in vivo *26*: 527-536 (2012)

Review

Exploring Anticarcinogenic Agents in a Rat Hepatocarcinogenesis Model – Focus on Selenium and Statins

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Abstract. *In this review, we describe a rat model for chemically induced hepatocarcinogenesis that can be used for studying the anticarcinogenic effects of different agents. In this model the process of carcinogenesis can be followed through the different stages of initiation, promotion and progression. Mechanistic studies of anticarcinogenic agents can be carried out and two examples are given by studies on selenium and statins as anticarcinogenic agents. These compounds suppress cancer via different mechanisms. In the case of selenium the induction of glutathione peroxidase 4 and inhibition of lipid peroxidation might be a part of the anticarcinogenic effect. In the case of statins, the inhibition of ubiquinone synthesis, as well as of the selenium-containing enzyme thioredoxin reductase 1 (TrxR1) might explain their anticarcinogenic properties. Interestingly, also in the case of selenium the inhibited carcinogenesis was associated with reduced TrxR activity, indicating an important role for this enzyme in carcinogenesis.*

Carcinogenesis

Malignant tumours consist of cells with dysfunction in their growth regulation, including defective contact inhibition, and dysfunction in their apoptotic machinery. Different cells within the same tumour can exhibit great and progressive genetic variability although they all, from the very beginning of carcinogenesis, originate from one single cell (1-3).

Chemical carcinogenesis is described as a multistep process and three major stages of this process have been identified: initiation, promotion and progression (1, 2, 4) (Figure 1). Prerequisite for this process is an initiated cell and the presence a factor in the environment – a so-called promotor, selecting for the growth of the initiated cell.

Usually the promotor is a toxic compound present in the environment that compromises growth and growth regulation. An altered cell which, under the influence of the selective pressure of a promotor, has a growth advantage during promotion, compared to the surrounding normal cells is called an "initiated" cell. During the promotion phase, all cells within the tissue are exposed to the promotor. The promotor may inhibit mitosis in the normal, non-mutated cells or may even be cytotoxic to these cells. The initiated cells that are resistant to these effects may instead proliferate and are selected for in the process of carcinogenesis. The initiated cells can respond to growth stimulatory signals appearing during regeneration after cell injury and are able to grow under circumstances where growth is inhibited in normal cells. As a consequence, focal proliferation may occur, generating clones of altered preneoplastic cells (1) (Figure 1).

Cell proliferation under toxic influence occurs with an obvious risk for new DNA lesions and genetic alterations. In the short term, proliferation of resistant cells may be beneficial to the organism and, in most situations, promotion is a reversible process dependent on the presence of the promotor. However, during promotion, new mutations may occur that give initiated cells the ability to maintain their growth advantage over the non-initiated cells even in the absence of the promotor. In this case, neoplastic, autonomously growing cells appear. The progression of the process from this point is called the "progression phase". Genetic instability facilitates further mutations and after

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Key Words: Hepatocarcinogenesis, liver cancer, rat model, carcinogenesis, selenium, statins, HMGCoA reductase inhibitors, thioredoxin reductase, glutathione peroxidase, review.

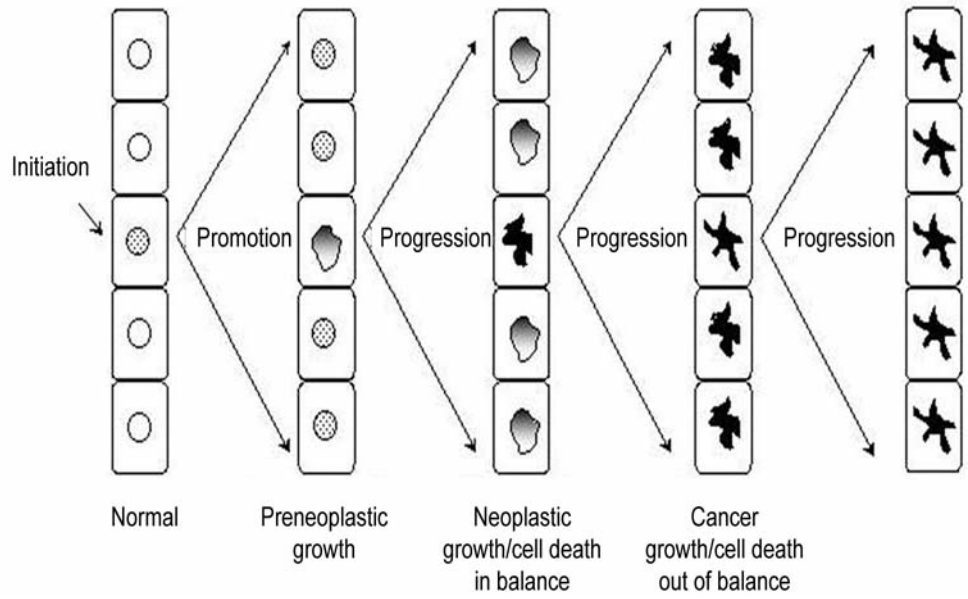

Figure 1. *Chemical carcinogenesis is a multistep process and can be divided into initiation, promotion and progression.*

several steps of genetic alteration and selection due to growth advantages, malignant tumour cells develop.

In experimental models, preneoplastic as well as neoplastic cells are characterized by changes in expression of drug-metabolizing enzymes, up-regulation of antioxidants and antioxidant regenerating systems, down-regulation of nuclear P53 protein and a capacity to avoid apoptosis (5-8). Carcinogenesis can be described as a chronic selection of cells with a resistant phenotype, at least for tumours that are developed as a consequence of environmental exposure to carcinogens and chronic cell injury.

A Hepatocarcinogenesis Model in Rat

A model for chemically induced hepatocarcinogenesis in the rat was initially described by Solt and Farber in 1976 (4). In this model, hepatocarcinogenesis can be followed through the initiation, promotion and progression stages in dose-response experiments, and tissues for histological, cytochemical and biochemical studies can be harvested. Initiation is performed using diethylnitrosamine (DEN) dissolved in saline (50 mg/ml) and injected intraperitoneally at a necrogenic dose of 200 mg/kg of rat body weight. During the third week, promotion is achieved by administration of 2-acetylaminofluorene (2-AAF). This can be administered as three intragastric injections of 2-AAF (20 mg/ml emulsified in agar) every second day, or as 2-AAF in the diet (0.02%) for 4 days. During the fourth week, 2/3 partial hepatectomy (PH) is performed to trigger cell proliferation. This is followed by another two intragastric injections of 2-AAF (20 mg/ml emulsified in agar) on day 2 and 4 after PH (Figure 2). It should be noted that in the original publication, promotion was achieved by two weeks of feeding a 0.02% 2-AAF chow-diet in combination with partial hepatectomy after one week of 2-AAF exposure.

During promotion, microscopic and macroscopic lesions appear in the tissue, referred to as preneoplastic liver foci and liver nodules, respectively. The growth of these lesions is dependent on the promotor and as many as 99% or more of these lesions will re-differentiate to normal-looking liver tissue after termination of the promotion (9). However, in fewer than 1% of the nodules, new local lesions will appear and progress further. During this progression phase, growth of the lesions is autonomous, independent of the promotor, and will progress until the development of malignant tumours, which eventually will kill the rat.

In another rat model using 2-AAF, described by Epstein *et al.* (10) and modified by Eriksson *et al.* (11), premalignant neoplastic liver nodules are developed in larger volumes. In this model, the liver nodules can be scooped out from the liver and used for biochemical studies consuming more material than produced, using the method of Solt and Farber. In the Epstein-Eriksson model, the intermittent feeding of 2-AAF is extended to 25 weeks.

These two models together are valuable for several reasons. First of all, they allow the opportunity to study the consecutive steps of carcinogenesis with dose-response experiments. Secondly, these liver models are well-

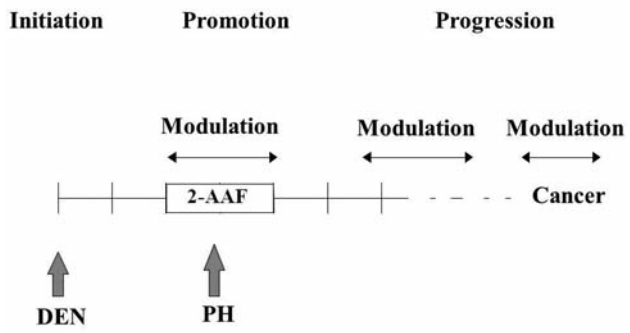

Figure 2. *Schematic presentation of the Solt-and-Farber model. 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; PH, partial hepatectomy.*

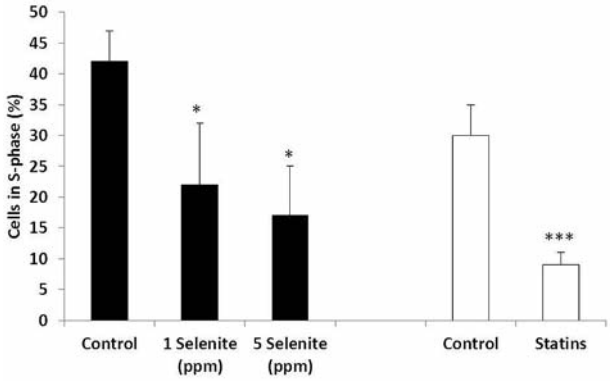

Figure 3. *Cell proliferation within liver nodules measured as 5-bromo-2'-deoxyuridine index (BrdU), i.e. the percentage of cells in the S-phase after selenium and statin treatment. Each value represents the mean±SEM of measurements from five rats. Statistical analysis using t-test give * p<0.05 and ***p< 0.001 for treated rats, compared to the respective control. The figure is based on raw data from studies (31) and (47).*

characterized in many aspects, involving toxicity, drug metabolism, enzyme activities and different cellular defence mechanisms. Finally, in studies on carcinogenesis, it is always a problem to obtain representative control tissue. In the models described here it is possible to use internal control tissue surrounding the lesions (in the Solt and Farber-model only), as well as age-matched normal liver, carcinogen-treated, non-neoplastic liver, and regenerating liver tissues.

Measurements of Liver Nodules and Cell Proliferation

Glutathione-S-transferase 7-7 (GST-π) is the placental form of GST. This isoenzyme is not present in normal rat liver but is highly and ectopically expressed in rat liver nodules (12, 13) and can be used as a marker for liver nodules (14, 15). Immunohistochemical staining of intracellular GST-π can therefore visualize initiated hepatocytes, liver foci and nodules. The relative volumes of these lesions can be calculated using a computerized morphometric analysis and is a good marker for carcinongenesis.

To investigate cell proliferation within the liver we have used antibodies against recombinant Ki-67 antigen (clone MIB-5) and 5-bromo-2'-deoxyuridine (BrdU) incorporation for various periods of time (2 h to 3 days) and calculation of mitotic figures. These methods are complementary to each other and give different but related information. In the studies described in this review we used administrated BrdU during 3 days before harvesting the livers. Nuclei staining positively for BrdU were in the S-phase, and by determination of the labelling index (percentage of BrdU-positive cells) in liver sections, the degree of cell proliferation during the period studied can be measured. The labelling index is determined in surrounding parenchyma and in liver nodules under guidance from consecutive GST-π -stained sections.

The BrdU index, as a measure of cell proliferation in liver nodules from our studies on selenium and statins, are shown in Figure 3.

Features of Preneoplastic Liver Nodules

In both rat models described here, liver lesions that express a specific phenotype appear to be resistant to the toxic effects of the promotor 2-AAF. During progression, the persisting neoplastic lesions constitutively express the resistant phenotype, even when 2-AAF is no longer present. The phenotype of the liver foci and nodules is characterized by a multitude of alterations that contribute to the increased resistance to toxic influences *via* different mechanisms (6). For example, drug metabolism is altered in a way that limits the toxic effect of lipophilic compounds. Furthermore, the multidrug-resistant protein P-glycoprotein (P-gp) is overexpressed (16, 17), which leads to a higher capacity for the hepatocytes to excrete toxic compounds out of the cell. Phase I activation of drugs is reduced by down-regulation of most cytochrome P450 enzymes, while phase II reactions are increased by overexpression of conjugating enzymes such as glucuronyltransferase, GST, γ-glutamyltransferase and aldehyde dehydrogenases (6, 12, 13, 18-20). Intracellular levels of water- and lipophilic-soluble antioxidants are also increased, such as ubiquinone and glutathione (21). Interestingly, the liver lesions are iron-deficient, reducing the oxidant and pro-oxidant effects of iron in cells exposed to oxidative stress (22). The increased cell-surface expression of transferrin receptor on sideropenic cells, increases the surface binding of diferric transferrin, saturating the need for extracellular electron acceptors in growing cells (23). The

Table I. *The phenotype of liver nodules compared to normal rat liver tissue.*

Enzymes/compound	Levels in liver nodules compared to normal liver
Phase I enzymes: Cytochrome P450 enzymes	Decreased
Phase II enzymes: Glucuronyltransferase, glutathione- *S*-transferase and aldehyde dehydrogenases	Increased
Iron	Decreased
Glutathione (GSH)	Increased
Ubiquinone	Increased
Thioredoxin reductase 1 (TrxR1)	Increased
Thioredoxin reductase 2 (TrxR2)	(Decreased)
P-glycoprotein (P-gp)	Increased

expression of the redox-active selenoenzyme Thioredoxin reductase 1 (TrxR1) is also increased, almost four-fold in these lesions, while mitochondrial TrxR2 is slightly reduced compared to normal liver (24). Taken together, all these changes (summarized in Table I) in the preneoplastic liver nodules contribute to their so-called resistant phenotype.

Here, two examples of studies on anticarcinogenic agents in a rat model will be described. The first agent to be discussed is selenium and the second is statins (3-hydroxi-3-metylglutaryl Coenzyme A-reductase inhibitors).

Selenium and Cancer

Selenium is a trace element that is essential for the activity of different selenoproteins within the body. Two important groups of selenoproteins are the TrxRs and glutathione peroxidases (GPx). These redox-active proteins are important in the defence against oxidative stress and detoxification of reactive oxygen species. In addition, TrxRs also have other functions, such as regulation of apoptosis, redox signalling, cell division and DNA synthesis. TrxRs are discussed in greater detail later in this review.

A number of epidemiological studies have shown a relationship between low selenium status and cancer (25). In animal models, both organic and inorganic selenium compounds have been shown to prevent or inhibit carcinogenesis (26, 27). A landmark in selenium-mediated tumour prevention research in humans was accomplished by Clark *et al*. and was presented in reports published in 1997 and 1998 (28, 29). This was a double-blinded, placebo-controlled trial where 1,312 US participants with history of basal squamous cell carcinomas of the skin were studied. A daily dose of 200 μg selenium, supplied as a high-selenium brewer's yeast, reduced the incidence of several forms of cancer. The most pronounced effect was seen on the incidence of prostate cancer, which was reduced by more than 60%.

Notably, the findings by Clark and co-workers initiated a large randomised, placebo-controlled, multicentre study with the aim of studing the effect of selenium, and vitamin E, in preventing prostate cancer, the so-called SELECT study (30). In this study, 35,533 men with prostate specific antigen (PSA) <4.0 ng/ml were included and randomised to placebo, vitamin E, selenium, or selenium plus vitamin E and were followed-up for 7-12 years. The study was published in 2011 and selenium in the form of the organic L-selenomethionine was not shown to have any preventive effect on the risk of developing prostate cancer. Unexpectedly, vitamin E supplementation resulted in an increased risk of developing prostate cancer (30).

Selenium as an Anticarcinogenic Agent in the Rat Model

Based on the findings by Clark *et al*., we hypothesized that selenium could prevent or ameliorate hepatocarcinogenesis. To test our hypothesis, we chose the Solt and Farber hepatocarcinogenic model in the rat since it constitutes a good model to study the different phases of carcinogenesis (initiation, promotion or progression). In this study selenite in supra-nutritional but subtoxic doses (1 and 5 ppm) was administered to the rats through the drinking water. Such supplementation during the promotion phase was found to reduce the volume fraction of preneoplastic liver nodules from 38% in control animals to 25% (1 ppm) and 14% (5 ppm) in the selenite-supplemented groups (9, 31) (Figure 4). In addition, the cell proliferation within the nodules markedly decreased in the selenite-treated rats, *i.e*. the potential for malignancy in the lesions was reduced (Figure 3).

Selenite treatment at 5 ppm during the progression phase, resulted in a significantly lower volume fraction of liver tumours along with a decrease of cell proliferation within the tumours (9, 31). Interestingly, selenite supplementation in the drinking water during the initiation phase when the first critical DNA-damage occurs did not affect carcinogenesis. Thus, we can conclude that the selenium-mediated improvement of DNA repair mediated by p53 (32), as previously reported, was not the major tumour preventive

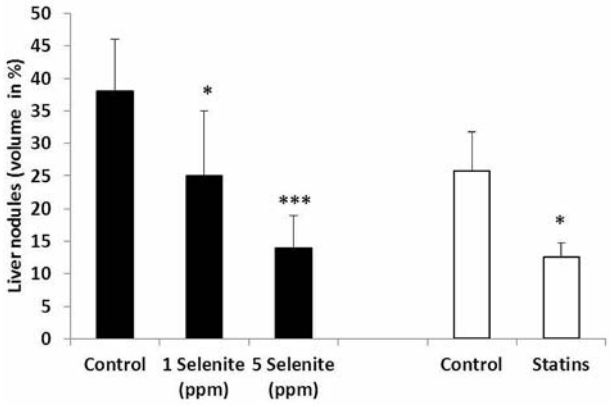

Figure 4. *Volume fraction of liver nodules, as a marker for carcinogenesis, after selenium and statin treatment. Each value represents the mean±SEM of measurements from five rats. Statistical analysis using t-test give *p<0.05 and *** p<0.001 for treated rats, compared to the respective control. The figure is based on raw data from studies (31) and (47).*

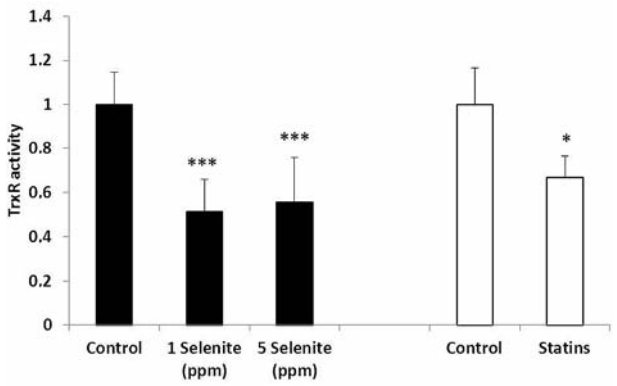

Figure 5. *Relative levels of Thioredoxin reductase (TrxR) activity in rat liver tissue after selenium and statin treatment. The values are percentage of activity compared to control tissue, obtained from non-treated rats in the same experiment. Each value represents the mean±SEM of measurements from five rats. Statistical analysis using t-test give *p<0.05 and ***p<0.001 for treated rats compared to the respective control. The figure is based on raw-data from the studies (31) and (59).*

mechanism of selenium in the hepatocarcinogenetic model used in our study. Instead, other mechanisms, important during promotion and progression, must be involved.

In a more recent study, materials and findings from our rat model experiments with selenium were used to investigate the mechanism behind the tumour-preventative effects of selenium in humans (33). Patients with liver cancer often have reduced selenium levels (34), which may cause lipid peroxidation and oxidative stress. Increased lipid peroxidation leads to enhanced activation of the transcription factor activator protein 1 (AP-1) and elevated expression of vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8), resulting in accelerated growth of liver tumours (35). The selenium-containing GPx4 is the only known enzyme that is able to reduce lipid peroxides (36). We showed that selenium inhibited lipid peroxidation by induction of the selenoprotein GPx4 in our rat model and this was also shown in human hepatocellular carcinoma cell lines. These findings were also corroborated in patients with hepatocellular carcinoma, where selenium levels were inversely correlated with VEGF, IL-8 and size of the tumour (33).

To conclude, selenium seems to have anticarcinogenic effects also in human hepatocellular carcinoma and a possible mechanism could involve the induction of GPx4.

Statins and Cancer

Statins (HMGCoA reductase inhibitors) are some of the most commonly prescribed drugs in the world today. Statins are competitive inhibitors of HMGCoA reductase enzyme in the liver, thus inhibiting endogenous synthesis of cholesterol in the body. In humans, statins are efficient at reducing serum cholesterol levels and reduce cardiovascular disease (37). During recent years, it has been proposed that statins have additional beneficial anticarcinogenic effects. The first report in this research area was published by Poynter *et al*. in 2005. In this case-control study (n=1,953 cases and n=2,015 controls), it was shown that statin-treated patients had a significantly lower risk of developing colon cancer compared to non-treated patients (38). In later epidemiological studies, preventative effects of statins on the development of prostate, lung and pancreatic cancer was reported (39-41). However, other epidemiological studies have failed to demonstrate anticarcinogenic effects (42, 43).

The liver is the main target organ for the action of statins. Thus, it is reasonable to believe that if statins had anticarcinogenic properties, this could be important for liver cancer. Indeed, interventional studies have been carried out to test this hypothesis and interesting data have been presented. In two small randomised, placebo-controlled trials the effect of statins on manifested liver cancer were studied. In the first trial (n=83), pravastatin treatment was associated with a longer median survival of patients with advanced hepatocellular carcinoma, with an increase from 9 months to 18 months in the pravastatin-treated patients (44). In the second study (n=183), addition of pravastatin treatment prolonged survival in patients with hepatocellular carcinoma from 12 months to 21 months compared to placebo (45).

Patients with chronic hepatitis B infections have a high risk of developing hepatocellular carcinoma. In a recent large

epidemiological study (n=33,413 patients; 328,946 person years), it was shown that patients suffering from chronic hepatitis B had a reduced risk of developing hepatocellular carcinoma if they were on statin treatment (46). Notably, the preventative effect of statin was also dose-dependent, with a larger effect being found with high statin doses.

The suggested anticarcinogenic effects of statins, and especially the pronounced positive effect in liver cancer, was the background for our mechanistic studies of statin in a rat model.

Statins as an Anticarcinogenic Agent in a Rat Model

In this study, we revealed a pronounced anticarcinogenic effect of lovastatin in our rat model (47). Lovastatin was administered to the rats through the diet at a rather low dose (approximate 13 mg lovastatin/kg bodyweight/day). The volume fraction of liver nodules was reduced by 50% compared to non-treated animals (Figure 4). Cell proliferation within the liver nodules was also reduced to one third, *i.e.* the potential for malignancy in the lesions was dramatically reduced with statin treatment (Figure 3).

By inhibiting the rate-limiting step (HMGCoA reductase) in the mevalonate pathway statins inhibit the entire mevalonate pathway and as a consequence, all cholesterol precursors are reduced. This includes inhibition of the synthesis of ubiquinone, a lipid that is crucial in the respiratory chain and also has antioxidative functions within the cell. Notably, the level of ubiquinone is up-regulated in preneoplastic liver nodules (21).

Our initial hypothesis was that inhibition of the ubiquinone synthesis could explain the anticarcinogenic effects of statins. Interestingly, in a very recent article, this hypothesis is supported by the findings that the mevalonate pathway is significantly up-regulated by mutant p53 in breast cancer (48). Consequently, it has been suggested that targeting the mevalonate pathway, for example by statins, could provide a way of preventing carcinogenesis and tumour growth.

To test the ubiquinone hypothesis, rats were treated with lovastatin, lovastatin plus ubiquinone, or ubiquinone alone and were compared with non-treated control rats. The hypothesis was that addition of ubiquinone to the statin treatment would reverse the anticarcinogenic effect of statins and thart these rats would subsequently have the same nodule density as the control rats. However, the addition of ubiquinone did not reverse the anticarcinogenic effect of statins. The nodule density remained unchanged compared to that of the statin treated animals. However, the addition of ubiquinone did reverse the inhibited cell-proliferation within the nodules to the same level as the control rats. We conclude that statins did have a pronounced anticarcinogenic effect in this model but that the inhibition of ubiquinone could only explain a part of this effect. Thus, other mechanisms must also be involved.

In follow-up studies, we investigated if statins could inhibit TrxR1, a specific enzyme of great importance for carcinogenesis.

Thioredoxin Reductases

TrxRs are redox-active selenoenzymes, having a selenocysteine residue in their active site and exceptionally broad substrate specificities (49). TrxR is also part of the thioredoxin system, comprising of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. This is an efficient reductase system for protein disulfide bonds. It plays an important role in a variety of cellular processes, including the formation of precursors for DNA synthesis, defence against oxidative stress and maintenance of the reduction potential of the intracellular environment (50). In general, high intracellular levels of TrxR are associated with a more efficient defence against oxidative stress. The Trx system is also involved in apoptosis by activation of p53, one of the key proteins in the apoptosis machinery (51). TrxR can also regenerate oxidized ubiquinone back to the active, reduced form (52). In the cell, there are two different isoenzymes of TrxR, the cytosolic TrxR1 and the mitochondrial TrxR 2.

We have previously shown that the levels of the cytosolic TrxR1 are increased almost four-fold in preneoplastic liver nodules, while the levels of mitochondrial TrxR2 are reduced compared to the normal, surrounding liver tissue (24). Thus, we have hypothesized that the increase in cytosolic TrxR1 is important for the survival of the neoplastic liver cell and that this factor gives an advantage in cell growth and cancer progression (24). It is interesting to note that TrxR1 in the rat liver model is selectively increased in the proliferating liver nodules that appear in the progression phase of the process and develop into dysplastic liver nodules and eventually hepatocellular carcinoma (9), but not in the remodelling nodules. This makes TrxR1 an interesting marker for liver cancer and liver cancer risk. Indeed, elevated TrxR1 levels have been reported not only in liver cancer cell but also in other types of cancer cells, such as malignant melanoma, breast, thyroid, prostate and colorectal cancer (53-56). Increased TrxR1 levels in tumour cells have also been associated with a poor prognosis and aggressive tumour growth (53, 56).

The hypothesis that TrxR1 is important for the carcinogenic process is further supported by a study in a lung cancer model in mice (57). In this study, injection of mouse lung cancer cells with knocked-down *TrxR1* resulted in pronounced reduction in tumour progression and metastasis compared to mice injected with control, malignant cells. Moreover, tumours arising in mice injected with *TrxR1* knock-down cells were much smaller in size (57).

In our rat studies with selenium supplementation, it might be expected that the levels of the selenoprotein TrxRs would increase, as is seen in normal rat liver (58). Interestingly, the level of TrxRs in liver nodules instead decreased, to about half of the activity of the untreated rats (Figure 5). This was also associated with inhibited carcinogenesis, further strengthening the hypothesis that TrxRs are important in the carcinogenic process.

Anticarcinogenic Effects of Statins and TrxR1

In our subsequent studies of statins in the rat model, we demonstrated that statin treatment was associated with a 45% reduction of TxR1 (Figure 5). There was a significant correlation between reduction of TrxR1-levels and inhibited carcinogenesis, as measured as a reduced volume fraction of liver nodules. Even when we adjusted for the decrease in volume of liver nodules developed during the statin treatment, there was a significant reduction in TrxR1. There was also a clear correlation between the HMGCoA-reductase inhibition, measured as lathosterol, and reduced TrxR1 levels (59).

Importantly, we confirmed that statin treatment was also associated with a reduction of TrxR1 in liver tissue from humans. In fact, the decrease in TrxR1 after statin treatment was even more pronounced in humans than in rats: a reduction of 85-90% was observed in the two cohorts studied (59). However, the two cohorts studied were small and the findings need to be confirmed in larger studies.

We suggest that the reduction of TrxR1, associated with statin treatment might be part of the anticarcinogenic effects of statins. Hence, TrxR1 might be a suitable molecular target for cancer therapy, which has also been proposed by others (60).

Conclusion

In conclusion, the rat model for chemically-induced hepatocarcingenesis described here is suitable for mechanistic studies on anticancer drugs during the different phases of initiation, promotion and progression. Carcinogenesis and cell proliferation can easily be measured and preneoplastic, neoplastic and control tissue can be obtained for biochemical and molecular studies. In this model, we have shown pronounced anticarcinogenic effects of selenium and statins, findings that we believe could form the platform for future interventional studies in humans.

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Received May 8, 2012
Revised June 8, 2012
Accepted June 11, 2012