Pro-inflammatory cytokines TNFα and IL6, and survival factor EGF positively regulate the mGSTA4 enzyme in hepatocytes.

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Running title : TNFα, IL6 and EGF up-regulate mGSTA4

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Summary

We hypothesized that glutathione transferases could be induced and may participate to cellular defenses against the oxidative stress occurring during liver regeneration. Here, we evidenced that mGSTA1, A4, Pi and Mu are up-regulated during mouse liver regeneration, exhibiting a biphasic pattern of induction correlating early G1 phase and G1/S transition of the cell cycle. Using confocal microscopy immunolocalization and subcellular fractionation, mGSTA4 was demonstrated in both mitochondria and cytosol and found preferentially increased in cytosol during liver regeneration. In addition, mGSTA4 was induced in vivo and in cultured hepatocytes by TNFα, IL6 and EGF, factors which play crucial roles in hepatocyte survival and proliferation during liver regeneration. However, the mitogenic effect of EGF was not responsible for the induction of mGSTA4. In transient transfections, IL6 and EGF, but not TNFα, transactivated hGSTA4 promoter cloned upstream the luciferase reporter gene suggesting that IL6 and EGF up-regulated hGSTA4 at a transcriptional level whereas TNFα could rather act at a post-transcriptional level. The inhibition of PI3K, p38MAPK and MEK/ERK signaling pathways, using specific inhibitors, prevented EGF-dependent induction of mGSTA4 and transactivation of hGSTA4 promoter. Altogether, these data favor the conclusion that, in regenerating hepatocytes, several GST isoforms are induced and that cytokines TNFα, IL6 and survival factor EGF positively regulate mGSTA4 via survival signaling pathways.
INTRODUCTION

Quiescent differentiated hepatocytes are able to re-enter the cell cycle and proliferate to restore the liver mass following liver deficits resulting from surgical removal or caused by chemicals and viruses. Entry into and progression through early G1 phase of the cell cycle, also called priming (1), are induced by the cytokines Tumor Necrosis Factor alpha (TNFα) \(^1\) (2,3) and Interleukin 6 (IL6) (4) and required for the hepatocytes to fully respond to growth factors (3). TNFα binding to its type 1 receptor (TNFRI) successively activates NFκB (5), IL6 expression and STAT3 (6,7) in the early G1 phase, which constitute a key signaling pathway during hepatocyte proliferation (4,8). The late G1 progression and commitment to DNA replication are controlled by growth factors (9-11) including Hepatocyte Growth Factor (HGF), Transforming Growth Factor alpha (TGFα) and Epidermal Growth Factor (EGF) (1) through the activation of the Mitogen Activating Protein Kinase Kinase (MEK)/Extracellular Regulating Protein (ERK) pathway (11).

Several lines of evidence indicate that partial hepatectomy (PH) is rapidly followed by an oxidative stress due to increased Reactive Oxygen Species (ROS) and nitric oxide (NO) production leading to lipid peroxidation (12-14).

With respect to ROS, TNFα is now recognized to play a crucial role in the production of these species during liver regeneration. In support to such a role, it is worth noting that the multimerization of TNFα type I receptor (TNFRI) following binding of TNFα has indeed been shown to lead to recruitment of TRAF (TNFR associated protein), a protein involved in signaling pathway regulating ROS production in mitochondria (15), probably through the inhibition of the complex III of the electron transport chain (16).

Both ROS and NO have been demonstrated to contribute significantly to induce hepatocyte proliferation. Indeed, in transgenic mice with targeted disruption of TNFRI genes, or of the type II NO synthase (iNOS), the enzyme that catalyzes the formation of NO from arginine,
TNFα, IL6 and EGF up-regulate GSTA4.

hepatocyte proliferation after PH is strongly impaired (2,12). In this context, it then appears that the TNFα–related oxidative stress functions as a signaling pathway rather than eliciting deleterious effects. Such a function is mediated through the activation of redox-sensitive proteins especially the transcription factor NFκB (17), which on one hand allows the occurrence of proliferation by transactivating cell cycle genes such as c-fos and c-jun controlling the G0/G1 transition, and on the other hand, attenuates deleterious responses resulting from oxidative stress (e.g. activation of caspases by trans-activating the anti-apoptotic gene Bcl-XL that prevents activation of caspases (18)).

NFκB also induces the mitochondrial uncoupling protein UCP-2 (13) and iNOS (19) that both contribute to reduce oxidative stress (1). UCP2, an inner mitochondrial channel for protons, plays a major role in limiting production of ROS (13) by dissipating the electrochemical gradient. The NO, whose production is enhanced by induction of iNOS, though participating to lipid peroxidation (14), also exhibits a cytoprotective effect by preventing apoptosis through S-nitrosylation of caspases that strongly inhibits caspase activities (20). This last mechanism is most likely involved in liver regeneration since in iNOS knock-out mice, PH is followed by a strong increased caspase 3 activity and hepatocyte death (12). Along with the TNFα-dependent activation of anti-oxidant defenses, it is worth noting that the cytokine IL6 and growth factors have also been shown to favor hepatocyte survival during liver regeneration, through stimulation expression of anti-apoptotic gene products (21, 22).

In normal hepatocytes, excess of ROS is also neutralized through the action of thiols, especially glutathione (GSH) of which content is increased during liver regeneration (21). In addition, glutathione transferases (GST) of the alpha class detoxify organic hydroperoxides and protect cells against oxidative stress (22). For instance, transfection of hGSTA2 in K562 cells results in cell protection towards H2O2-induced lipid peroxidation (23). The GSTA4 enzyme, the alpha subunit that exhibit the highest activity against 4-hydroxynonenal (4-HNE) (24,25), also efficiently protects against oxidative damage mediated by this cytotoxic product of lipid.
peroxidation generated by ROS overproduction (26). In addition, we recently demonstrated that ROS overproduction induced by hepatic iron overload was correlated with an increase in mGSTA4 expression (27).

These observations suggest that GSTs enzymes could participate to defenses against oxidative stress during liver regeneration. However, to date, little is known about regulation of GST expression and activity during liver regeneration. Lee and Boyer (28) have reported a decrease in mRNA levels of several GSTs 12 h post-partial-hepatectomy (PH), while Mori et al. (29) have shown a higher expression of GSTPi after 2 and 3 days post-PH. To our knowledge, no study has attempted to correlate expression of GSTs with the oxidative stress occurring during the first hours following PH.

The aim of this paper was first to study the expression and activities of several GST subunits, including the mGSTA4 isoform, during the liver regeneration after two-third hepatectomy in mouse. Then, we investigated whether the oxidative stress, the pro-inflammatory cytokines TNFα and IL6 and the growth/survival factor EGF, involved in proliferation and survival of hepatocytes during liver regeneration, may regulate the expression of mGSTA4 both in vivo and in hepatocytes in primary culture.
EXPERIMENTAL PROCEDURES

Antibodies

The rabbit polyclonal antibody against a mGSTA4 peptide was characterized in our laboratory (30). The other antibodies were purchased: rabbit polyclonal anti-human GSTA1, GSTMu and GSTPi antisera (Biotrin, Dublin, Ireland), rabbit polyclonal anti-cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-cytochrome c oxidase (Molecular Probes, Eugene, OR), monoclonal anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204) and polyclonal anti-p44/42 MAP Kinase (Cell Signaling Technology, Beverly, MA), anti-phosphorylated-AKT (Ser 473) (New England Biolabs, Beverly, MA) and anti-albumin (ICN Pharmaceuticals, Orsay, France) antibodies; horseradish peroxidase (HRP)-linked rabbit antiserum (Biorad, Ivry sur Seine, France); mouse and goat anti-rabbit rhodamine conjugated IgG (Santa Cruz Biotechnology).

Animal experiments

Balb/c male mice (8 weeks old, Janvier laboratories, Le Genest, France) were subjected to 70% partial hepatectomy under ether anesthesia and were killed at various times post-PH. As controls, animals underwent sham-operation consisting in laparotomy without tissue resection. L-NIL (L-N6-1-Iminoethyl-lysine dihydrochloride; Calbiochem, Nottingham, UK) was injected intraperitoneally at the dose of 40 µg/g of body weight in normal and hepatectomized animals 1 h before the surgery. Animals were killed 2 h after NIL-injection. TNFα, IL6 (Promocell, Heidelberg, Germany) and EGF (Promega, Saint Quentin Fallavier, France) were given intraperitoneally in 0.2 ml of sterile pyrogene-free saline (0.9% NaCl) with 0.1% of BSA at the doses of 40 ng/g, 80 ng/g and 125 ng/g of body weight, respectively. Control animals received the corresponding sterile saline vehicle. Animals were killed 2 h after TNFα and IL6 injection and 6 or 24 h after EGF injection.
**Isolation and culture of hepatocytes**

Mouse hepatocytes were isolated by the two-step perfusion procedure using 0.025 % collagenase D (Roche Diagnostic, Meylan, France) as previously described (31). Isolated hepatocytes were resuspended in Williams’ medium E (Gibco-BRL, Paisley, Scotland) containing penicillin G / streptomycin (100 IU/ml), 0.2 mg/ml serum bovine albumin, 5 µg/ml bovine insulin, 10 % fetal calf serum (FCS; Gibco-BRL), plated at a density of 6.10^4 / cm² and maintained at 37°C under atmosphere of 5% CO₂/95% air. EGF was added at 50 ng/ml and TNFα and IL6 at 20 ng/ml, 24 h after cell seeding. Specific inhibitors Ly 294002, SB 203580 (Calbiochem) and U0126 (Promega) of PI3K, p38 MAPK and MEK, respectively, were added one hour prior to EGF stimulation at the final concentrations of 15 µM, 12µM and 50 µM, respectively.

**RNA extraction and Northern blot analysis**

Total RNA from cells or liver biopsies were isolated using SVRNA extraction Kit (Qiagen, Valencia, CA). Ten µg total RNA from each sample were used for Northern blot analysis. Blots were hybridized with the corresponding ^32^P-labeled cDNA probe (][α-^32^P]dCTP, 3000Ci/mmol, Amersham, UK) at 65°C overnight. Blots were washed at moderate stringency and exposed to radiograph films. The 18s ribosomal probe was used as control.

**Protein extraction and Western blot analysis**

Cultured hepatocytes and liver biopsies were homogenized in lysis buffer (HEPES pH 7.5 50 mM; NaCl 150 mM; EDTA 1 mM; EGTA 2.5 mM; Tween-20 0.1 %, glycerol 10 %, β-glycerophosphate 10 mM, sodium fluoride 1 mM, sodium orthovanadate 0.1 mM, PMSF 0.1 mM, leupeptin 10 µg/ml and aprotinin 10 µg/ml). CDK1 was purified from liver extracts using p9CKS^ts1 beads and recovered with sample buffer as described previously (9). Protein concentrations were determined using the Bradford’s method. Proteins were fractionated by SDS-PAGE (12.5 %) and transferred to a PVDF membrane further incubated for 2 h in PBS containing 3 % BSA, then overnight with primary antibody. Membranes were washed twice
before incubation with secondary antibody for 1 h. Proteins of interest were visualized using chemiluminescence reagent (ECL, Interchim).

**Determination of intracellular MDA and 4-HNE levels**

MDA and 4-HNE levels were determined using lipid peroxidation assay kits (Calbiochem, UK). Liver biopsies were washed in ice-cold 0.9 % NaCl and sonicated in 20 mM Tris-HCl, pH 7.4, to approximately 10 % (w/v). Liver extracts were centrifuged at 3000g for 10 min at 4°C and the supernatant was collected prior to determination of total protein concentration and the MDA and 4-HNE colorimetric assay.

**Mitochondrion isolation**

Liver mitochondria were isolated from freshly harvested livers by differential centrifugations in ice-cold H medium containing 210 mM mannitol, 70 mM sucrose and 2 mM HEPES buffer (pH 7.4) (32). The purified pellets were suspended in 200µl of buffer containing 150 mM KCl, 0.5 mM malonate, 0.1 mM oxoglutarate and 10 mM HEPES.

**GST activity assays**

GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) were determined by spectrometry analysis (33,34) on total proteins. GST activity toward 4-hydroxynonenal (4-HNE) was quantified using an HPLC method as previously described (27).

**Immunohistochemistry**

Mice were anesthetized and perfused through the portal vein with 4 % paraformaldehyde in 0.1 M sodium cacodylate for 15 min at a flow rate of 10 ml/min. Tissue fragments were washed in 0.1 M PBS for 4 h, in 10% glycerol-PBS overnight and frozen in liquid nitrogen-cooled isopentane. Frozen tissue sections were mounted on glass slides coated with 10% of gelatin in PBS and incubated in PBS containing 3 % BSA for 30 min. They were then covered with a solution of anti-rabbit GSTA4 (1/100) or anti-rabbit cytochrome c (1/100) antiserum for 1 h at room temperature. Sections were washed with PBS, incubated for 2 h with goat anti-rabbit IgG conjugated to rhodamine (1/200) washed and mounted. Serial z-axis optical analysis of
sections was done at 1 µm intervals using a laser scanning confocal microscope (Confocal Leica TCS NT).

$[^3]H$-thymidine incorporation

The rate of DNA synthesis was measured by incubating cultured hepatocytes with [methyl-$^3H$]-thymidine (5Ci/mmol, Amersham, UK) at 2µCi per 35 mm Petri dish for 24 h. Cells were sonicated in PBS and [methyl-$^3H$]-thymidine incorporation was measured after DNA precipitation in 15% trichloroacetic acid.

Reporter gene constructs and transient transfection in primary cultured mouse hepatocytes

Two DNA fragments corresponding to the 5'-flanking region of the hGSTA4 gene (1571 and 165 pb upstream exon 1) previously characterized (35) were cloned into the pGL3 basic vector (Promega) upstream the firefly luciferase reporter gene. The pRL-CMV vector encoding Renilla luciferase, and PGL3-GSTA4(-1571) or PGL3-GSTA4(-165) were co-transfected in 24 h cultured hepatocytes using cationic lipids according to Gilot et al (36). Dual luciferase assays (firefly and Renilla) were done using a Promega Kit. PGL3 basic (promoterless pGL3-luciferase construct) and PGL3 promoter (pGL3-luciferase construct with the SV40 promoter) vectors were transfected providing negative and positive firefly luciferase controls.

Statistical analysis.

Values were expressed as the mean ± SD. The Student's t test was used for the estimation of statistical significance. A p-value less than 0.05 was considered statistically significant.
RESULTS

Induction of several GST isoforms during liver regeneration

The expression of the different GST isoforms was analysed during liver regeneration, over a 96 h period after two/third hepatectomy (PH) of Balb/c mice. To ensure that liver regeneration occurred as previously described, the relative mRNA levels of the two cell cycle genes, cyclin D1 and CDK1, were analyzed. A clear induction of cyclin D1 transcripts was observed between 30 and 96 h while the level of CDK1 mRNA was increased between 48 and 96 h post-PH with a maximal expression at 72 h (Figure 1A) as previously reported (37).

Levels of mGSTA4, A1 and Pi mRNAs were augmented during liver regeneration exhibiting a biphasic induction of expression (Figure 1B). A first increase was observed within one hour post-PH and mRNA levels remained high for 12 to 24 h. The second peak of induction took place at 40-48 h and the increase in transcript levels was observed until 72 to 96 h. In sham-operated mice, mGSTA4, A1 and Pi mRNA levels showed a transient and moderate increase between 1 and 8 h post-PH but not thereafter. mGSTMu mRNAs were detected at very low levels and no significant change was observed during the first 96 h of regeneration.

mGSTA4, A1, Pi and Mu proteins were further investigated by Western blot (Figure 2A). Expression of mGSTA4, A1, Pi and Mu proteins exhibited a biphasic pattern as observed for mRNAs. The first induction occurred between 0.5 and 4 h after PH, mGSTA1 being very transiently induced at 0.5 h while up-regulation of mGSTA4, Mu and Pi remained elevated between 0.5 and 4 h. The second induction of the four GST proteins took place between 24 and 72 h depending upon the GST isoforms. In sham-operated mice, a slight increase of mGSTA4, A1, Mu and Pi proteins was also detected at 1 and 12 h post-PH, as observed with the corresponding mRNAs, which returned to control values thereafter, except for mGSTPi that remained elevated until 48 h.
To confirm the induction of several GSTs, during the first hours post-PH, we measured GST activities using three different substrates: CDNB, a common substrate for mGSTA4, A1, Mu and Pi, 4-HNE and EA, specific substrates for GSTA4 and GSTPi respectively (22). GST activities, measured with CDNB, 4-HNE and EA, were all significantly increased between 0.5 and 2 h after PH compared to sham-operated animals. The induction of GST expression observed between 24 and 72 h was also correlated with a strong induction of GST activities using CDNB as a substrate (Figure 2B).

*mGSTA4 is located in the cytosol and mitochondria of hepatocytes*

The subcellular localization of mGSTA4 was analyzed in normal mouse liver using the indirect immuno-fluorescence technique and confocal microscopy (Figure 3A). A punctuated staining typical of a mitochondrial distribution was observed for cytochrome c while mGSTA4 exhibited both a punctuated and an intense homogeneous cytosolic staining.

To confirm these results, mGSTA4 protein expression was analyzed by Western blot in the cytosol and mitochondria of normal or regenerating livers after cell fractionation. The reliability of the cell fractionation was demonstrated in cell extracts from normal and 24 h post-PH regenerating livers, by performing Western blots of the mitochondrial cytochrome c and cytochrome oxidase proteins which were mainly detectable in mitochondrial fractions while albumin was observed only in cytosols (Figure 3B).

By western blot, the mGSTA4 protein was detectable in both mitochondrial and cytosolic fractions from normal liver, regenerating livers 1 and 24 h post-PH and sham-operated livers 24 h post-laparotomy (Figure 3C). The signal obtained in cytosols was much higher than in mitochondria and was increased at 1 and 24 h post-PH, compared to normal and sham-operated animals. No change in mGSTA4 level was found in mitochondrial extracts during liver regeneration, even after a longer exposure time of the blots (data not shown).
TNFα, IL6 and EGF, but not lipid peroxidation, induce mGSTA4 expression in vivo

During the first hours post-PH, ROS are produced in mitochondria (13) while nitric oxide (NO) is released. These compounds contribute to the enhanced lipid peroxidation (14).

To determine whether 4-HNE and MDA, two metabolites known to induce GSTA4 (38), could be responsible for the early induction of mGSTA4 following PH, the levels of 4-HNE and MDA, measured in 1 h regenerating livers, were increased by a 3-fold factor (p<0.001) when compared to normal (Figure 4A) or sham-operated animals (data not shown). We also measured lipid peroxidation by analyzing 4-HNE/MDA levels and mGSTA4 expression by Western blot in mice injected with the selective inhibitor of iNOS, L-N6-(1-Iminoethyl)lysine (NIL) which blocked the rise of NO production and lipid peroxidation in heparatectomized liver (14). The level of lipid peroxidation was strongly decreased in both non heparatectomized (25-fold; p<0.01) and one hour regenerating (3.9-fold; p<0.001) livers of NIL-treated mice when compared to normal and heparatectomized animals which have not been injected with NIL, respectively (Figure 4A). The levels of mGSTA4 were not affected by NIL treatment in regenerating liver while, in normal liver, injection of NIL increased mGSTA4 expression (Figure 4B). These results therefore favor the conclusion that the increase in 4-HNE and MDA content after PH did not trigger mGSTA4 induction.

Thus, we postulated that the cell cycle priming factors, TNFα and IL6, produced within 15 min post-PH and survival/growth factor EGF which cooperate with TNFα and IL6 to induce progression through the cell cycle and favor survival of hepatocytes (1), could be involved in this induction. TNFα and IL6 injections to normal mice induced expression of mGSTA4 but not that of mGSTA1 used as control, compared to mice injected with the vehicle only (Figure 4C).

In normal livers at 6 and 24 h after EGF administration, expression of mGSTA4 was significantly induced compared to the levels of mGSTA4 in livers of mice injected with the vehicle and non injected animals (Figure 4D). To determine whether EGF also induced
proliferation of liver cells, expression of CDK1 protein, a cell cycle marker of S, G2 and M phases (9,10), was studied and compared to its expression in regenerating livers at 40 and 72 h post-PH. In livers of control and EGF-treated mice, CDK1 was not detected while its expression was strongly induced in regenerating livers. These results indicated that TNFα, IL6 and EGF up-regulated mGSTA4 expression in normal liver.

**TNFα, IL6 and EGF induce mGSTA4 expression in primary cultures of mouse hepatocytes**

Primary cultures of mouse hepatocytes were used to confirm the up-regulation of mGSTA4 by TNFα, IL6 and EGF and analyze the level of regulation.

Addition of TNFα and IL6 to the culture medium at 24 h, led to induction of the mGSTA4 protein, while the levels of mGSTA1 and albumin, used as controls, were unaffected (Figure 5A). Stimulation by EGF at 4 or 24 h after plating transiently induced mGSTA4 at 24 and 48 h, respectively, whereas the level of mGSTA1 was not increased after EGF stimulation (Figure 5B). To verify if stimulation by these factors resulted in an increase in mGSTA4 mRNA levels, a Northern blot analysis using mGSTA4 cDNA was performed on EGF-, TNFα - and IL6-treated hepatocytes. Induction of mGSTA4 mRNAs by EGF, TNFα and IL6 was confirmed (figure 5C).

In order to determine whether the induction of mGSTA4 by these soluble factors occurred at the transcriptional level, transfection experiments were performed using the human GSTA4 promoter, that we previously isolated and characterized (35). A significant increase in luciferase activities was observed after stimulation with EGF and IL6, but not with TNFα, when cells were transfected with the PGL3-GSTA4(-1571) plasmid carrying 1571 base pairs of the hGSTA4 promoter upstream the luciferase reporter gene (Figure 5D), indicating that induction of hGSTA4 by EGF and IL6 was most likely due to a transcriptional activation of the GSTA4 gene.

**Proliferating and/or survival pathways are involved in the induction of mGSTA4 by EGF**
TNFα, IL6 and EGF up-regulate GSTA4

To confirm that proliferating and/or survival pathways were involved in the induction of mGSTA4 expression by EGF, we used inhibitors of different signaling pathways, namely Ly 294002, SB 203580 and U0126 which inhibit PI3K, p38MAPK and MEK, respectively, and analyzed mGSTA4 expression by Western blot.

Treatments of EGF-stimulated hepatocytes by Ly 294002, SB 203580 and U0126 led to a strong decrease in mGSTA4 protein expression while GSTA1 was not modified (Figure 6A). Since interferences between PI3K and MEK/ERK pathways has been evidenced in many cell types, the effects of these inhibitors on the phosphorylation of AKT (AKT-P) and ERK1/2 (ERK-P), respective substrates of PI3K and MEK, were investigated by Western blot (Figure 6A). Ly 294002 was found to strongly diminish AKT-P and ERK-P levels while U0126 almost completely abolished ERK-P and slightly decreased AKT-P. SB 203580 affected neither AKT-P nor ERK-P. In addition, these three molecules strongly inhibited DNA replication in EGF-stimulated hepatocytes (Figure 6B).

The ability of these inhibitors to down regulate hGSTA4 transcriptional activity by transient transfection of PGL3-GSTA4(-1571) plasmid was investigated. Four hours after transfection, hepatocytes were treated with SB 203580, Ly 294002 and U0126 in absence or presence of EGF, during 24 h. As expected, luciferase activity was enhanced in presence of EGF. SB 203580 slightly decreased luciferase activities in both non-stimulated and EGF-stimulated cells while treatments by Ly 294002 and U0126 strongly down-regulated reporter gene activity in both non stimulated and EGF stimulated cultures (Figure 6C).
DISCUSSION

Liver regeneration following partial hepatectomy is associated with an overproduction of ROS that probably play a critical role in the induction of hepatocyte proliferation (1). Several mechanisms of defense are activated to neutralize this ROS excess (12,13). Among the protective defense systems in hepatocytes, GSTs, and particularly GSTA4, are recognized to play an important role in the elimination of lipoperoxidation products in various physiopathological situations (39,40).

Only a few studies have dealt with the regulation of GSTs during liver cell growth without detailed kinetics of GST expression during the first 3 days post-PH (28,29). Our results clearly demonstrated for the first time that several GSTs belonging to distinct classes, in particular mGSTA4, exhibited a biphasic pattern of induction concomitant with the two critical steps of liver regeneration, i.e. the entry into the cell cycle, the so-called priming, and the commitment to DNA synthesis, controlled by the pro-inflammatory cytokines TNFα and IL6 and growth factors, respectively (1).

An oxidative stress, demonstrated by H₂O₂ production in mitochondria (13), and increased levels of MDA and 4-HNE products in cytoplasm detected in this study, is observed in early G1 phase during liver regeneration. We postulated that this increased in lipid peroxidation products was associated with an increase in mitochondrial and/or cytosolic mGSTA4 content. Several recent studies have dealt with immunolocalization of GSTA4 in hepatocytes and led to contradictory results. This enzyme was reported to be located either in both mitochondria and cytosol (41), only in mitochondria (42) or predominantly at or near the plasma membrane (43). Our observations based on both immunofluorescence confocal microscopy and subcellular fractionation support a distribution of mGSTA4 in both mitochondria and cytosol and a preferential increase of mGSTA4 content in the cytosol of regenerating hepatocytes. The mitochondrial and cytosolic localization of mGSTA4 is compatible with the formation of ROS in
mitochondria and lipid peroxidation-derived aldehydes in membranes that diffuse within the cell and attack targets far from the site of their original production.

The induction of mGSTA4 during the first h post-PH could result from an oxidative stress, since 4-HNE is known to be substrate and inducer of this GST isoform (25,38). However, our results show that, in both normal and regenerating liver, NIL, a specific inhibitor of iNOS, diminished the levels of 4-HNE and MDA while mGSTA4 protein content was unchanged or augmented in regenerating and normal liver, respectively. These findings favored the conclusion that the expression of mGSTA4 was not strictly correlated to the levels of lipid peroxidation and more precisely to the contents of 4-HNE and/or MDA. Nevertheless, it cannot be totally excluded that free radicals rather than lipoperoxidation products, may contribute to the up-regulation of mGSTA4 during liver regeneration.

In the regenerating liver, induction, through NFkB activation (17), of cellular defenses involved in the neutralization of the excess of ROS and lipid peroxidation products, such as the mitochondrial UCP-2 protein (13), iNOS (14) and anti-apoptotic proteins Bcl-XL and Akt (44,45), are dependent upon stimulation by the pro-inflammatory cytokines TNFα and IL6, and the growth/survival factors TGFα, HGF and EGF. Here, we show that injection of TNFα, IL6 and EGF to normal mice resulted in an induction of mGSTA4 while mGSTA1 remained unchanged. The biphasic induction of mGSTA4 during the first hours after PH could result from the overproduction of the pro-inflammatory cytokines TNFα and IL6 involved in the cell cycle priming and activation of survival pathways by growth factors such as EGF. This hypothesis is strongly reinforced by our in vitro data, demonstrating that mGSTA4 expression was also increased after stimulation by TNFα, IL6 and EGF. The second peak of induction of mGSTA4 occurring between 24 and 48 h post-PH could be due to the increase in growth factors in plasma that promotes the G1/S transition (11). However, our data indicated that mGSTA4 induction by EGF is not necessarily correlated to progression in late G1 or S phases. Indeed, in vivo injection of EGF induced mGSTA4 while hepatocytes did not proliferate, as previously reported (1) and
confirmed in this study by the absence of CDK1 expression, a cell cycle marker of S, G2 and M phases (9).

Previous studies have demonstrated modulation of GST expression by cytokines and growth factors in the liver. Increase in hGSTA1 and A2 by IL4 in cultured human hepatocytes (46) and marked decrease in rGSTA2 and M1 mRNA levels by IL1β in rat hepatocytes (47) have been evidenced. Moreover, rGSTP1 expression is strongly induced by EGF in cultured rat hepatocytes (48). However, this is the first time, to our knowledge, that induction of several GSTs was evidenced during liver regeneration and that a correlation was established between this induction and the soluble factors essential for hepatocyte survival and proliferation.

Our results also suggested that IL6 and EGF positively regulate hGSTA4 expression at the transcriptional level, whereas TNFα could rather act at a post-transcriptional level as previously shown for IL1β on rGSTA2 and M1 regulation (47) in rat hepatocytes. Another hypothesis can be proposed to explain why TNFα did not induce luciferase activity after transfection with the 1.5 kb hGSTA4 sequence promoter. Indeed, in a previous study, we found several putative binding sites for AP1, Sp1, STAT as well as NFκB within the 1.5 kb sequence upstream the transcription start site of the hGSTA4 gene (35). More recently, we localized several other putative binding sites for transcriptional factors, including NFκB, AP1 and CREB, within the 0.5 kb upstream the promoter region used for our transfection assays (data not shown). Therefore, we cannot rule out that TNFα could activate hGSTA4 transcription via the putative NFκB binding site located upstream the 1.5 kb promoter sequence.

The p42/44ERK1/2 (11), p38MAPK (49) and PI3K (44) signaling pathways, activated during liver regeneration, are essential for both proliferation and survival of hepatocytes during the hepatic regenerative process. Recently, several reports have indicated that MAP Kinase pathways are involved in the regulation of GST enzymes. Indeed, Kang et al, (50) have demonstrated that the activation of p38MAPK and PI3K during the oxidative stress leads to the
induction of rGSTA2. In addition, Yin et al (51) have demonstrated that GSTPi had protective effects against H₂O₂-mediated cell death via activation of p38MAPK, ERK and NFκB and repression of JNK signaling pathways. Here, we showed that the use of specific inhibitors Ly 294002, U0126 and SB 203580 of the protein kinases PI3K, MEK and p38MAPK, respectively, prevented the induction of mGSTA4 expression and the transcriptional activation of the hGSTA4 promoter-luciferase construct by EGF.

Altogether, these data demonstrate the induction of several GST isoforms, including mGSTA1, A4, Mu and Pi, during liver regeneration. They also strongly suggest that pro-inflammatory cytokines, TNFα and IL6, and growth/survival factor, EGF, which control hepatocyte survival and proliferation during liver regeneration, might be involved in the up-regulation of mGSTA4 via PI3K and/or MAPKinase pathways. Thus, mGSTA4 could be a target gene induced by survival factors, and could contribute to cellular defenses against oxidative stress in hepatocyte.
REFERENCES


**Footnotes**

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1 Abbreviations used: TNFα, Tumor Necrosis Factor alpha; IL6, Interleukin 6; NFkB, nuclear factor for the kappa chain of B cells; STAT3, signal transducer and activator of transcription protein 3; AP1 activating protein-1; CREB, cAMP response element-binding protein; HGF, Hepatocyte Growth Factor; TGFα, Transforming Growth Factor alpha; EGF, Epidermal Growth Factor; CDK1, cyclin-dependent kinase 1; MEK/ERK, Mitogen Activating Protein Kinase Kinase /Extracellular Regulating Protein; PI3K, Phosphoinositide 3 Kinase; MAPK, Mitogen-Activated Protein Kinase; JNK, c-jun N-terminal kinase; ROS, Reactive Oxygen Species; iNOS, inducible nitric oxide synthase; GSH, glutathione; GST, glutathione transferase; 4-HNE, 4-hydroxynonenal; MDA, malonaldehyde; CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; PH, partial hepatectomy.
FIGURE LEGENDS

FIG. 1. **Expression of cell cycle markers and GSTs during mouse liver regeneration.**
Northern blot analysis of cyclin D1 and CDK1 mRNAs (A) and mGSTA4, A1, Mu and Pi mRNAs (B). Total RNAs were prepared from livers of partially hepatectomized and sham-operated animals at the indicated times and from normal liver (NL). As controls of total RNA loading, corresponding 18S mRNAs are presented.

FIG. 2. **GSTs expression and activity during liver regeneration.** A, Detection of mGSTA4, A1, Mu and Pi proteins. Western blots were performed on total proteins using antibodies directed against each GST isoform. B, (a-d) CDNB (1-chloro-2,4-dinitrobenzene, a common substrate of GST), (b) 4-HNE (4-hydroxynonenal, a specific substrate of GSTA4) and (c) EA (ethacrynic acid, a specific substrate of GSTPi) activities measured in cytosols of livers after partial hepatectomy (dark squares) or sham operation (dark circles). These data are representative of three different experiments (Student’s t test; *p < 0.05).

FIG. 3. **mGSTA4 immunolocalization in liver.** A, mGSTA4 immunolocalization in mitochondria and cytosol of normal liver using confocal microscopy : (a) control reaction with purified immunoglobulins of the pre-immune serum and then with anti-rabbit immunoglobulins conjugated to rhodamine. (b-c) Immunostaining with rabbit anti-GSTA4 (b) and cytochrome c (c) antibodies. Bars : 10 µm. B-C, Western blot analysis of mitochondrial markers and mGSTA4 expression. Equal amounts of mitochondrial (M) and cytosolic (C) proteins of normal (NL), 1 or 24 h regenerating (PH1, PH24) and 24 h "sham" (S24) livers were fractionated by SDS-PAGE (12.5%) and blotted with specific cytochrome c, cytochrome c oxidase and albumin (B) and mGSTA4 (C) antibodies.

FIG. 4. **Effects of lipid peroxidation, IL6, TNFα and EGF on mGSTA4 expression.**
A, Lipid peroxidation assay was carried out by measuring MDA+4-HNE levels in liver extracts of normal and hepatectomized mice 1 h post-PH, injected or not with NIL (40 µg/g of body
TNFα, IL6 and EGF up-regulate GSTA4

weight) 1 h prior to PH. B, Western blot analysis of mGSTA4 expression in the same livers. Total proteins were prepared from regenerating or quiescent livers of control and NIL-injected mice. C, Western blot analysis of mGSTA4 and A1 expression in livers of control (injected with saline buffer) and TNFα- or IL6-injected mice. D, Western blot analysis of mGSTA4 and CDK1 in normal livers (NL) and in livers of control (injected with saline buffer) and EGF-injected mice. Expression of mGSTA4 and CDK1 was studied 6 and 24 h after EGF administration. As positive control of proliferation and CDK1 expression, extracts of regenerating livers at 40 and 72 h post-PH were used. The expression of mGSTA4 in controls versus treatments was quantified by densitometry: * p<0.01; ** p<0.001.

FIG. 5. Up-regulation of mGSTA4 expression by EGF, IL6 and TNFα in primary cultures of mouse hepatocytes. A, Western blot analysis of mGSTA4, A1 and albumin expression in untreated hepatocytes (control) or exposed to IL6 (20 ng/ml) or TNFα (20 ng/ml). Cytokines were added 24 h after cell seeding and hepatocytes were harvested 6 h after treatments. B, Western blot analysis of mGSTA4 and A1 expression in hepatocytes treated (+) or not (-) by EGF (50 ng/ml) and analyzed at the indicated times after plating. Stimulations by EGF were either performed at 4 or 24 h after plating. C, Northern blot analysis of mGSTA4 in untreated hepatocytes or stimulated by EGF, TNFα or IL6. Treatments were performed for 6 h starting 24 h after plating. These data are representative of three independent experiments. D, Hepatocytes were transfected with plasmids carrying the 1.5 kb proximal promoter (PGL3-GSTA4(-1571)) or deleted promoter (PGL3-GSTA4(-165)) of hGSTA4 upstream luciferase reporter gene. Cells were exposed to EGF for 24 h after 4 h transfection, and to TNFα or IL6, for 6 h, 18 h after transfection. At 48 h of culture, cells were harvested and lysed for luciferase activity assays. Values are means ± SD of three independent experiments; statistical significance was calculated between control and treated cells transfected with PGL3-GSTA4(-1571) plasmid. p<0.01 with EGF and IL6 treatments. Inset: negative (PGL3 basic) and positive (PGL3 prom) firefly luciferase controls.
FIG. 6. Inhibition of mGSTA4 expression by inhibitors of PI3K (Ly 294002), P-p38MAPK (SB 203580) and MEK (U0126) in EGF-stimulated hepatocytes. A, Western blot analysis of mGSTA4, A1, P-AKT, P-ERK and total ERK expression. Twenty-four hours after plating, hepatocytes were treated with 15 µM of PI3K inhibitor Ly 294002, 12 µM of P38 inhibitor SB 203580 or 50 µM of MEK inhibitor U0126. One hour later, the cells were stimulated with EGF and harvested at 48 h of culture. P-ERK membranes was reprobed with anti-total ERK. B, [Methyl-\(^{3}\)H]thymidine incorporation between 48 and 72 h of culture into DNA of unstimulated hepatocytes (control) or stimulated by EGF for 72 h in presence or absence of Ly 294002, SB 203580 or U0126 inhibitors. C, Hepatocytes transfected with the PGL3-GSTA4(-1571) plasmid were treated or not with 50 ng/ml EGF in absence (control) or in presence of Ly 294002, SB 203580 or U0126 at the concentrations indicated above. Luciferase activity was normalized to that of the cotransfected plasmid expressing firefly luciferase. Data are presented as means of three experiments ± SD; p<0.001, and p<0.01 between controls and Ly 294002 or U0126 and SB 203580, respectively, in basal condition without EGF. P<0.001 between control versus Ly 294002 and U0126, p<0.01 between control and SB 203580, in EGF-stimulated cells.
Figure 1

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Figure 2

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HEPATECTOMY | SHAM-OPERATED

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B

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c-

d-

Figure 2
Figure 3

**A**

- a-control
- b-anti-mGSTA4
- c-anti-cytochrome c

**B**

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Figure 4
Figure 5
Figure 6
Pro-inflammatory cytokines TNFα and IL6, and survival factor EGF positively regulate the mGSTA4 enzyme in hepatocytes

Fabienne Desmots, Mary Rissel, David Gilot, Dominique Lagadic-Gossman, Fabrice Morel, Christiane Guguen-Guillouzo, Andre Guillouzo and Pascal Loyer

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