Aim: This study tests the hypothesis that statins (HMGCoA reductase inhibitors) inhibit carcinogenesis and that this effect may be mediated by the statin-induced inhibition of ubiquinone synthesis. Materials and Methods: The effects of lovastatin, with and without addition of ubiquinone, were studied in a rat model for chemically induced hepatocarcinogenesis. Intermediates in the mevalonate pathway were measured. Results: Lovastatin treatment reduced the volume fraction of liver nodules by 50% and the cell proliferation within the liver nodules was reduced to one third. Ubiquinone (Q10) treatment reversed the statin-induced inhibition of cell proliferation. Lathosterol levels were reduced significantly in the statin-treated rats, indicating inhibition of the mevalonate pathway, but cholesterol levels were not affected. Conclusion: Lovastatin inhibits carcinogenesis in a rat model for liver cancer, despite unaffected cholesterol levels. The statin-induced inhibition of cell proliferation may, at least in part, be explained by the inhibition of ubiquinone synthesis.

Statins are some of the most commonly prescribed drugs in the world today. Statins are competitive inhibitors of HMGCoA reductase enzymes in the liver, thus inhibiting the endogenous synthesis of cholesterol in the body. In humans, statins are efficient at reducing serum cholesterol levels and are protective against cardiovascular diseases (1). During recent years it has been proposed that statins have additionally beneficial anticarcinogenic effects. Several large epidemiological studies have shown a preventative effect of statins on the development of colorectal, prostate and lung cancer (2-4). However, other studies have failed to demonstrate this effect (5, 6).

Liver cancer is the fifth most common cancer in the world. In a randomised controlled trial (n=83), pravastatin treatment was associated with a longer median survival of patients with advanced hepatocellular carcinoma, an increase from 9 months to 18 months in the pravastatin-treated patients (7). In a more recent cohort study (n=183), addition of pravastatin treatment prolonged survival in liver cancer patients from 12 months to 20.9 months (8).

The tumour preventative effect of statins has been suggested by some researchers to be associated with a cholesterol-lowering effect (9). Several groups have reported an increase in apoptotic activity during statin treatment (10-12), suggesting that a statin-mediated inhibition of prenylation of key proteins in apoptotic pathways such as p21ras and ErB2 might be of importance (12, 13). However, the mechanism behind the tumour preventative effects of statins is still not fully elucidated.

By inhibiting the rate-limiting step (HMGCoA reductase) in the mevalonate pathway, statins inhibit the entire mevalonate pathway and as a consequence all the cholesterol precursors are reduced (Figure 1). In addition, the synthesis of ubiquinone is inhibited. Ubiquinone (Q) is a lipid that is crucial in the respiratory chain in addition to performing an antioxidative function. Earlier reports have shown that the levels of ubiquinone are elevated in preneoplastic liver tissue (liver nodules) in rats (14).

In rat, the most prevalent ubiquinone is ubiquinone-9 (Q9), and ubiquinone-10 (Q10) represents only a small fraction of the total Q (14). In humans, Q10 is the most
prevalent ubiquinone and Q9 constitute only a small fraction of the total Q (14). However, Q9 and Q10 have the same function in the cell and are interchangeable (14).

In the present work, the hypothesis that part of the tumour preventative effect of statins could be related to inhibition of ubiquinone synthesis has been tested. Thus the effect of lovastatin (given orally at a relatively low dose) and ubiquinone have been studied in a sequential initiation-promotion model for experimental hepatocarcinogenesis in rat. The model used here is originally described by Solt and Farber (15) and is called “the resistant hepatocyte model”, implementing the appearance and selection of resistant hepatocytes with up-regulated cellular defence. The model has been modified for gastric feeding by the same group and only slightly modified in this study. In addition, this study measured different intermediates in the mevalonate pathway, including ubiquinone, within the rat liver tissue after lovastatin and Q10 administration.

Materials and Methods

Animal experiments. Male Fischer-344 rats, weighing 160 g, were purchased from Charles River, Germany. Animals were maintained at a 12 h light and 12 h dark cycle and fed standard chow diet R36 (Lactamin AB, Linköping, Sweden) until the start of the experiment (week 1). The chow diet used during the experiment contained lovastatin (150 mg/kg R36) or Q10 (1 g/kg R36) or both lovastatin (150 mg/kg R36) and Q10 (1 g/kg R36), produced by Lactamin AB, Linköping, Sweden. The animals were allowed to acclimatise in the animal room for four days before the start of the experiment. Temperature, humidity and ventilation in the animal room were controlled in accordance with international standards.

The animals were divided into four groups (1-4) with nine rats in each group (Figure 2). In group 1 (control rats) the rats were fed on the standard chow diet. In group 2 the rats were fed on a chow diet containing 150 mg/kg lovastatin, in group 3 the chow diet contained 1 g/kg Q10 and in group 4 the chow diet contained 150 mg/kg lovastatin and 1 g/kg Q10. The carcinogenic process was induced with diethylnitrosamine (DEN) dissolved in saline (50 mg/ml) and injected intraperitoneally in a necrogenic dose of 200 mg/kg of rat body weight. During the second week, promotion was performed by 3 intragastric injections of 2-AAF (20 mg/ml emulsified in agar) every second day. During the third week 2/3 partial hepatectomy (PH) was performed followed by another 2 intragastric injections of 2-AAF (20 mg/ml emulsified in agar) on day 2 and 4 after PH. The promotor was injected intragastrically due to lovastatin and ubiquinone was administration in the diet. This design also guaranteed the same dose of promotor to all rats.

Rats were sacrificed at 7 days (four rats in each group) and 21 days post PH (five rats in each group).
Dose and administration of Lovastatin. Lovastatin has a low bioavailability; taken orally, only 5% of an administered Lovastatin dose reaches the systemic circulation in humans (16). In earlier experiments in rodents, statins were given intraperitoneally (12, 17, 18). The bioavailability for this type of administration is not known, but is likely to be higher than that after oral administration.

The maximal recommended dose of Lovastatin in humans is 80 mg/day. This corresponds to approximately 1 mg Lovastatin/kg bodyweight. The dose of Lovastatin given to the rats was 150 mg/kg of chow diet. The rats consumed about 20 g of chow diet per day corresponding to a dose of 3 mg/day. The mean weight of the rats was 225 g at the end of the study. The dose was therefore 13 mg/kg bodyweight/day. Since rodents have a much quicker metabolism of statins compared to humans, this is likely to be an adequate dose, with an exposure similar to or less than that in humans. Similar or higher doses have been used in previous work (12, 17, 18). The biological effects noticed in the statin groups indicate that the dose given was not at a subtherapeutic level.

Determination of liver nodule density. Rats were sacrificed after exsanguination via the abdominal aorta under isoflurane anaesthesia. The livers were removed, weighed and cut into 4 mm-thick slices. Representative slices were harvested, fixed in 4% buffered formaldehyde and processed for histological investigation. Paraffin sections were incubated with a monoclonal antibody towards intracellular glutathione-S-transferase 7-7 (antiGST-Yp/Yf subunit 7) to visualise initiated hepatocytes, liver foci and nodules as described previously (19). Liver foci and nodules were quantified by number and size using morphometric densitometry, expressing numbers and volumes in relation to surface area of the section and volume fraction, respectively. The relative volumes of the lesions were calculated as described previously (19).

Labelling index and apoptosis. Three days before sacrifice, the rats were administered 5-bromo-2'-deoxyuridine (BrdU) using Alzet micro-osmotic pumps (1 μl per hour, 3 days) implanted subcutaneously on the back of the animals. Incorporated BrdU was detected by BrdU-immunohistochemistry. BrdU-labelled and unlabelled nuclei were counted in 6 image fields (×250) corresponding to 600-800 cells, which were sufficient according to cumulative mean. Labelling index (LI) was calculated for the surrounding parenchyma as well as in the liver nodules. LI measures the number of labelled hepatocytes expressed as a percentage of all hepatocytes and is a way to estimate cell proliferation during the period of BrdU labelling (20, 21).

For identifying apoptotic cells in situ, TUNEL staining was carried out as described elsewhere (22). Rat thymus sections were used as positive control as well as rat liver sections treated with DNAase to confirm that the method could detect apoptosis and fragmented DNA.

Determination of cholesterol and cholesterol precursors. Liver tissue (50-70 mg from each rat) was dissolved in 2 ml of Folch solution (chloroform/MeOH 2:1; v/v) with argon on top and sealed with a teflon cap. After 24 h at room temperature, 100 μl and 1800 μl of (chloroform/MeOH 2:1; v/v) with argon on top and sealed with a teflon cap. After 24 h at room temperature, 100 μl and 1800 μl of the extract were transferred into new vials for assay of cholesterol and other cholesterol precursors, respectively. Internal standards were added; 2 μg d6-cholesterol for cholesterol analysis and 150 ng d6-sitosterol + 150 ng d4-lathosterol for the other cholesterol precursors. After hydrolysis, cyclohexane (3 ml) was added, the upper organic phase was transferred to new glass vials and the extraction was repeated once more with 3 ml of cyclohexane. Extracts were pooled and evaporated under a stream of argon in heating blocks at 60°C for derivatization. Samples were analysed with a gas chromatography-mass spectrometry (GC-MS) method described previously (23). This method allows separation and quantification of nine structurally related cholesterol intermediates (squalene, desmosterol, 7-dehydrocholesterol, lathosterol, zymosterol, dihydro-lanosterol, lanosterol, FF-MAS, T-MAS) and two plant sterol (campesterol and sitosterol).

Analysis of serum cholesterol was performed on a modular autoanalyser (Roche) using a cholesterol CHOD-PAP reagent (Roche, cat. no 1875540).

The values given in Table I represent mean levels from all the rats in each group (five rats for all values except for the serum-cholesterol levels 7 days post PH, where there were only four rats in each group).

Ubiquinone analysis. Liver tissue (100-200 mg) was dissolved in 10 ml of Folch solution (chloroform/MeOH 2:1; v/v) with argon on top and sealed with a teflon cap. After 24 h at room temperature, 500 μl of the extract was transferred into a new vial and evaporated under a stream of argon in heating blocks at 35°C. The dry extract was dissolved in 150 μl of MeOH and 50 μl of ethyl ether and transferred to HPLC glass vials. Oxidised and reduced forms of Q9 and Q10 were quantified by reversed-phase HPLC (Dionex P680 HPLC pump, Gilson 234 autoinjector and Jasco UV-1570 UV/VIS detector) using an ACE 5 C18 SIN 150×4.6 mm column at room temperature. An isocratic flow of 95% MeOH and 5% 2-propanol at 1.2 ml/min was employed with a program time of 60 min at 275 nm and 295 nm for oxidised and reduced ubiquinones, respectively and with retention times of 16.5 (Q9 red), 23.5 (Q10 red), 25.5 (Q9ox) and 40.0 min (Q10ox). Q9 and Q10 standards (Sigma) were reduced with NaBH4 to obtain Q9 red and Q10 red. Standard curves in the linear range was used to calculate ubiquinone content in the samples.

Statistical analysis. Values are presented as means±standard error of the mean (SEM). Significances were tested by calculation of one-way ANOVA, followed by planned comparisons according to LSD test (least significant difference test). When appropriate, variances were stabilised by log transformations.

Ethical approval. Animal experiments received full approval from the Animal Stockholm Experimentation Ethics Committee.

Results

Liver nodule density. To test the hypothesis, the development of preneoplastic liver tissue (liver nodules) in non-treated rats was compared with rats treated with statin, Q10 or both. After 21 days post PH, the relative nodule density in the non-treated rats (group 1) was approximately 25% and in the statin-treated rats (group 2) only 12% (p<0.05) (Figure 3). The rats treated with both statins and Q10 (group 4) also exhibited a reduction of liver nodule density compared to the controls (p<0.05). There was no statistically significant difference between the animals treated with only Q10 (group 3) and the control animals (group 1).
Labelling index and apoptosis. To investigate further the effect of statin treatment the cell proliferation within the liver tissue was monitored. LI measures the numbers of cells in S-phase in a selected sample and is thus a way to measure cell proliferation in tissues. In rats fed with a statin-containing diet (group 2), the LI in liver nodules was significantly lower compared with the control rats (group 1) 21 days after PH ($p<0.001$) (Figure 4). In the rats, fed with both Q10 and statins (group 4), the statin-induced decrease in cell proliferation in liver nodules was completely reversed and there was no statistically significant difference compared to the control rats (group 1). There was also no statistically significant difference between control rats (group 1) and rats fed with Q10 only (group 3).

In the surrounding liver tissue, Q10 treatment resulted in an inhibiting effect of cell proliferation compared to controls ($p=0.01$) (Figure 4).

The statin-induced inhibition of cell proliferation first became apparent 21 days post PH. In rats sacrificed 7 days post PH, when the mito-inhibition of 2-AAF on heptocytes is still active, there was a tendency of lower cell proliferation in the rats fed with statins compared with the control rats but the difference did not reach statistical significance (data not shown).

To determine the possible effect on apoptotic index in the statin- and Q10-treated animals TUNEL staining was carried out. However, there were only negligible numbers of apoptotic cells in any of the liver sections from any of the animals in any of the groups 7 or 21 days post PH (data not shown).

Body weight and liver weight. At the end of the experiment, 21 days post PH, the statin-treated animals (groups 2 and 4) had a significantly lower mean bodyweight compared with the non-statin treated animals (groups 1 and 3) (199 g versus 249 g, $p<0.01$). The rats treated with statins (group 2 and 4) also had lower levels of plant sterols in their livers (sitosterol and campesterol), indicating a lower food intake compared with the non-statin treated rats (data not shown). The mean liver weight in the statin treated animals (group 2 and 4) was somewhat smaller than in the non-statin treated rats (group 1 and 3) but this difference was not statistically significant (8.90 g versus 9.49 g).

Cholesterol and cholesterol precursors. To investigate wether the lovastatin treatment was sufficient in blocking the mevalonate pathway in the rats, cholesterol precursors were determined (Table I, Figure 5). Lathosterol levels have previously been shown to be a good marker for determination of the flux through the mevalonate pathway (24). Lathosterol levels were markedly reduced in all statin-treated animals ($p<0.001$) and were not affected by
Figure 3. Volume fraction of liver nodules 21 days after PH. A: Images show representative liver section from one rat in each group. B: The values represent means from 5 rats±SEM. p<0.05 for rats treated with statin or statin+Q10 compared with non-statin treated rats (group 1).
ubiquinone treatment indicating that the statin treatment was efficient in blocking the mevalonate pathway (Figure 5). The levels of other cholesterol precursors were also markedly decreased (Table I). In contrast, the cholesterol levels in liver and serum were not affected by statin treatment. The difference in cholesterol levels in serum between the different groups was not statistically significant.

Ubiquinone analysis. To confirm the statin-mediated effect on ubiquinone and the absorption of the supplemented Q10, measurements of ubiquinone in liver tissue were performed. There were no statistically significant differences in the levels of Q9 in the different groups (Table I). The levels of Q10 were below the levels of quantification with the HPLC method applied in the non-supplemented animals (group 1 and 2) but were markedly increased in the supplemented animals (group 3 and 4). The proportion of reduced Q10 vs. oxidised Q10 was significantly larger 21 days post-PH (29%) compared with 7 days post PH (15%), $p<0.001$ (data not shown for the rats 7 days post-PH). There were no detectable Q9 or Q10 levels in serum with the method employed.

Discussion

This study shows that lovastatin, given orally in a low dose, reduces efficiently the volume fraction of preneoplastic liver tissue (liver nodules) in a rat liver model for chemical carcinogenesis. The cell proliferation within the liver nodules was reduced to one third compared to controls. The low cell proliferation within the liver nodules in statin treated rats can be expected to slow down the carcinogenic process and postpone or completely abolish tumour progression and malignant transformation in the liver nodules.

The levels of extramitochondrial but not mitochondrial ubiquinone are increased in preneoplastic liver tissue (liver nodules) and is regarded as a component of the resistant cell phenotype (14). Ubiquinone synthesis can be expected to be inhibited by statins, and lower levels of ubiquinone have also been reported in liver tissue after statin treatment in rats (18). In the rats in this study, the Q9 values in liver homogenate were not significantly affected. It is only the small fraction of Q in the cytosol and in the cytoplasmic membranes that has antioxidative properties (14). Since only the total level of Q could be measured, changes in the small extramitochondrial Q fraction may be undetected. In accordance with this hypothesis, addition of Q reversed the statin-induced inhibition of cell proliferation completely. However the volume fraction of liver nodules was not reversed by Q10 treatment. Therefore, it can be concluded that there must be some additional effects of statin treatment explaining the tumour preventative effect. One possible explanation is that the reduced flux in the mevalonate pathway may suppress generation of isoprenoid units of importance for isoprenylation of proteins, which is important for the tumour development.

The relative starvation observed in statin treated animals is noticeable. Previous work has shown that prolonged diet restriction in rats (45 weeks) partly inhibits tumour development in this model (25). However, it is not likely that the moderate starvation for 6 weeks among the statin-treated animals can explain the 50% reduction in liver nodules.

It is well established that statins do not have the same capacity to reduce cholesterol levels in rat as in man (17, 18, 19).
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per se that causes the tumour preventative effect in this model. Thus, the precise mechanism behind the tumour preventative effect of statins remains elusive. It could be speculated that a low intracellular level of ubiquinone, leading to an impaired intracellular oxidoreductive capacity, may trigger apoptosis within the preneoplastic cells. Several reports have demonstrated an increased apoptotic index in statin-treated tissues and cells (10-12). In this rat model, apoptosis within the tissue is not expected since the liver undergoes massive regeneration after PH. This study investigated the possible statin-mediated effect of apoptosis but significant apoptosis was not detected in any of the animals.

In two randomised controlled trials in patients with manifest liver cancer, statin treatment was associated with a prolonged survival (7, 8). Thus it seems that statins are able to slow down tumour progression in the liver. However, liver cancer in humans may differ in many ways from the chemically induced hepatocarcinogenesis studied here. Still it is interesting that the findings reported from the clinical studies are in accordance with the findings of this study, namely that statins decrease cell proliferation within preneoplastic liver nodules and slow down tumor growth. Observations in this rat model may provide a platform for further and larger clinical trials in patients, exploring the possible cancer protective role of statins in liver cancer.

In conclusion, these results demonstrate a pronounced tumour preventative effect of lovastatin in a rat model for hepatocarcinogenesis, despite there being no change in cholesterol levels. The results also show that the statin-induced inhibition of cell proliferation within preneoplastic tissue was reversed fully by ubiquinone supplementation, indicating that part of the anticarcinogenic effect may be mediated by ubiquinone inhibition.

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