Growth Regulation via p38 Mitogen-activated Protein Kinase in Developing Liver*

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During normal development in the rat, hepatocytes undergo marked changes in the rate of proliferation. We have previously observed transient G1 growth arrest at term, re-activation of proliferation immediately after birth, and a gradual transition to the quiescent adult hepatocyte phenotype after postnatal day 4. We hypothesized that these changes in proliferation are due in part to growth inhibitory effects mediated by the p38 mitogen-activated protein kinase pathway. p38 kinase activity measurements showed an inverse relationship with hepatocyte proliferation during the perinatal and postnatal transitions, whereas p38 content remained constant. Anisomycin activated the p38 pathway in fetal hepatocyte cultures while inducing growth inhibition that was sensitive to the p38 inhibitor, SB203580. Activation of p38 in these cultures, via transient transfection with a constitutively active form of its upstream kinase MKK6, also inhibited DNA synthesis as well as reducing cyclin D1 content. Transfection with inactive MKK6 did neither. Furthermore, MKK6-induced growth arrest was sensitive to SB203580. Finally, administration of SB203580 to near-term fetal rats in utero abrogated the transient hepatocyte growth arrest that occurs at term. These findings indicate a role for the p38 mitogen-activated protein kinase pathway in the physiological regulation of hepatocyte proliferation during normal development in the rat.

The developing liver undergoes marked changes in growth and during and after the perinatal period. In the rat, a burst of hepatocyte proliferation results in the tripling of liver mass over the last 3 days prior to term (1). This is followed by a marked decrease in hepatocyte proliferation during the 1st postnatal week (2). Although late gestation fetal hepatocytes are highly proliferative both in vivo and in culture (2), the hepatocytes in adult liver are quiescent, with only 1 in 20,000 showing cell cycle activity. In addition to this fetal-to-adult transition, we have observed a period of temporary hepatocyte growth arrest that occurs just prior to parturition in the rat (2, 3). This is followed by a brief period of synchronized hepatocyte proliferation in the immediate postnatal period and a subsequent transition to the quiescent adult rat hepatocyte phenotype beyond the 1st postnatal week. The mechanisms that regulate these developmental changes in hepatocyte proliferation have not been characterized. Our work was motivated by the assumption that the elucidation of these mechanisms would contribute to understanding the pathophysiology of liver injury and carcinogenesis.

The present series of experiments was based on studies demonstrating that perinatal rat hepatocytes are arrested in the G1 phase of the cell cycle and that this cell cycle arrest coincides with a decline in cyclin D1 content. Based on evidence indicating that control of cyclin D1 abundance may be downstream from the p38 mitogen-activated protein (MAP) kinase pathway (4), we examined the role of this signaling pathway in the perinatal and post-neonatal regulation of hepatocyte proliferation. p38 is a mammalian homologue of the yeast osmost-sensing MAP kinase, Hog1 (5). Activation of p38 in response to non-physiologic mediators such as H2O2, UV light, and x-rays and physiological mediators such as TGF-β and inflammatory cytokines (e.g. interleukin-1β and tumor necrosis factor-α) has been shown to cause a variety of cellular responses in vitro. These include the promotion of apoptosis in various cellular systems (6, 7), maintenance of cell quiescence in fibroblasts (8), stimulation of B and T lymphocyte proliferation (9), neuronal differentiation (10), cardiac myocyte hypertrophy (11), and platelet aggregation (12). Recent studies utilizing transgenic mice expressing dominant-negative p38 or a constitutively active form of the p38-activating kinase MKK6, as well as mice lacking MKK3, have begun to establish the role of p38 in normal physiology. In particular, these studies have indicated involvement of the p38 MAP kinase pathway in cytokine production by immune cells (13–15), proliferation and differentiation of thymocytes in vivo (16), and regulation of T cell apoptosis (17). Mice that lack MAPKAP kinase 2, a downstream effector of p38, show resistance to lippopolysaccharide-induced endotoxic shock associated with attenuated production of tumor necrosis factor-α (18). However, the potential involvement of the p38 pathway in hepatocyte growth regulation and liver development remains unexplored.

The components of the p38 MAP kinase pathway have been partially characterized. Proximal kinases involved in the activation of p38 include the MAP kinase kinase kinase (MAPKKK) family members TAK1, ASK1, and MTK1 (19–21). Two intermediate kinases, MKK6 and MKK3, serve as specific ac-

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1 The abbreviations used are: MAP, mitogen-activated protein; MAPKKK, MAP kinase kinase kinase; MEM, minimal essential medium; PBS, phosphate-buffered saline; EGFP-F, farnesylated enhanced green fluorescent protein; PCNA, proliferating cell nuclear antigen; JNK, c-Jun NH2-terminal kinase; TGF-β, transforming growth factor-β; BrdUrd, bromodeoxyuridine.
tivators of p38 (22). Downstream effectors of p38 include MAP-KAP kinase-2, Mnk1/2, and the transcription factors ATF2, Elk-1, CHOP, MEF2C, and CREB (22–27). As noted above, cyclin D1 expression has been identified as an additional target for p38 regulation. In CCL39 cells overexpressing the p38 kinase MKK3 together with p38, a significant decrease in mitogen-induced cyclin D1 expression was observed in vitro using a cyclin D1-luciferase reporter construct (4), suggesting a possible transcriptional mechanism for p38-mediated growth arrest. In addition, there is evidence for post-transcriptional regulation of gene expression by p38 (28). This raises the possibility that p38 could be involved in either transcriptional or post-transcriptional regulation of hepatic cyclin D1. The following studies were undertaken in order to establish a role for p38 in the regulation of hepatocyte proliferation through the perinatal and neonatal-to-adult transitions.

**EXPERIMENTAL PROCEDURES**

**Animals**—Pregnant Harlan Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) of known gestational age (day of conception designated as E0, preterm as E19, and term as E21) were used. Given the importance of precisely determining timing at the end of gestation, term animals were identified by frequent observation for onset of parturition. Cesarean sections were performed using pentobarbital anesthesia (50 mg/kg body weight, given by intraperitoneal injection). For postnatal studies, mothers were allowed to deliver spontaneously. Pups were reared with their mothers and weaned on postnatal day 21–22.

**Hepatocyte Isolation and Primary Culture**—Fetal rat hepatocytes were isolated by collagenase digestion as described previously (3). Immunocytochemical analyses (3) have demonstrated that these preparations consist of ~90% hepatocytes, with the remaining cell population representing a mixture of nonparenchymal cell types. This level of hepatocyte predominance persists for up to 72 h in culture under the defined, mitogen-free conditions used for all experiments.

Hepatocytes were cultured on Falcon Primaria plates (Becton Dickinson, Franklin Lakes, NJ) at a density of 2 × 10⁶ cells per 100-mm plate. Cells became attached within 2 h in minimum essential medium (MEM) containing 5% fetal bovine serum and supplemented with nutrients and cofactors as described previously (3). After cell attachment, all studies were done after maintaining the cultured hepatocytes for 18 h (defined serum-free) and supplemented MEM.

**Preparation of Hepatocyte Lysates and Whole Liver Homogenates**—For preparation of hepatocyte lysates, cultured cells were rinsed twice with 10 ml of cold phosphate-buffered saline (PBS) and then scraped into 2 ml of immunoprecipitation buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10 mM NaF, and 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% Tween-20, 10% glycerol, 144 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin). Lysates were sonicated at 4 °C (full microtip power, two times for 10 s; Ultrasonic Homogenizer 4710 Series, Cole-Parmer, Chicago) and clarified by centrifugation at 10,000 × g for 5 min at 4 °C.

Where indicated, anisomycin and SB203580 hydrochloride (water-soluble form; catalogue number 559395, Calbiochem) were added directly to culture media to achieve the desired final concentrations. This was done without changing the media.

For preparation of whole liver homogenates, the pooled livers from one litter were combined in 1 ml of cold immunoprecipitation homogenization buffer (without Tween 20) per 100 mg of tissue and homogenized for 10 strokes at 700 rpm using glass-Teflon homogenization vessels. Tween 20 was then added to a final concentration of 0.1%. Homogenates were clarified by centrifugation at 10,000 × g for 10 min at 4 °C, frozen immediately on dry ice, and stored at −70 °C.

**BrdUrd Incorporation Studies**—BrdUrd incorporation and detection was performed as described previously (2) using reagents obtained from Roche Molecular Biochemicals. BrdUrd was added directly to culture medium to achieve a final concentration of 10 μM. Cells in the presence of BrdUrd were fixed for 12 h, then heated at 70 °C for 10 min, fixed with 100% cold methanol for 20 min at −20 °C. Incubation with mouse anti-BrdUrd antibody and fluorescein-conjugated secondary antibody followed the manufacturer's directions.

**Transient Transfection of Cultured Fetal Hepatocytes**—Hepatocytes were cultured in serum-free supplemented MEM for 18 h prior to transfection. After this period, expression vectors (10 μg each) for MKK6 (22) and a farnesylated enhanced green fluorescent protein (EGFP-F; CLONTECH, Palo Alto, CA) were combined with 50 μl of GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA) and added to the culture medium. Hepatocytes were incubated at 37 °C for 5 h. This was followed by addition of 10% fetal calf serum. After an interval appropriate for specific experiments, cells were fixed in 100% cold methanol for 20 min at −20 °C.

**p38 Activity Determinations**—p38 kinase activity was determined in p38 immunoprecipitates of hepatocyte lysates and liver homogenates (23). The p38 polyclonal rabbit antibody used for all experiments was raised against a mouse GST-p38α fusion protein. The level of phospho-p38 was determined by phospho-specific Western blot analysis using the polyclonal rabbit p38 antibody. This p38 polyclonal antibody was used to determine the level of phospho-p38 in mediating developmental changes in hepatocyte proliferation. The aforementioned p38 antibody was used to assess total p38 content. A phospho-specific p38 rabbit polyclonal antibody (New England Bio-labs, Beverly, MA) was also used as an indirect indicator of p38 activation state. Detection employed chemiluminescence (ECL, Amersham Pharmacia Biotech).

**Immunohistochemistry**—Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed as described previously (2). For cyclin D1 immunohistochemistry, formalin-fixed liver sections (6 μm) were dehydrated with increasing concentrations of ethanol and heat-treated at 95 °C for 5 min. Sections were treated for 15 min at room temperature with avidin/biotin blocking solutions (Vector Laboratories, Burlingame, CA) and then with 5% normal horse serum in PBS for 15 min at room temperature (Life Technologies, Inc.). Sections were then incubated with 20 μg/ml cyclin D1 primary antibody (sc-8396, Santa Cruz Biotechnology, Inc.) in PBS for 30 min at room temperature, followed by horse anti-mouse secondary antibody (1:500 dilution; Vector Laboratories). Signal was detected by incubation with fluorescein-streptavidin conjugate (Vector Laboratories). Sections were counterstained with 4,6-diamidino-2-phenylindole in order to count the total number of nuclei.

**In Situ Injections of Fetal Rats**—For in vivo experiments aimed at examining the role of p38, the p38 inhibitor SB203580-HCl was administered to fetuses in utero by intraperitoneal injection. An initial laparotomy was performed on E20.5 timed pregnant rats, and fetuses were exteriorized in utero under sterile conditions. Alternate fetuses were given intraperitoneal injections of either SB203580 (50 μg in 50 μl of normal saline) or 50 μl of saline vehicle as control. The fetuses were replaced, the laparotomy incision was closed, and gestation was allowed to continue for 24 h. At that time, Cesarean section was performed, and fetal livers were harvested, formalin-fixed, and sectioned for immunohistochemical analysis.

**Data Analysis**—For kinase assays, the level of substrate phosphorylation was determined by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Where noted, automated image analysis was used to quantify immunohistochemistry results. Images were acquired, and positive cells were counted using a ST56 camera (Dage-MTI, Michigan City, IN) and Percepts Frame Grabber with Biovision software (Percepts Corp., Knoxville, TN). In all cases, analyses were done with the observer unaware of the experimental conditions.

**RESULTS AND DISCUSSION**

**p38 Activity Correlates with Hepatocyte Growth Arrest during Liver Development**—To examine the possible involvement of p38 in mediating developmental changes in hepatocyte proliferation, p38 activity was determined in whole liver homogenates derived from perinatal and adult rat livers (Fig. 1). p38 activity was highest in livers in which hepatocyte proliferation was lowest (term [E21] and mature (28-day-old and 125–150-g adult) rats). Activity was lowest in preterm and intermediate postnatal liver samples in which there is significant hepatocyte proliferation. This pattern of p38 activity was corroborated by a Western immunoblot using phospho-specific anti-p38 (not shown). p38 protein content did not change appreciably in liver throughout the developmental period studied, as determined by Western immunoblot analysis (Fig. 1).

**Anisomycin Results in In Vitro, p38-dependent Fetal Hepatocyte Growth Arrest**—In order to study the effect of p38 activation in cultured hepatocytes on growth in vitro, the level of basal p38 activity in cultured hepatocytes was measured...
vates the stress-activated protein kinases, c-Jun NH2-terminal kinase that are subinhibitory for protein synthesis (29), potently activated by anisomycin. Anisomycin is a protein synthesis inhibitor that, even at concentrations subinhibitory for protein synthesis, can activate the stress-activated protein kinases, c-Jun NH2-terminal kinase (JNK) and p38 (30–32). Results (Fig. 2B) showed that a single dose of 0.2 μM anisomycin administered to cultured fetal hepatocytes resulted in maximal activation of p38 within 30 min. Activity decreased rapidly and was back to base line within 4 h. A higher dose of anisomycin (20 μM) resulted in a similar maximum level of p38 activation, although activation was sustained for at least 4 h. Of note, the basal activity that we observed in E19 hepatocytes was only 10–20% of the activity attained within 30 min of exposure to anisomycin at two concentrations, 0.2 or 20 μM (Fig. 2B).

Since we had confirmed potent p38 activation by anisomycin in our cultures, we sought to activate maximally the pathway in cultured fetal hepatocytes. We accomplished this using anisomycin. Anisomycin is a protein synthesis inhibitor that, even at concentrations subinhibitory for protein synthesis, can activate the stress-activated protein kinases, c-Jun NH2-terminal kinase (JNK) and p38 (30–32). Results (Fig. 2B) showed that a single dose of 0.2 μM anisomycin administered to cultured fetal hepatocytes resulted in maximal activation of p38 within 30 min. Activity decreased rapidly and was back to base line within 4 h. A higher dose of anisomycin (20 μM) resulted in a similar maximum level of p38 activation, although activation was sustained for at least 4 h. Of note, the basal activity that we observed in E19 hepatocytes was only 10–20% of the activity attained within 30 min of exposure to anisomycin at two concentrations, 0.2 or 20 μM (Fig. 2B).

Since we had confirmed potent p38 activation by anisomycin in our cultures, we tested the hypothesis that anisomycin-mediated growth arrest in fetal hepatocytes is mediated, at least in part, by p38 activation. Cultured E19 hepatocytes were exposed to anisomycin at the lower concentration of 0.2 μM in order to examine the effect of p38 activation on proliferation. Frequency of anisomycin exposure was varied and combined with SB203580 pretreatment (Fig. 3A). Hepatocytes were monitored for changes in proliferation by calculating nuclear labeling indices (the percentage of cells incorporating BrdUrd over a 12-h period in culture). Changes in cellular viability were assessed by gross morphology. Both anisomycin-treatment regimens resulted in similarly reduced rates of hepatocyte proliferation compared with controls (Fig. 3B). There was no morphological evidence of hepatocyte apoptosis. The effect of the specific p38 inhibitor, SB203580 (33, 34), was examined as an indicator of the proportion of anisomycin-mediated growth inhibition that could be attributed to p38 activation. Exposure to 10 μM SB203580 in both experimental designs reversed the anisomycin-induced growth arrest by half. Treatment with SB203580 alone did not significantly affect hepatocyte proliferation.

**Fig. 1.** p38 kinase activity in whole liver homogenates. Immunoprecipitation kinase assays were performed using ATF-2 as substrate. The results, shown graphically as mean of three independent experiments, represent the PhosphorImager analysis of resulting autoradiograms. The upper panels show a representative kinase assay autoradiogram and an accompanying Western immunoblot for total p38 content. Ad, adult.

**Fig. 2.** p38 kinase activity in cultured fetal hepatocytes. A, immunoprecipitation kinase assays were performed on whole cell lysates from E19 hepatocytes (solid bars) and E21 hepatocytes (hatched bars) prepared between 0 and 78 h after cell attachment. B, the time course of p38 activation in E19 hepatocytes was examined following addition of anisomycin at two concentrations, 0.2 (gray bars) or 20 μM (black bars). Basal activity is shown by the open bar at time 0. The results shown in both panels were confirmed in replicate experiments.
interpreted as indicating that p38 activation led to fetal hepatocyte growth arrest in vitro.

In an additional experiment (Fig. 5), we were able to demonstrate that the inhibition of cell proliferation secondary to transient transfection with MKK6(Glu) was completely reversed by the addition of SB203580 at the time of transfection. The complete reversal of MKK6-mediated growth arrest by SB203580 indicates that, while p38 activation accounts for a significant proportion of anisomycin-mediated growth arrest, anisomycin also attenuates proliferation through other pathways. Presumably, these include the inhibition of protein synthesis that is considered the primary effect of this agent (29). Although anisomycin also activates the JNK pathway, this is unlikely to account for fetal hepatocyte growth arrest in vitro given that this pathway is constitutively active in late gestation fetal hepatocytes in vivo (35).

In order to assess the effect of p38 activation on cyclin D1 content in hepatocytes, we performed immunocytochemistry for cyclin D1 on hepatocytes transfected with the active and inactive forms of MKK6. Results were similar to those obtained using BrdUrd incorporation. Cyclin D1 staining was prominent in approximately 50% of hepatocytes transfected with MKK6(Ala) plus EGFP-F or with EGFP-F alone (Fig. 6). Transfection with MKK6(Glu) resulted in a marked decrease in cyclin D1 staining that paralleled those results obtained using BrdUrd incorporation as an index of cell proliferation.

Administration of SB203580 Reverses Hepatocyte Growth Arrest in Vivo—In order to test whether p38 activation is involved in perinatal hepatocyte growth arrest in vivo, SB203580 was administered to fetal rats in situ. Saline vehicle or SB203580 were injected into alternate E20.5 fetuses in utero. Gestation was allowed to continue for another 24 h. Cesarean section was performed just prior to parturition (E21.5). Fetal livers were harvested, sectioned, and analyzed for PCNA staining (Fig. 7). Results obtained by image analysis and automated counting were confirmed by direct observation. Livers from saline-in-
Fig. 6. Effect of MKK6 transfection of fetal hepatocyte cultures on cyclin D1 content. E19 fetal hepatocytes in primary culture were co-transfected with EGFP-F and either inactive MKK6 (MKK6(Ala)); A) or constitutively active MKK6 (MKK6(Glu)); B), as for the experiment shown in Fig. 4. However, BrdUrd incorporation was omitted, and cultures were analyzed by immunocytochemistry for cyclin D1. The photomicrographs show dual immunofluorescence for EGFP-F (green) and cyclin D1 (red). The data shown in the graph represent nuclear labeling index ratios for two independent experiments (shown as solid and gray bars). For each experiment, between 150 and 300 EGFP-F-positive cells were counted as either cyclin D1-positive or cyclin D1-negative. Asterisks denote significant differences (p < 0.0001) from both control conditions, MKK6(Ala) and No Vector, by χ² analysis.

In order to understand the physiology of hepatocyte growth regulation, it will be important to identify the proximal and distal factors that participate in the p38 signaling pathway during development. Several physiological mediators have been described to activate p38 in vitro including TGF-β, cytokines (tumor necrosis factor-α and interleukin-1β), and oxidative stress. TGF-β has been postulated to be an activator for p38 via the MAPKKK family member TAK1 and its co-activator TAB1 (12, 36). TGF-β1 mRNA levels have been demonstrated to be at peak levels during late gestation in liver development (37). p38 has also been shown to be regulated by changes in the reduction-oxidation status of cells in vitro (38). Differences in the transmembrane potential and oxidative status of hepatocytes have been described in fetal versus adult liver in the mouse (39). Thus, redox changes occurring at the end of gestation may be involved in the activation of hepatic p38 in the term rat fetus. Of note, CCl4-induced metabolic oxidative stress resulted in activation of JNK but inactivation of p38 in adult liver (38). This may indicate that the mechanisms for transient hepatic p38 activation at term and tonic activation in the adult differ.

The cyclin D1 promoter may also be a downstream target for p38 activity, but the mechanism of this interaction has not been characterized (4). However, our own data suggest that up-regulation of cyclin D1 in fetal hepatocytes is post-transcriptional (40). Therefore, it is possible that p38-mediated regulation of cyclin D1 abundance involves one or more post-transcriptional mechanisms. Cyclin D1 has been demonstrated to be a target of direct phosphorylation and subsequent ubiquitination and proteolysis (41). Alternatively, the eukaryotic initiation factor, eIF-4E, has been implicated in cell cycle control by binding to and facilitating the translation of the cyclin D1 message (42, 43). These represent two potential pathways whereby p38 activation might modulate hepatocyte cyclin D1 content.

Perhaps the most unexpected result of the present studies is the finding that in vivo inhibition of p38 resulting from direct administration of SB203580 was sufficient to stimulate hepatocyte cell cycle activity at term. These results are consistent with our earlier studies indicating that the temporary hepatocyte growth arrest that occurs at the end of gestation is a result of growth inhibition, not diminished growth stimulation (2). We had arrived at the conclusion based on the finding that term hepatocytes, once isolated and cultured under defined conditions, spontaneously entered the cell cycle in a synchronous manner without growth factor stimulation. This is also consistent with findings indicating that, although activation of the extracellular signal-regulated kinase (ERK) pathway may be required for accumulation of cyclin D in growth-
stimulated adult hepatocytes (44), this pathway is uncoupled and inactive in late gestation fetal liver (45). Finally, our results indirectly support the conclusion that the high level of p38 activity in adult liver represents a tonic growth inhibitory influence. Thus, the regulation of p38 and its role in hepatocyte cycle regulation should be considered in studies aimed at understanding the physiology and pathophysiology of liver growth, carcinogenesis, and response to injury.

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