Inhibition of the Ras oncoprotein reduces proliferation of hepatocytes in vitro and in vivo in rats

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ABSTRACT

Ras oncoproteins are probably implicated in normal and malignant cell growth in various organs. Inhibition of Ras interferes with cell proliferation of non-hepatic cells in vitro and in vivo. A potential role for Ras in normal and malignant hepatocyte proliferation prompted us to evaluate the impact of Ras inhibition by FTS (S-farnesylthiosalicylic acid) on hepatocyte proliferation in vitro in the human hepatic tumour cell line HepG2 and in vivo after PH (partial hepatectomy) in rats. Rats were administered with FTS intraperitoneally (1, 8 and 16 h after PH) and killed 12, 24 and 48 h after PH. Cell proliferation, phosphorylation of members of the MAPK (mitogen-activated protein kinase) pathway and levels and activity of cell cycle effectors (cyclin D, cyclin E, Cdk2 and Cdk4) were assessed in FTS-treated rats compared with controls. FTS significantly decreased overall cell count, PCNA (proliferating-cell nuclear antigen) expression and BrdU (bromodeoxyuridine) incorporation into HepG2 cells after 7 days of culture. FTS treatment significantly reduced BrdU incorporation and PCNA expression in hepatocytes after PH. Unlike control rats, cell membrane expression of Ras was decreased in FTS-treated animals after PH, resulting in decreased Raf membrane recruitment and phosphorylation and in reduced phosphorylation of ERK1/2 (extracellular-signal-regulated kinase 1/2). The antiproliferative effect of FTS was linked to a decrease in expression and activity of the cyclin E/Cdk2 complex, without affecting cyclin D and Cdk4. Ras inhibition by FTS significantly decreased proliferation of HepG2 cells and normal hepatocytes after a strong and highly synchronized proliferation stimulus elicited by PH. The inhibitory effect was at least partially mediated by inhibition of Ras/Raf/MAPK signalling. It appears worthwhile to evaluate the impact of Ras inhibition on the development of hepatocarcinomas in vivo in adequate animal models.

INTRODUCTION

There is a general consensus that the Ras oncoprotein may play an important role not only in the regulation of normal cellular proliferation and differentiation, but also in carcinogenesis [1,2]. Ras activates several intracellular signalling pathways, including the MAPK (mitogen-activated protein kinase) pathway, which play an important role in promoting DNA synthesis and cell proliferation [3]. In vitro, studies have shown that expression...
of Ras increased between the G1- and S-phases of the cell cycle [4,5] and its inhibition resulted in a cell-cycle block in G1-phase. [6,7]. \textit{In vivo} in the liver, it remains, however, ill defined whether interfering with Ras activation could lead to reduced liver cell proliferation. An interesting model to study the impact of targeted inhibition of intracellular pathways in the liver is liver cell proliferation following PH (partial hepectomy). A cascade of events moves cells from their resting state into cell cycle progression in order to restore liver mass [8]. Several intracellular signalling pathways are involved, including activation of the small GTPase Ras, in response to mitogens [8,9]. Ras mRNA expression increases in the regenerating liver in a close relationship with the peak of DNA synthesis [10], and appears to be specifically related to cellular proliferation and cell cycle progression in various animal models of liver cell proliferation [11,12]. In addition, modulation of Ras expression in the liver influences the course of liver regeneration in mice [13]. Very recently, it has also been shown that Ras and the MAPK pathway might play a role in the development of hepatocellular carcinomas in humans [14,15], a cancer with poor survival and scarce therapeutic options. However, additional and unequivocal findings are needed before targeting Ras as a future approach in preventing cellular transformation and malignant liver cell growth can be recommended.

Farnesylation of Ras is important for its membrane anchorage and activation [16], and inhibition of this process arrests cell growth [17,18]. The Ras antagonist FTS (S-farnesylthiosalicylic acid) acts on the three isoforms of Ras, and appears to achieve inhibition of their membrane anchorage and activation by two distinct mechanisms: (i) inhibition of the enzyme PPMTase (prenylated protein methyltransferase) [19,20], and (ii) the antagonism of the mature form of the Ras oncoprotein [20,21]. As FTS exhibits antitumoral effects in non-liver cell tumours \textit{in vivo} in nude mice and prevents cirrhosis development in rats [22,23], the aim of the present study was to evaluate the impact of inhibition of Ras activation through FTS on normal hepatocyte proliferation in rats after PH \textit{in vivo} as well as on a hepatic tumour cell line \textit{in vitro}. The principal aim of the study was to determine whether targeted inhibition of the Ras oncoprotein \textit{in vivo} by such small molecules as FTS has an impact on hepatocyte proliferation and, therefore, could be a potential target for cancer chemoprevention in the liver.

**MATERIALS AND METHODS**

**Cell culture**

HepG2 cells (2.25 × 10⁵ cells/100-mm-diameter dish), a human hepatoblastoma-derived cell line, were cultured in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10% (v/v) fetal bovine serum, 1% streptomycin and penicillin (Gibco), 1% sodium pyruvate (Gibco) and 1% (v/v) MEM (minimal essential medium) (Gibco) over 7 days. DMSO (the solvent for FTS) or FTS at a final concentration of 100 µmol/l was included in the culture medium from the beginning of the experiment. BrdU (bromodeoxyuridine) was added to the culture medium at a final concentration of 10 µmol/l from 48 h of culture onwards. Culture medium was changed every other day. Cells were harvested by trypsinization at the end of the experiment for FACS analysis and cell counting using a Burker cell. In a subset of dishes, cells were lysed directly and the supernatant was recovered for Western blot analysis. Cell viability was checked using Trypan Blue staining. At least three independent experiments were performed for each analysis.

**Animals**

Male Wistar rats (body weight, 180–250 g) were obtained from the rat breeding facilities at the Catholic University of Louvain Medical School, Brussels, Belgium. Animals were kept in a temperature- and humidity-controlled environment with a 12 h light/dark cycle. They were allowed free access to water and a standard food pellet diet (Usine d’Alimentation Rationnelle). The animals were handled according to the guidelines established by the Catholic University of Louvain, Brussels, Belgium.

**Surgical procedures and experimental design**

All operations were carried out under light ether anaesthesia at room atmosphere using a clean, but not, sterile technique. PH was performed as described by Higgins and Anderson [24]. Rats were injected intraperitoneally with 50 mg of BrdU/kg of body weight 2 h before being killed. Animals were killed at 12, 24 and 48 h after PH. Livers were removed, weighed and snap-frozen or preserved in formal.

The initial approach consisted testing the toxicity of FTS in our animal model by injecting a dose of 75 mg/kg of body weight per injection, the lower limit of the reported LD₅₀ in rats, initially to non-hepatectomized rats and then to rats subjected to two-thirds PH. Three intraperitoneal injections were administered to each animal during a 24 h period with an interval of 8 h between each injection. Death of the animals was considered as the primary end point for this part of the study. According to the results obtained during assessment of FTS toxicity, the highest dose of FTS that did not produce any death in hepatectomized rats during the first 24 h after PH was chosen for the final experiments. Consequently, the regime that was used for the final experimental procedure comprised three intraperitoneal injections of 50 mg of FTS/kg of body weight at 1, 8 and 16 h after PH. Controls received a similar volume of DMSO (vehicle for
FTS) at similar time points. At least three, and a maximum of six, animals per experimental group were used.

**Synthesis of FTS**

FTS was prepared as described previously [19]. Briefly, thiosalicylic acid (2.65 g; 6 mmol), guanidine carbonate (3.82 g; 7 mmol) and trans,trans-farnesyl bromide (5 g; 6 mmol) were mixed overnight in 220.6 ml of acetone at room temperature (20°C). After the acetone had evaporated, chloroform was added together with a few drops of 2 mol/l HCl. The mixture was washed with water, and the organic phase was separated and dried on magnesium sulfate and evaporated. FTS was purified on silica gel with mixtures of chloroform and ethyl acetate. FTS was dissolved in DMSO (20 mg of FTS/500 µl of DMSO) for injection.

**Preparation of liver homogenates, cell lysates and nuclear extracts**

Livers were homogenized and cells were lysed (HepG2) in ice-cold buffer [0.1 % Triton X-100, 50 mmol/l Hepes (pH 7.5), 150 mmol/l NaCl, 10 % (v/v) glycerol, 1.5 mmol/l MgCl₂, 1 mmol/l DTT (dithiothreitol), 1 mmol/l sodium fluoride, 1 mmol/l PMSF, 100 mg/ml leupeptin, 10 mg/ml aprotinin and 0.1 mmol/l sodium orthovanadate]. Nuclear extracts were prepared from frozen liver using a commercially available nuclear extract kit (Active Motif), according to the manufacturer’s instructions.

**Preparation of rat liver cytosolic and membrane fractions**

Liver membrane fractions were prepared as described by Wheeler et al. [25], with minor modifications. Livers were homogenized in a buffer containing 10 mmol/l Tris/HCl (pH 7.4), 1 mmol/l EDTA, 0.25 mol/l sucrose, 2 µg/ml PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mmol/l DTT and 1 mmol/l benzamidine, and were centrifuged at 10 000 × g for 10 min at 4°C. The supernatant was removed and centrifuged at 100 000 × g for 1 h at 4°C to prepare cleared cytosol. The resulting pellet was solubilized in 1 ml of lysis buffer containing 20 mmol/l Tris/HCl (pH 7.4), 2.5 mmol/l EDTA, 1 % (w/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.1 % SDS, 100 mmol/l NaCl, 100 mmol/l sodium fluoride and 1 mmol/l sodium orthovanadate, and was centrifuged at 100 000 × g for 1 h at 4°C. This was used as the membrane fraction.

**Western blotting**

Proteins were separated by SDS/PAGE and transferred onto PVDF transfer membranes, according to standard techniques. Membranes were incubated with primary antibodies [mouse monoclonal anti-PCNA (proliferating-cell nuclear antigen; 1:750 dilution overnight; Dako), mouse monoclonal anti-Ras (1:1000 dilution for 2 h; Upstate), rabbit polyclonal antiphospho-Raf-1 (extracellular-signal-regulated kinase 1; 1:500 dilution for 2 h; Santa Cruz Biotechnology), rabbit polyclonal anti-(phospho-p44/42 MAPK) (1:500 dilution overnight; Cell Signaling), mouse monoclonal anti-ERK1 (extracellular-signal-regulated kinase 1; 1:500 dilution overnight; BD Biosciences) and mouse monoclonal anti-β-actin (1:80 000 dilution for 1 h; Sigma)] and secondary antibodies [HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG (1:20 000–1:40 000 dilution for 1 h; Jackson ImmunoResearch) and HRP-conjugated goat anti-rabbit IgG (1:20 000–1:100 000 dilution for 1 h; Jackson ImmunoResearch)]. The antigen–antibody reaction was visualized using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) detection system. Membranes were stripped using Restore Western blot stripping buffer (Pierce Chemicals) and reprobed whenever possible following the manufacturer’s instructions. Quantification of immunoreactive proteins was obtained by densitometry using the Gel Doc 2000 device and software (Bio-Rad Laboratories).

**Immunoprecipitation and Cdk kinase assays**

Immunoprecipitation and Cdk kinase assays were performed as described previously [11]. The histone H1 kinase assay was used to assess Cdk2 activity, and the Rb (769) fusion protein (Santa Cruz Biotechnology) was used as a substrate for Cdk4.

**Histology and immunohistochemistry**

Hepatic sections were stained with haematoxylin/eosin for conventional histology, or incubated with a mouse anti-BrdU antibody (1:100 dilution overnight; Dako) and a secondary HRP-conjugated polymer anti-(mouse IgG) antibody (1 h; EnVision™+ System; Dako) to visualize cell proliferation.

**Flow cytometry**

Incorporation of BrdU into DNA was quantified by flow cytometry, essentially as described by Borbath et al. [26].

**Reverse transcription and quantitative PCR**

cDNA was prepared by reverse transcription of total hepatic RNA, as described previously by Stärkel et al. [27]. Quantitative PCR analysis was performed with the GeneAmp 5700 Sequence Detection System and software (Applied Biosystems), according to the standardized thermal profile of the system set previously by the manufacturer with SYBR Green for detection. All primers (Table 1) were designed using the Primer Express design software (Applied Biosystems). RPL19 (ribosomal protein L19) RNA was chosen as an internal standard. Expression of mRNA was quantified using the ΔΔCT method as specified by the manufacturer.
Table 1  Primers used for quantitative PCR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene Forward</th>
<th>Reverse</th>
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<tr>
<td>p21</td>
<td>CCTGTTCCACACAGGAGCA</td>
<td>CATGAGCGCATCGCAATC</td>
</tr>
<tr>
<td>p27</td>
<td>CTTCCGCCTGCAGAAAC</td>
<td>CTTCTCCAAGTCCCGGGTCTAG</td>
</tr>
<tr>
<td>Ras</td>
<td>AGCATAAACTGCGGAACTCAGA</td>
<td>CATGAGCTGCAAGTGTGCT</td>
</tr>
<tr>
<td>RPL19</td>
<td>CAAGCGGATTCTCATGGAAAC</td>
<td>TGGTCAGCCAGGAGCTTCT</td>
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Statistical analysis
Results are expressed as means ± S.E.M. The statistical differences between the groups were tested using a Student’s t test. Statistical significance was assumed for P values < 0.05.

RESULTS

FTS inhibits liver tumour cell proliferation in vitro
First, we examined whether FTS was able to inhibit proliferation of the hepatic tumour cell line HepG2 in vitro. After 3 days of culture in the presence of FTS, a slight non-significant decrease in cell proliferation was observed compared with untreated cell cultures (results not shown). However, after 7 days of culture, FTS-treated cells had a marked reduction in overall cell density (Figure 1A), and a total cell count using a Burker cell confirmed a significant decrease in the number of cells/dish (Figure 1B). Trypan Blue staining of the cells showed viability of > 98 % of the recovered cells. Furthermore, a significant decrease in BrdU incorporation into the cells (Figure 1B) as well as a reduced expression of PCNA by Western blot (Figure 2) was found in the FTS-treated cultures compared with controls. These observations clearly show that FTS inhibits proliferation of tumour-derived hepatocytes in vitro.

FTS significantly reduces hepatocyte proliferation in vivo after PH
As FTS effectively inhibited hepatocyte proliferation in vitro, we next examined whether FTS was also able to inhibit hepatocyte proliferation in vivo. To study the in vivo effect of FTS on hepatocytes, the PH model was chosen as it induces a synchronous, vigorous and reproducible proliferation of hepatocytes in rats.

Injection regime of FTS and mortality
Considering death as the primary end point, we treated rats subjected to PH or not with up to three consecutive injections of 75 mg of FTS/kg of body weight at 1, 8 and 16 h. No mortality was observed in the non-hepatectomized rats (n = 6/group) receiving either FTS or DMSO (vehicle for FTS). By contrast, three injections of 75 mg of FTS/kg of body weight caused death within 24 h in 57 % of the rats subjected to PH (four out of seven
rats), but not in rats receiving the DMSO alone (n = 8). Therefore we decreased the dose of FTS to 50 mg/kg of body weight and repeated the experiment in partial-hepatectomized rats. At that time, three consecutive injections of FTS at 1, 8 and 16 h after PH did not induce any mortality in the animals (n = 8). These results suggest that, in our experimental conditions, only the association of three injections of 75 mg of FTS/kg of body weight with PH elicits death in rats.

PCNA expression
PCNA is widely used as a cell proliferation marker [28]. Therefore homogenates from DMSO- and FTS-treated rats, killed at 12, 24 and 48 h after PH, were subjected to Western blot analysis with a PCNA-specific antibody. Low PCNA expression was found in DMSO-treated animals at 12 h. PCNA peaked at 24 h and decreased at 48 h (Figure 3A). FTS treatment significantly reduced PCNA expression at 12 and 24 h after PH compared with the DMSO group. At 48 h, PCNA expression was similar in FTS- and DMSO-treated animals. However and in contrast with the DMSO group, no prominent peak of PCNA expression was observed during the first 48 h after PH in the FTS-treated animals (Figure 3B).

BrdU incorporation
To confirm the impact of FTS treatment on DNA synthesis, we determined the incorporation of BrdU into DNA of hepatocytes during S-phase of the cell cycle by immunohistochemistry and quantified this incorporation by FACS. BrdU incorporation was negligible at 12 h after PH. BrdU incorporation peaked in DMSO-treated animals at 24 h and decreased thereafter (Figure 4A). As with PCNA, FTS strongly reduced BrdU incorporation at 24 h after PH predominantly in hepatocytes (Figure 4) and returned thereafter to levels observed in DMSO-treated animals (Figure 4A). In parallel with PCNA expression, BrdU incorporation was also blunted in FTS-treated animals over the first 48 h after PH compared with their DMSO-treated littermates, which exhibited a clear peak at 24 h. Taken together, the results obtained for PCNA expression and BrdU incorporation strongly suggest that FTS impairs hepatocyte proliferation.

Liver-to-body mass index
The liver-to-body mass index reflects recovery of the liver mass proportional to the weight of the animal. In accordance with decreased cellular proliferation in FTS-treated animals at 24 h after PH, the liver-to-body mass index was significantly lower in FTS-treated rats compared with DMSO controls (1.5 ± 0.08 compared with 1.7 ± 0.03 % respectively; P < 0.001). The differences between both groups were attenuated with partial recovery of cellular proliferation at 48 h after PH (2.4 ± 0.01 compared with 2.5 ± 0.08 % in FTS- and DMSO-treated animals respectively).
BrdU incorporation into DNA in DMSO- and FTS-treated animals at various time points after PH

(A) Quantification of BrdU-positive hepatocytes by flow cytometry. (B) Representative immunohistochemical staining of BrdU-positive nuclei in hepatocytes 24 h after PH. BrdU incorporation into hepatocyte nuclei was hardly detectable 12 h after PH and was significantly decreased at 24 h in FTS-treated animals.

FTS dislodges Ras from the cell membrane and interferes with downstream signalling through the MAPK pathway

As FTS decreased cell proliferation, we next investigated whether this effect was indeed due to functional inhibition of the Ras protein.

Subcellular Ras localization

Cytosolic and membrane fractions from DMSO- and FTS-treated animals were analysed for Ras expression by Western blotting. During the first 48 h after PH, Ras expression was principally confined to the cell membranes in DMSO-treated animals (Figure 5). No Ras immunoreactive protein was detected at 12 h (results not shown), and only small amounts of Ras protein were found in the cytosol beyond 12 h after PH (Figure 5). By contrast, Ras membrane expression decreased progressively in FTS-treated animals, and substantial amounts of Ras protein were demonstrated in the cytosolic fractions from the 12 h time point onwards after PH (Figure 5). In addition, FTS did not affect Ras mRNA expression, which significantly and similarly increased in both groups after PH (results not shown).

Expression of membrane-bound phospho-Raf

Activation of the Ras oncoprotein recruits cytosolic Raf to the plasma membrane, where it becomes activated by phosphorylation [29]. Therefore we subjected membrane fractions from DMSO- and FTS-treated animals to Western blot analysis for phospho-Raf expression. Phospho-Raf was selectively detected at the membranes of DMSO- but not of FTS-treated animals at 24 h after PH (Figure 6A). No phospho-Raf expression was detected at any time during the first 48 h after PH in FTS-treated rats (results not shown), suggesting that FTS treatment interferes with activation of Raf by Ras.

Activation of the MAPK ERK1/2

The activation of Ras leads to downstream activation of the MAPK cascade, which includes the phosphorylation and activation of ERK1/2 [7,30]. Thus the presence of phospho-ERK1/2 might serve as a marker for Ras activation. Therefore we assessed the expression of phospho-ERK1/2 in homogenates of DMSO- and FTS-treated rats by Western blotting. Phospho-ERK1/2 was significantly decreased in FTS-treated animals during the first 48 h of the experiment (Figure 6B). The largest decrease was found during the first 24 h in the FTS-treated group. With interruption of the treatment 16 h after PH, phospho-ERK1/2 levels increased again, but did not reach the level of expression observed in the DMSO-treated animals (Figure 6C).

Taken together, these results confirm the functional inhibition of Ras by FTS and suggest that decreased cell proliferation might be mediated by inhibition of downstream signalling through the MAPK pathway.
Effect of FTS is connected to the cell cycle principally through decreased cyclin E/Cdk2 expression and activity

The cyclin D/Cdk4 and cyclin E/Cdk2 complexes constitute the principal factors involved in controlling the G0/G1 and the G1/S checkpoints of the cell cycle respectively. It has been proposed that activation of the Ras oncoprotein might directly influence both cell cycle checkpoints [31–33]. We therefore examined the expression of both components of these complexes in the nucleus as well as the activity of their kinase subunits.

Nuclear cyclin D and Cdk4 expression and activity

Cyclin D expression did not change significantly during the first 48 h after PH regardless of whether the animals were treated with DMSO or FTS (Figure 7A). Although, FTS decreased the expression of Cdk4 during the first 24 h after PH (Figures 7A and 7B), this decrease did not translate into a significant impact on the activity of this kinase (Figure 7C).

Nuclear cyclin E and Cdk2 expression and activity

FTS treatment decreased cyclin E expression during the first 48 h after PH compared with DMSO-treated animals (Figure 8A). FTS treatment also decreased Cdk2 expression during the first 24 h after PH, in contrast with DMSO-treated animals in which a steady and progressive increase in Cdk2 levels occurred throughout the experiment (Figures 8A and 8B). As a consequence of decreased cyclin E and Cdk2 levels, Cdk2 activity remained strikingly lower in FTS-treated animals during the first 48 h after PH (Figure 8C). Interestingly, Cdk2 activity at 48 h after PH, i.e. more than 24 h after the last dose of FTS, did not recover in the FTS-treated group, despite an increase in nuclear Cdk2 in both DMSO- and FTS-treated animals (Figure 8). These results suggest that FTS exerts an inhibitory effect on Cdk2 kinase activity even 24 h after drug withdrawal.

p27 and p21 mRNA expression

We next investigated whether decreased Cdk2 activity was related to increased expression of the Cdk inhibitors p27 and p21. No significant differences in p27 mRNA expression was found at any time point in FTS-treated animals compared with the DMSO-treated group (results not shown).

A strong peak of p21 mRNA was observed 24 h after PH and remained elevated until 48 h after PH in DMSO-treated animals. Compared with the kinetics of
DNA synthesis, the peak in p21 expression followed the up-regulation of Cdk2 activity as well as the first wave of DNA synthesis in DMSO-treated animals. By contrast, p21 levels were significantly higher in FTS-treated animals at 12 h after PH, preceding the increase in Cdk2 activity observed in DMSO-treated animals as well as the first wave of DNA synthesis, and remained elevated until 48 h after PH (Figure 9). In parallel with the strong decrease in DNA synthesis in the FTS-treated group, peak levels of p21 expression were also lower in FTS-treated animals compared with the DMSO-treated group. These results suggest that p21 might contribute to down-regulate Cdk2 activity and subsequently DNA synthesis in FTS-treated animals.

**DISCUSSION**

The Ras oncoprotein is thought to play an important role in cell proliferation mechanisms, including those of hepatocytes. The aim of the present study was to examine whether interfering with the activation of the mature Ras oncoprotein would affect liver cell proliferation, an approach that could be exploited in liver carcinogenesis. Therefore we studied the effect of FTS on HepG2 cells, a human hepatoblastoma-derived cell line, *in vitro* and on normal hepatocytes *in vivo* after PH in rats. FTS has been shown to inhibit the activation of the Ras oncoprotein *in vitro* [18–21]. This inhibiting effect is either due to
inhibition of Ras farnesylation, which is essential for its membrane anchorage [16], or results from binding of FTS to the specific Ras membrane-binding site acting as a real competitive inhibitor of Ras membrane anchorage [20]. Our present results clearly show that FTS inhibits proliferation of the hepatic tumour cell line HepG2 \textit{in vitro}, which suggests that the treatment acts on transformed hepatocytes. Similarly, it has been shown that inhibition of Ras by FTS induces inhibition of the proliferation of human tumour cell Panc-1 \textit{in vitro} and \textit{in vivo} [17,22]. Low dose chronic administration of FTS to rats also inhibited stellate cell proliferation, thereby preventing the occurrence of cirrhosis [23]. When FTS was administered to rats during the first 24 h after PH, a delay in recovery of the liver mass as well as a highly significant decrease in hepatocyte proliferation was also observed, which was confirmed by two independent proliferation markers, PCNA expression and BrdU incorporation. Although cell proliferation in FTS-treated rats remained similar to levels observed in the DMSO-treated group at 48 h after PH, a prominent peak of PCNA expression and BrdU incorporation that usually occurs early after PH was not seen in FTS-treated animals. This observation suggests that a delay in hepatocyte proliferation persisted at 48 h after PH and this despite withdrawal of the Ras inhibitor at 16 h after PH. Interestingly, incomplete recovery of cell proliferation at 48 h after PH occurred in spite of an increase in cell cycle effector proteins such as cyclin D, Cdk4 and Cdk2 to levels observed in untreated animals. However, neither phospho-ERK 1/2 levels nor Cdk2 kinase activity fully recovered after FTS withdrawal, which might, at least in part, explain the prolonged effect of FTS on cell proliferation. In addition, the Cdk inhibitor p21 increased before initiation of DNA synthesis in FTS-treated animals and remained elevated until 48 h after PH. These findings suggest that the block in G1- S-phase transition and the prolonged down-regulation of Cdk2 activity could be related to persistently elevated p21 levels in FTS-treated animals. As later time points have not been examined in our present study, we cannot exclude that the cells might finally escape from the block leading to complete liver regeneration in the long term. Recent findings confirm that FTS acts in a specific manner on the active GTP-bound forms of Ras proteins. It principally competes with Ras-GTP for binding to specific binding sites in the plasma membrane preventing active Ras from activating intracellular downstream signalling pathways [34]. Although FTS might have some effects on the farnesylation of Ras through interaction with PPMTase, the physiological impact of these interactions is rather minor. Therefore it is unlikely that effects on other farnesylated molecules besides Ras account for inhibition of cellular proliferation in our present study. The Ras/ERK pathway is primarily involved in cell proliferation in the regenerating liver; however, we cannot exclude that other Ras-dependent downstream signalling pathways, e.g. the Akt or SAPK (stress-activated protein kinase) pathway, might have influenced the proliferative response elicited by PH to some extent.

As shown previously by our group [35], Cdk2 kinase activity is essential for transition from G1- to S-phase and inhibition or reduction in its activity blocked hepatocytes in late G1-phase. It has been shown that Ras expression increases at a critical time point in the regenerating rat liver [10,36]. In addition, interference with Ras gene expression also reduced liver regeneration, presumably through reduction of cyclin E/Cdk2 expression [13]. Our results of strongly reduced liver cell proliferation using the Cdk2 inhibitor roscovitine [35] and the Ras inhibitor FTS in the present study adds further evidence for a critical role of the Ras–cyclin E/Cdk2 axis in hepatocyte proliferation. It has been suggested that Ras signalling via the MAPK pathway may be important in this process. The observations in FTS-treated animals in our present study maintain an important role for the Ras/Raf/MAPK pathway in liver cell proliferation. Reduced Ras membrane expression, increased cytosolic Ras retention and decreased activation of Raf and ERK1/2 in FTS-treated animals suggest that the antiproliferative effect of FTS is at least partially mediated by inhibition of the Ras/Raf/MAPK pathway. How this pathway is connected to the cell cycle still remains poorly understood. It has been shown that the Ras protein activates the transcription of the cyclin D1 gene [33]. Our present results are not in favour of a major effect on this cell cycle effector, since cyclin D1 nuclear protein levels did not change significantly after administration of the Ras inhibitor FTS. In addition, the activity of its preferred kinase partner, Cdk4, was also not affected in the nucleus by the treatment, making it unlikely that
the inhibitory effect is a result of decreased activation of the cyclin D/Cdk4 complex. By contrast, FTS treatment decreased cyclin E and Cdk2 nuclear expression with a prolonged inhibitory effect on Cdk2 kinase activity in the nucleus. These results suggest that the Ras/Raf/MAPK pathway principally modulates the activity of the cyclin E/Cdk2 complex in our experimental conditions. A similar observation has been made by Lüdde et al. [13], who showed that Ras activation, in vivo, has a direct impact on the hepatocyte proliferation by modulating the activity of the cyclin E/Cdk2 complex.

The PH model has been chosen as it allows vigorous and synchronous hepatocyte proliferation to be studied under strictly controlled and reproducible conditions. The observation that the inhibitory effect of FTS on cell proliferation extended beyond drug withdrawal in this model is of particular interest from an oncology point of view. Tumour cells have less vigorous non-synchronized proliferation patterns over a longer time period. Given the prolonged effect of FTS, these cells might therefore remain susceptible to the antiproliferative effect of the compound. With our demonstration that FTS does inhibit the proliferation of hepatic tumour cells in vitro as well as proliferation of normal hepatocytes in vivo, it is tempting to speculate that FTS might have antiproliferative effects in hepatocellular carcinoma in vivo. Indeed, the Ras/Raf/MAPK signalling pathway appears to be important in liver tumour development, and Ras mutations or activation of the MAPK pathway have been demonstrated in hepatocellular carcinoma [14,15].

Taken together, our present results show that interfering with the activation of the Ras oncoprotein in hepatic tumour cells in vitro as well as in normal hepatocytes in vivo that have acquired a high proliferative competence dramatically reduces their ability to proliferate. This effect lasted for at least 48 h after PH, even though the inhibitor was withdrawn 16 h after PH. Mutations of Ras appear to be involved in the development of many cancers, including those in the liver [1,2]. Having shown in the present study that the Ras inhibitor FTS is capable of inhibiting normal and malignant hepatocyte proliferation with minimal toxic manifestations, it appears worthwhile to test the preventive and/or therapeutic effects of this compound in suitable animal models of hepatocellular carcinomas. Such an approach may potentially lead to the development of new therapeutic strategies in humans.

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