGT-positive nodules, 6 and 14 months after initiation. However, 24–28 months after initiation relatively low MI was found. SAM treatment caused a 53–57% fall in LI and MI, 6 months after initiation. However, several months after interruption of this treatment (14th and 24th-28th months) no changes in LI and/or MI could be found.

Histology. Remodeling has been proposed as the cause of the disappearance of early preneoplastic lesions, either spontaneously (15) or as a consequence of SAM treatment (5). Thus, preneoplastic lesions were analyzed for their histochemical and histological patterns in order to evaluate if remodeling of preneoplastic cells into normal appearing liver could account for the decrease in tumor yield in SAM-treated rats.

Relatively few focal lesions could be observed in the liver of the two rat groups studied 6 months after initiation. GGT-positive foci represented 13.2 ± 1.8 and 9.03 ± 0.7% of liver parenchyma, in PTS and SAM groups, respectively (mean ± SD, n = 10; P < 0.001). Very few GGT-positive foci, representing 6–8% of liver parenchyma, were seen in the liver of SAM-treated and control rats, 14 and 24–28 months after initiation. All foci were small and showed a nonuniform pattern of GGT histochemistry and irregular boundaries with the surrounding parenchyma. All of these foci belonged to the clear and acidophilic cell types.

In the rats not treated with SAM, most nodules observed 6–28 months after initiation were GGT positive. Although many of them exhibited a nonuniform pattern of GGT histochemistry, especially at the 6th month (Fig. 3, A and D), they were still recognizable as typical nodules by hematoxylin and eosin staining (Fig. 3C), with cells showing large nuclei and prominent nucleoli, constituting irregular plates of hepatocytes not exhibiting major atypias (Fig. 3, B, E, F). This behavior was not modified by SAM treatment, which did, however, increase by about 3-fold the percentage of PNs with a nonuniform pattern of GGT histochemistry (nonuniform nodules, at the 6th month: 25 ± 6.5 and 70.4 ± 8.6% of total, without and with SAM, respectively; n = 10 and 6 without/with SAM, P < 0.001). Microscopic examination revealed that 6–14 and especially 24–28 months after initiation, many lesions, which macroscopically appeared as large nodules, resulted from the coalescence of smaller and sometimes cytologically heterogeneous nodules (Fig. 3, C-F). An analysis of the cytological features of nodules present 6–28 months after initiation (Table 3) showed that most nodules belonged to the clear and acidophilic cell types. Smaller percentages of mixed cell nodules and basophilic cell nodules could be observed. No major changes in the percentages of clear and acidophilic cell and mixed cell nodules occurred throughout the study in PTS-treated rats, except for a progressive decrease in the percentage of basophilic nodules between the 6th and 28th month. The distribution pattern of various histological types of nodules was apparently not influenced by SAM treatment, with the exception of basophilic nodules which underwent about 50% decreases 6 and 14 months after initiation. No basophilic nodules were found at the 24th-28th month in SAM-treated rats.

A detailed analysis of the lesions present in control and SAM-treated rats 24–28 months after initiation showed some nonneoplastic lesions, consisting of pelycosis hepatis, oval cell hyperplasia, fatty change, and bile duct hyperplasia, in the rats of both groups. No extensive necrosis and fibrosis were observed. Nodules showing atypical cells with a glandular pattern were observed in two rats (Fig. 4, A and B). Eight carcinomas, in the control group, were well differentiated tumors: four trabecular carcinomas (Fig. 4C), and four adenocarcinomas (Fig. 4D). Poorly differentiated tumors (Fig. 4E) were found in two rats of the PTS group. The carcinomas were constituted of multiple, confluent nodules, which occupied large parts of liver.
parenchyma, in five rats. In one rat only two carcinomatous nodules were present, and they did not extend to a large portion of the liver.

A different situation characterized SAM-treated rats. Trabecular carcinomas were found in two rats, and a small nodule showing atypical cells and glandular pattern was seen in another rat (Fig. 4F). In three rats there occurred a leukemic infiltration of liver which appeared, microscopically, to be of the lymphohcytic type in two rats, and of the myelomonocytic type in the third one (Fig. 4G). In these rats a diffuse leukemic infiltration of the spleen red pulp (Fig. 4H), associated with a destruction of the spleen architecture, was also observed. Leukemic infiltration, restricted to only the red pulp, was observed in the spleen of five control rats. No pulmonary metastases of liver carcinomas were seen in PTS-treated and SAM-treated rats.

DISCUSSION

Results from various laboratories indicate that lipotropic compounds, such as SAM, methionine, choline, and betaine, prevent the development of liver and mammary gland carcinomas, or sarcomas induced in rats or mice by various carcinogens (3, 17–23). Lipotrope-enriched diet increases the survival of
AKR/J mice bearing spontaneous thymic lymphomas (24). Moreover, inhibition of PN progression to carcinoma has been reported in Fischer rats fed a diet containing extra choline amounts (25). Accordingly, SAM given to rats after the development of PNs inhibits the growth of these lesions (5, 26). Interestingly, methionine, added to a suspension of human bone marrow cells, impairs the utilization of labeled precursors for DNA synthesis (27). In the majority of the above researches (17, 19–23), methionine, choline, and betaine were administered orally in mM daily doses per kg of body weight, while SAM was injected i.m. in μM daily doses per kg of body weight. Moreover, all lipotropic compounds used were given continuously to rats, during the entire experimental period, and cancer incidence was studied at a maximum 14 of months after initiation of carcinogenesis. The possibility that lipotropic compounds simply induced a delay in the appearance of neoplastic lesions cannot be excluded.

The results in the present paper show that a relatively short treatment (6 months) with 384 μmol/kg/day of SAM causes a decrease in incidence and size of preneoplastic nodules and neoplastic lesions, during a period of time, after initiation (28 months), roughly corresponding to more than two-thirds of the rat life span. It should be noted that no interference of SAM with death rate, and body and liver weights was recorded, suggesting that the SAM dose used is not toxic under our experimental conditions.

In the liver of rats subjected to initiation/selection ± PB, early preneoplastic lesions undergo a rapid increase in number and size, followed by a spontaneous decrease (5, 15, 28), that has been at least partially attributed to spontaneous remodeling (15, 28). However, between the 6th and the 28th month after initiation, nodules showing a nonuniform pattern of GGT histochemistry still maintained their histological features, suggesting that at least some of these nodules may persist even if they lose one (or more?) biochemical marker. Nevertheless, our results show that in this period of time, there is a progressive fall in PN number per rat, in the absence of SAM treatment. This decrease could be, at least in part, accounted for by confluence of nodules undergoing rapid size increase as well as nodule evolution to cancer. A different behavior characterizes SAM-treated rats. At the end of SAM treatment nodules were absent in 55% of these rats, and in the remaining rats PN number per rat liver was 33% lower than in control rats. This could be a consequence of inhibition of preneoplastic tissue growth and rise in cell loss by apoptosis during SAM administration (5), which should shift the equilibrium between cell production and cell loss, in favor of cell loss (6). Thereafter PN number underwent only a slight decrease and, consequently, it became higher than in control rats. This may be explained by both a reduced progression of nodules to tumor, as suggested by the absence of carcinomas in some nodule-bearing rats 14–28 months after initiation, and by a decrease in nodule confluence, as suggested by the smaller diameter of nodules in SAM-treated rats. This interpretation seems to be substantiated by the observation that carcinomas developing in SAM-treated rats are smaller than in control rats, and no poorly differentiated carcinomas were found in SAM-treated rats. Moreover, although there is a fall in the growth rate of PN at the end of SAM treatment, there was apparently no effect of previous SAM treatment on nodule growth 14 months after initiation. However, the relatively small nodule diameter, at this time, suggests a slow recovery of DNA synthesis and/or an increased cell loss for a presumably long period of time, after arresting SAM treatment. This phenomenon cannot be explained at this stage of our study. SAM does not exhibit any cytotoxic effect on liver cells, and evidence has instead been provided indicating that it affords a cytoprotective effect against toxic liver injury (7).

The various histological types of nodules reflect variations in glycogen metabolism and growth rate (29). Basophilic nodules exhibit a higher DNA synthesis with respect to other preneoplastic lesions and have been suggested as being more prone to carcinous transformation than the other histological types of nodules (29, 30). It should be noted, however, that changes in nodule cytological features could be largely influenced by environmental factors which interfere with carbohydrate metabolism and protein and DNA synthesis. Our findings indicate that no major changes in nodule histological type, and thus presumably in the metabolic reactions which influence histological features, occur during PN progression between 6 and 28 months after initiation. SAM does not greatly modify the histological patterns of nodules, except for a marked fall in basophilic nodules. The relationships between this fall and the decrease in tumor yield in SAM-treated rats should be the object of further investigation.

The mechanisms underlying the SAM chemopreventive effect are far from being completely elucidated. Recent data exclude that this effect depends on production of 5'-methylthioadenosine, a SAM catabolite that inhibits growth (31). The chemopreventive effect of SAM has been tentatively attributed to the methylation and inhibition of expression of growth-related genes, such as c-myc, c-Ha-ras, and c-Ki-ras, which are highly hypomethylated and overexpressed in PNs, in the absence of SAM (24, 32). However, there is no definitive proof that the methylation of regulatory sequences of gene promoters is responsible for inhibition of expression of growth-related genes and nodule growth in SAM-treated rats. Nonetheless the role of DNA methylation in the SAM chemopreventive effect is proved by the finding that 5-azacytidine, a known inhibitor of DNA methyltransferases (33), largely overcomes the inhibitory effect of SAM on the growth of preneoplastic tissue (34).

SAM, a naturally occurring, nontoxic and nonmutagenic compound (35) which enters liver cells (36–42), is used in humans for the treatment of liver injury, including cirrhosis, chronic hepatitis (7, 43–45), etc. Multistage models of experimental carcinogenesis are artificial situations, largely different from the human condition. Recent evidence (46), however, strongly supports the view that human hepatocarcinogenesis is a multistep process. Moreover, liver macronodular cirrhosis,

<table>
<thead>
<tr>
<th>Time (mo)*</th>
<th>Treatment</th>
<th>Total no. of nodules</th>
<th>Clear/acidophilic</th>
<th>Mixed</th>
<th>Basophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Control (10)</td>
<td>291</td>
<td>83.0</td>
<td>12.1</td>
<td>3.7</td>
</tr>
<tr>
<td>SAM (6)</td>
<td>120</td>
<td>87.3</td>
<td>11.0</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Control (12)</td>
<td>237</td>
<td>83.6</td>
<td>13.5</td>
<td>2.5</td>
</tr>
<tr>
<td>SAM (6)</td>
<td>99</td>
<td>89.9</td>
<td>9.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>24–28</td>
<td>Control (10)</td>
<td>108</td>
<td>86.7</td>
<td>11.1</td>
<td>1.9</td>
</tr>
<tr>
<td>SAM (5)</td>
<td>79</td>
<td>90.0</td>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Time after initiation.

**SAM** (384 μmol/kg/day) was started at the end of selection (4th week) and was continued up to the 24th week.

1 Number of nodules found in all nodule-bearing rats. Numbers in parentheses, number of rats with nodules.

2 Mixed cell nodules are constituted essentially of clear cells and some basophilic cells.

### Table 3 Percentage of various histological types of nodules in SAM-treated and control rats

<table>
<thead>
<tr>
<th>Time (mo)*</th>
<th>Treatment</th>
<th>Total no. of nodules</th>
<th>Clear/acidophilic</th>
<th>Mixed</th>
<th>Basophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Control (10)</td>
<td>291</td>
<td>83.0</td>
<td>12.1</td>
<td>3.7</td>
</tr>
<tr>
<td>SAM (6)</td>
<td>120</td>
<td>87.3</td>
<td>11.0</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Control (12)</td>
<td>237</td>
<td>83.6</td>
<td>13.5</td>
<td>2.5</td>
</tr>
<tr>
<td>SAM (6)</td>
<td>99</td>
<td>89.9</td>
<td>9.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>24–28</td>
<td>Control (10)</td>
<td>108</td>
<td>86.7</td>
<td>11.1</td>
<td>1.9</td>
</tr>
<tr>
<td>SAM (5)</td>
<td>79</td>
<td>90.0</td>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Time after initiation.

**SAM** (384 μmol/kg/day) was started at the end of selection (4th week) and was continued up to the 24th week.

1 Number of nodules found in all nodule-bearing rats. Numbers in parentheses, number of rats with nodules.

2 Mixed cell nodules are constituted essentially of clear cells and some basophilic cells.
Fig. 4. Histological pattern of liver and spleen lesions in the rat, 24–28 months after initiation. A, clear/acidophilic cell nodule showing a central area (arrowheads) with a glandular pattern and some atypias; B, clear cell nodule showing scattered areas of atypical cells arranged in a glandular array, compatible with early adenocarcinomatous transformation; C, trabecular carcinoma; D, adenocarcinoma; E, poorly differentiated carcinoma; F, atypical liver nodule showing glandular array; G, leukemic infiltration of the liver; H, leukemic infiltration of the spleen red pulp. A-E, control rats; F-H, SAM-treated rats. A, x 139; B, x 139; C, x 307; D, x 139; E, x 512; F, x 139; G, x 307; H, x 139.
positive for HBV antigen, has been suggested as being a pre-cancerous condition (47). In transgenic mice, various HBV genes are expressed only if hypomethylated (48, 49), and a correlation between methylation and expression of HBV genes has been found in human primary liver tumors (50). It could be of interest to investigate if long-term treatment with relatively high SAM doses of individuals with liver macronodular cirrhosis, positive for HBV antigen, prevents later development of HCC and is associated with methylation of HBV antigen genes. Twenty-eight months after initiation all of the rats showed leukemic infiltration of liver and/or spleen. Hematopoietic lymphoreticular tumors are relatively frequent in old rats (12), and their incidence seems to increase in DENA-initiated rats (51). This phenomenon does not seem to be influenced by SAM.

REFERENCES
45. Micalli, M., Chiti, D., and Balestra, V. Double-blind controlled clinical trial
46. Sakamoto, M., Hirohashi, S., and Shimosato, Y. Early stages of multistep
hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular car-
47. Blumberg, B. S., and London, W. T. Hepatitis B virus and the prevention of
48. Araki, K., Akagi, K., Miyazaki, J., Matsubara, K., and Yamamura, K. Cor-
relation of tissue-specific methylation with gene inactivity in hepatitis virus
49. Pourcel, C., Tiollais, P., and Farza, H. Transcription of the S gene in trans-
genric mice is associated with hypomethylation of specific sites and with
50. Ghen, J. Y., Hsu, H. C., Lee, C. S., Chen, D. S., Zuckerman, A. J., and
Harrison, T. J. Detection of hepatitis B virus DNA in hepatocellular carci-
noma: methylation of integrated viral DNA. J. Virol. Methods, 19: 257–263,
choline on liver tumor promotion by phenobarbital and DDT in diethylnit-
Chemoprevention of Rat Liver Carcinogenesis by \textit{S}-Adenosyl-l-methionine: A Long-Term Study

Rosa M. Pascale, Vincenzo Marras, Maria M. Simile, et al.