Up-regulated Transcriptional Repressors SnoN and Ski Bind Smad Proteins to Antagonize Transforming Growth Factor-β Signals during Liver Regeneration*

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Transforming growth factor-β (TGF-β) functions as an antiproliferative factor for hepatocytes. However, for unexplained reasons, hepatocytes become resistant to TGF-β signals and can proliferate despite the presence of TGF-β during liver regeneration. TGF-β is up-regulated during liver regeneration, although it is not known whether it is active or latent. TGF-β activity may be examined by assessing Smad activation, a downstream signaling pathway. Smad pathway activation during liver regeneration induced by partial hepatectomy or CCl4 injury was examined by assessing the levels of phospho-Smad2 and Smad2-Smad4 complexes. We found that Smad proteins were slightly activated in quiescent liver, but that their activation was further enhanced in regenerating liver. Interestingly, TGF-β/Smad pathway inhibitors (SnoN and Ski) were up-regulated during regeneration, and notably, SnoN was induced mainly in hepatocytes. SnoN and Ski are transcriptional repressors that may render some cells resistant to TGF-β via binding Smad proteins. Complexes between SnoN, Ski, and the activated Smad proteins were detected from 2 to 120 h during the major proliferative phase in regenerating liver. Inhibitory Smad complexes decreased after liver mass restitution (5–15 days), suggesting that persistently activated Smad proteins might participate in returning the liver to a quiescent state. Our data show that active TGF-β/Smad signals are present during regeneration and suggest that SnoNSki induction might explain hepatocyte resistance to TGF-β during the proliferative phase.

Transforming growth factor-β (TGF-β) has an amazing range of effects depending on the cellular and environmental context, including control of growth and differentiation, modeling of the extracellular matrix (ECM), and modulation of the immune response (1, 2). In the liver, TGF-β and activin A are important regulatory factors, controlling cell proliferation and death; but under abnormal conditions, these cytokines may cause liver fibrosis (3, 4). Initially, TGF-β was found to inhibit DNA synthesis in primary cultures of rat hepatocytes stimulated by epidermal growth factor or hepatocyte growth factor (5, 6). Later on, TGF-β was demonstrated to reversibly inhibit the early proliferative response to partial hepatectomy in the rat (7). Blocking TGF-β or activin A signals in vivo causes proliferation of hepatic cells. All three TGF-β isoforms and activin A are up-regulated during the regenerative response to partial hepatectomy (8–10). Interestingly, during liver regeneration, hepatocytes become refractory to TGF-β signals, and the liver can still grow despite the presence of this cytokine, although the mechanisms involved are not known (25, 60). Liver regeneration after partial hepatectomy is an extensively studied model of hepatic cell proliferation (11–13). After surgical removal of liver lobes, the remaining cells proliferate to restore the mass of the removed tissue. DNA synthesis is mostly complete by 72 h, but the whole process may last up to 2–3 weeks. Similarly, regenerative growth is also observed after toxic liver injury, e.g. by carbon tetrachloride (CCl4). Although many cytokines are involved in the onset of liver regeneration, TGF-β is a premier candidate to stop it once the liver has regained its appropriate functional mass (13).

Signaling by TGF-β and activin A occurs through transmembrane Ser/Thr kinase receptors. Ligand binds the type II receptor to recruit the type I receptor into a heteromeric complex (14). Smad proteins are the critical downstream targets of TGF-β superfamily signals (15). Members of the receptor-activated subgroup of Smad proteins such as Smad2 and Smad3 are phosphorylated by the type I receptors after TGF-β or activin A signaling (16–19). Phosphorylated Smad2 and Smad3 form heteromeric complexes with the common Smad4 protein and then translocate to the nucleus (20, 21). In the nucleus, Smad proteins associate with different DNA-binding partners, depending on the cellular context, such as Fast, transcription factor-E3, Jun, Fos, etc. TGF-β regulates numerous transcriptional responses, including the regulation of several ECM and cell cycle genes (22–24).

Down-regulation of TGF-β signaling is carried out in part by a feedback mechanism involving induction of expression of the inhibitory Smad6 and Smad7 proteins, which, through receptor binding, can prevent receptor-activated Smad activation (26, 27). Alternatively, Smad binding to different transcription factors may also modulate TGF-β signaling. Corepressors TGF, Ski, and Sno recruit histone deacetylases to Smad proteins to repress transcription (28). Ski and SnoN (Ski-related novel gene) are two closely related members of the Ski family of nuclear oncoproteins that were identified on the basis of homology v-Ski, the transforming protein of the Sloan-Kettering virus (29–31). When SnoN and Ski are overexpressed, they...
cause oncogenic transformation of chicken and quail embryo fibroblasts as well as muscle differentiation of quail embryo cells (32). Many types of human cancer cells express high levels of Ski or SnoN (33, 34). Ski and SnoN regulate transcription in conjunction with other cellular proteins, and the consensus sequence GTCTAGAC is a high affinity binding site for them. Coincidentally, the same sequence was later found to be a high affinity binding site for Smad proteins. Recently, diverse types of studies converged on the conclusion that Ski and SnoN bind directly to the Smad2-Smad3-Smad4 complexes and negatively regulate the Smad pathway activation. This results in the inability of the cell to induce growth inhibitory proteins following TGF-β addition. Because TGF-β and activin inhibit the growth of many cell types, it has been observed that overexpression of SnoN and Ski inhibitors may render some cells resistant to TGF-β. Many types of human cancer cells express high levels of TGF-β receptor (8). In addition, hepatocytes become refractory to TGF-β signals during liver regeneration (37–41).

TGF-β is always secreted as a latent complex and is found mainly as a latent form in vivo. Latency appears to be a critical step in the control of TGF-β activity because enhanced TGF-β expression does not always correlate with increased levels of active TGF-β (42). During liver regeneration, there is an increase in TGF-β mRNA and protein levels, but it is not known whether the TGF-β is active or latent (8). In addition, hepatocytes become refractory to TGF-β signals during liver regeneration (25, 60). In this study, we set about to investigate the activation state of the TGF-β/Smad pathway during liver regeneration and to explore the mechanisms of hepatocyte resistance to TGF-β during this process. We determined that there is activation of TGF-β/Smad pathway during liver regeneration. We also observed up-regulation of the inhibitors SnoN and Ski, potentially explaining the reason for hepatocyte resistance to TGF-β signals.

EXPERIMENTAL PROCEDURES

Materials—TGF-β was obtained from R&D Systems. 8-Bromo-cAMP, phorbol 12-myristate 13-acetate (PMA), phorbol 12, 13-dibutyrate (PDBu), retinoic acid, and anisomycin were obtained from Sigma. Interleukin-6 (IL-6) was a gift from Dr. Gennaro Giliberto (Istituto di Ricerche di Biologia Molecolare, Rome, Italy). The ECL kit was from Amersham Biosciences. CCl4 was from ICN Biomedicals.

Animals—Studies were performed on wild-type C57BL6 mice 12–16 weeks of age. Animals were maintained on an ad libitum diet of rodent laboratory chow 5008 (Ralston-Purina Co., St. Louis, MO).

Partial Hepatectomy Model—Mice ranging from 12 to 16 weeks old were anesthetized under isofluorane and subjected to a ventral laparotomy, and the anterior two-thirds of the liver were removed. Animals were killed at 0, 0.5, 2, 6, 12, 24, 36, 48, 72, 96, and 120 h and at 6, 7, 10, and 15 days following partial hepatectomy. Usually, three or four animals were used for surgeries at each time point. The livers were harvested and processed for protein and/or RNA analysis as previously described (43).

CCl4 Injury Model—In the acute model, mice ranging from 12 to 16 weeks old were injected intraperitoneally with 0.4 μg of CCl4 (diluted in mineral oil/g weight of animal. Under isofluorane anesthesia, the animals were killed at 0, 0.5, 2, 6, 12, 24, 36, 48, 72, 96, and 120 h and at 1 and 2 weeks following treatment with CCl4. Usually, three or four animals were used for treatment at each time point. The livers were harvested and processed for protein and/or RNA analysis (44).

Hepatic Cell Isolation—Cells were isolated using the collagenase perfusion method adapted from Thorgersson and co-workers as previ-
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RESULTS

Smad Proteins Are Slightly Active in Quiescent Liver, but Their Activation Is Further Enhanced during Hepatic Regeneration following Partial Hepatectomy—TGF-β and activin A are up-regulated during liver regeneration, but it is not clear whether they are active or latent. To assess their activity, it was critical to evaluate the activation of the Smad pathway in the course of liver regeneration induced by partial hepatectomy by examining the levels of Smad2 and Smad4 expression as well as levels of phospho-Smad2. Total RNAs, from mouse livers harvested at the indicated times after PH, were analyzed by Northern blotting using full-length Smad2 and Smad4 cDNAs as probes. We observed a major Smad2 (2.8 kb) and two main Smad4 (2.5 and 6.0 kb) mRNA bands on the gel as previously described (58). Smad2 mRNA levels showed a slight increase during the 12–48 h after surgery (0 h = 1-fold and 48 h = 2-fold induction), whereas Smad4 levels did not show significant changes during the course of regeneration (Fig. 1, A and B).

Smad4 protein levels did not change during the course of regeneration, whereas Smad2 and Smad3 proteins seemed to decrease slightly during the first 2 days after PH (0 h = 1-fold and 24 h = 0.4-fold increase). However, levels of phospho-Smad2 protein were detected in nuclear extracts from quiescent livers, but they were increased at early hours after surgery (0 h = 1-fold, 2 h = 3-fold, and 120 h = 1.9-fold increase) (Fig. 1C). Smad2 phosphorylation was elevated through the first 5 days and then decreased to basal levels (Fig. 1D).

Transcriptional Repressors SnoN and Ski Are Induced during Liver Regeneration—At this point, our data showed that signals mediated by TGF-β or activin were present in quiescent liver and that activity was increased following PH. The presence of phospho-Smad2 is strong evidence for TGF-β pathway activation during the regenerative process. However, because TGF-β usually opposes cell proliferation, we hypothesized that up-regulation of inhibitors of TGF-β antiproliferative action could be necessary during the regenerative process.

Hepatocytes become insensitive to TGF-β under certain conditions. In fact, the liver is able to grow despite increasing levels of this antiproliferative cytokine during the course of liver regeneration. To find the mechanism by which hepatocytes become refractory to TGF-β signals during regeneration, we examined the expression of TGF-β pathway inhibitors. Microarray analysis of gene expression in livers at 2 h post-hepatectomy showed that SnoN is induced as an immediately-early gene after PH (43). Because SnoN and Ski represent potential candidates to block TGF-β signals during regeneration, we set about to examine SnoN and Ski expression in regenerating livers at different stages by Northern blotting and immunoblotting.

Northern blot analysis of SnoN and Ski confirmed that SnoN mRNA was up-regulated as an immediate-early gene during regeneration. We observed that SnoN mRNA (6.2 kb) was increased after PH, with a peak of expression at 2 h after PH

Institute of Technology, Cambridge, MA); and TGF-β1 cDNA was a gift of Dr. Gloria Gutiérrez-Venegas (Facultad de Odontología, Universidad Nacional Autónoma de México). Densitometry analysis was carried out with Foto/Eclipse imaging analyzer and Collage Version 2.0 software. Densities are expressed as relative -fold induction.

Immunohistochemistry—Control or TGF-β-treated paraformaldehyde-fixed cultured hepatocytes and Formalin-fixed, paraffin-embedded liver sections were studied. Liver sections from mice subjected to partial hepatectomy (PH) or CCl4 injury and cultured hepatocytes were used for Smad2, Smad3, and Smad4 nuclear localization studies and were evaluated by immunocytochemistry. The anti-Smad primary polyclonal antibodies were diluted 1:250. The horseradish peroxidase-labeled anti-rabbit secondary antibody was diluted 1:1000. Slides were mounted with Vectashield (Vector Laboratories, Inc.).

mRNA Analysis—For RNA preparation, a small piece of liver was harvested, and total liver RNA preparation was performed as described (43). Total RNA was also purified from fresh isolated hepatic cells. mRNA expression of SnoN, Ski, Smads, ATP synthase, c-Fos, and TGF-β1 was assessed by Northern blot assay using 20 µg of total RNA/lane. Total RNA preparation, Northern blotting, and hybridizations were performed as described previously (43, 44). ATP synthase was used as a loading control. Smad cDNAs were a generous gift of Dr. Jeffrey L. Wrana (Mount Sinai Research Institute, Toronto) and Dr. Liliana Attisiano (University of Toronto, Toronto); SnoN and Ski cDNAs were kindly provided by Dr. Harvey F. Lodish (Massachusetts Institute of Technology, Cambridge, MA); and TGF-β1 cDNA was a gift of Dr. Gloria Gutiérrez-Venegas (Facultad de Odontología, Universidad Nacional Autónoma de México). Densitometry analysis was carried out with Foto/Eclipse imaging analyzer and Collage Version 2.0 software. Densities are expressed as relative -fold induction.

Immunoprecipitation and Immunoblot Analyses—Preparation of liver cell nuclear extracts, immunoprecipitations, protein electrophoresis, protein transfer, and protein detection by Western blotting and ECL were carried out as previously described (46). Nuclear extracts (0.5–1.5 mg of protein) were used for the immunoprecipitations. The following primary polyclonal antibodies (at a dilution of 1:1000) were used: anti-SnoN (catalog no. sc-9141), anti-Ski (catalog no. sc-9140), anti-Smad2/3 (catalog no. sc-6032), and anti-Smad4 (catalog no. sc-1909) antibodies from Santa Cruz Biotechnology and anti-phospho-Smad2 (catalog no. 06-829) and anti-Smad4 (catalog no. 06-693) antibodies from Upstate Biotechnology, Inc. Horseradish peroxidase-conjugated anti-rabbit or anti-goat secondary antibodies (Zymed Laboratories Inc.) were used for Smad2, Smad3, and Smad4 nuclear localization studies and were labeled anti-rabbit secondary antibody was diluted 1:1000. Slides were evaluated by immunocytochemistry. The anti-Smad primary polyclonal antibodies were diluted 1:250. The horseradish peroxidase-labeled anti-rabbit secondary antibody was diluted 1:1000. Slides were mounted with Vectashield (Vector Laboratories, Inc.).

mRNA Analysis—For RNA preparation, a small piece of liver was harvested, and total liver RNA preparation was performed as described (43). Total RNA was also purified from fresh isolated hepatic cells. mRNA expression of SnoN, Ski, Smads, ATP synthase, c-Fos, and TGF-β1 was assessed by Northern blot assay using 20 µg of total RNA/lane. Total RNA preparation, Northern blotting, and hybridizations were performed as described previously (43, 44). ATP synthase was used as a loading control. Smad cDNAs were a generous gift of Dr. Jeffrey L. Wrana (Mount Sinai Research Institute, Toronto) and Dr. Liliana Attisiano (University of Toronto, Toronto); SnoN and Ski cDNAs were kindly provided by Dr. Harvey F. Lodish (Massachusetts Institute of Technology, Cambridge, MA); and TGF-β1 cDNA was a gift of Dr. Gloria Gutiérrez-Venegas (Facultad de Odontología, Universidad Nacional Autónoma de México). Densitometry analysis was carried out with Foto/Eclipse imaging analyzer and Collage Version 2.0 software. Densities are expressed as relative -fold induction.
Fig. 3. Activated Smad2-Smad4 complexes interact with the repressors SnoN and Ski during liver regeneration following PH. Nuclear extracts from mouse livers in different stages of regeneration after PH were divided into five portions, and each one was immunoprecipitated with different polyclonal antibodies: anti-SnoN, anti-Smad2/3, anti-Smad4, anti-Smad1, and anti-Ski. Protein levels were detected by immunoblotting and ECL assays. A, nuclear extracts from regenerating livers were immunoprecipitated with anti-SnoN antibody, and the levels of Ski, Smad2, and Smad4 proteins coprecipitated with SnoN were detected by immunoblotting. B, nuclear extracts from regenerating livers were immunoprecipitated with anti-Ski antibody, and the levels of SnoN, Smad2, and Smad4 proteins coprecipitated with Ski were detected by immunoblotting. C, nuclear extracts from regenerating livers were immunoprecipitated with anti-Smad2/3 antibody, and the levels of SnoN, Smad2, and Smad4 proteins coprecipitated with Smad2/3 were detected by immunoblotting. D, nuclear extracts from regenerating livers were immunoprecipitated with anti-Smad4 or anti-Smad1 antibodies, and the levels of SnoN coprecipitated with them and the levels of Smad4 were detected by immunoblotting. E, nuclear extracts from regenerating livers at later time points were immunoprecipitated with anti-Smad2/3 antibody, and the levels of SnoN and Smad4 proteins coprecipitated with Smad2/3 were detected by immunoblotting. F, nuclear extracts from regenerating livers at later time points were immunoprecipitated with anti-Ski antibody, and the levels of SnoN proteins coprecipitated with Smad4 were detected by immunoblotting. Each panel shows representative data from two to three individual experiments. CO-IP, co-immunoprecipitation; P-Smad2, phospho-Smad2.

h = 1-fold and 2 h = 6-fold induction). In contrast, the two main bands of Ski mRNA (4.7 and 6.0 kb) were induced at later stages of regeneration (0 h = 1-fold and 48 h = 2-fold induction) (Fig. 2A), showing that ski is a delayed-early gene as previously described (30, 31).

SnoN and Ski protein levels were induced during the process of regeneration. Nuclear extracts prepared at the indicated times were used to immunoprecipitate SnoN and Ski, and protein levels were detected by Western blot assays using anti-SnoN and anti-Ski polyclonal antibodies. SnoN protein levels were increased in the early stages of regeneration, during the first 2–6 h, and remained elevated until 48 h after PH. Two SnoN protein isoforms (~70 and 80 kDa) were present in the mouse liver nuclear extracts, and both were increased at early times of liver regeneration from 2 to 48 h after surgery (0 h = 1-fold and 2 h = 3-fold increase) (Fig. 2B). Ski protein levels were induced as well, but at later times points, 24–48 h after PH (0 h = 1-fold and 48 h = 7-fold increase). Ski protein analysis showed the presence of at least two main isoforms of ~90 kDa (Fig. 2B).

Ski and SnoN Interact with Smad Proteins during Hepatic Regeneration—We assessed whether activated Smad2-Smad4 complexes interact with SnoN and Ski during liver regeneration. Nuclear extracts from mouse livers at different times after PH were also analyzed by Western blotting. Ski protein was induced late in regeneration around 24–48 h after PH (0 h = 1-fold and 2 h = 7-fold increase). However, SnoN interacted with Ski in the early hours after PH and remained associated until 4–5 days after surgery. Interestingly, although Smad proteins were associated with Ski in quiescent and regenerating livers, there was no change in the level of their association during the course of the regeneration (Fig. 3B). Nuclear extracts from regenerating livers were also immunoprecipitated with anti-Smad2/3 antibodies. Smad2 and Smad3 protein levels showed discrete changes during the course of regeneration, and association of phospho-Smad2 with Smad4 increased during the process. The Smad2-Smad3 complex associated with
SnoN early after PH and remained associated for >5 days, whereas the Smad2-Smad3 complex associated with Ski later in the process after 48 h (Fig. 3C).

Nuclear extracts from regenerating livers were also immunoprecipitated with anti-Smad4 or anti-Smad1 antibodies. Smad4 levels and the levels of SnoN coprecipitated with Smad4 or Smad1 were detected by immunoblotting. Smad4 levels did not change during regeneration, but they showed an enhanced association with SnoN at early stages of the process. Smad1 protein, which is regulated by bone morphogenetic proteins, but not by TGF-β/activin, did not show association with SnoN or Smad4 during the course of regeneration, supporting the specificity of the interaction between Smad4 and SnoN (Fig. 3D).

Finally, we also evaluated the duration of the Smad-SnoN complexes in hepatectomized livers at 5, 6, 7, 10, and 15 days after surgery, when the liver mass was getting restored. During this period of time, we still saw some levels of phospho-Smad2 like in quiescent liver, but we also observed a gradual decrease in Smad-SnoN complexes at 5, 6, 7, 10, and 15 days after PH. Our data agree with a role for TGF-β signals in maintaining hepatic cells in the G0 phase of the cell cycle when the liver mass has been regained (Fig. 3, E and F). In summary, our data support the idea that hepatocytes may become refractory to TGF-β actions during regeneration through up-regulating the TGF-β/Smad pathway inhibitors SnoN and Ski.

**FIG. 4.** TGF-β/Smad pathway inhibitor SnoN is up-regulated during regeneration after CCl4 hepatic injury. A. SnoN mRNA levels were induced early during hepatic regeneration. Total RNA was isolated from mouse regenerating livers at different hours after CCl4 treatment. Northern blot analysis of SnoN expression was carried out using full-length SnoN cDNA as a probe. An ATP synthase (ATPsyn) probe was used as a control to normalize RNA loading. B, levels of SnoN protein and its association with Smad proteins were enhanced during liver regeneration after CCl4 injury. Mouse regenerating livers were harvested at different hours after acute CCl4 treatment, and liver nuclear extracts were prepared. Nuclear extracts were used to immunoprecipitate SnoN, SnoN proteins, and corepressing proteins were detected by immunoblotting using polyclonal antibodies and ECL. C, Smad4 protein levels were steady, but Smad4-SnoN complexes were enhanced during liver regeneration after CCl4 injury. Levels of Smad4 protein were detected in nuclear extracts from mouse regenerating livers after acute CCl4 treatment. Coprecipitation of Smad4 with SnoN, Ski, and phospho-Smad2 (P-Smad3) was also detected in the same nuclear extracts. Proteins were detected by immunoblotting using polyclonal antibodies and ECL. Each panel shows representative data from two or three individual experiments. CO-IP, co-immunoprecipitation.

**FIG. 5.** SnoN is induced mainly in hepatocytes during mouse liver regeneration. Hepatocytes and non-parenchymal cells were isolated from quiescent and regenerating livers. Total RNA and nuclear extracts were obtained from livers harvested 2 h after PH or CCl4 treatment. Expression of SnoN mRNA was induced mainly in hepatocytes from regenerating livers following PH or CCl4 treatment. Northern blot analysis of SnoN expression was carried out using full-length SnoN cDNA as a probe, and c-Fos and TGF-β1 were used as controls. TGF-β1 was expressed mainly in non-parenchymal cells, and c-Fos was induced in both hepatocytes and non-parenchymal cells after regeneration. Each panel shows representative data from two or three individual experiments.
Hepatocytes become refractory to TGF-β actions during the course of regeneration. Therefore, up-regulation of SnoN and Ski could provide a mechanism to block TGF-β signals and thus favor their proliferation in regeneration. We investigated whether SnoN is induced in hepatocytes or in non-parenchymal cells during the course of regeneration. Hepatocytes and non-parenchymal cells were isolated from quiescent and regenerating livers 2 h after PH or CCl₄ intoxication, and total RNA was obtained. The data showed that SnoN mRNA was induced mainly in hepatocytes, but to a lesser degree in non-parenchymal cells from regenerating livers. As controls, TGF-β₁ was expressed mainly in non-parenchymal cells, and c-Fos was induced in both hepatocytes and non-parenchymal cells during regeneration (Fig. 5) as previously described (8).

**TGF-β Induces Smad2 Activation and SnoN Expression in Primary Cultures of Mouse Hepatocytes.—**To begin to understand the TGF-β signals in hepatocytes and how SnoN is regulated in hepatocytes, we explored the induction of SnoN by some of the different signal transduction pathways involved in the onset of liver regeneration and by TGF-β. Recently, TGF-β was described as able to induce SnoN in the hepatoma cell line Hep3B (38). IL-6 is required for liver regeneration and induces many immediate-early genes during regeneration (43, 47–49). Isolated mouse hepatocytes and HepG2 cells, another hepatoma cell line, were cultured and treated for 1 h with 1 nM TGF-β₁, 10 μg/ml IL-6, 100 μM 8-bromo-cAMP (cAMP), 10 nM PMA (protein kinase C activator), 100 nM retinoic acid (RA), 100 nM anisomycin (Ani), or 5% fetal bovine serum (Ser). Total RNA was isolated from cultured mouse hepatocytes or HepG2 cells, and Northern blot analysis of SnoN and c-Fos cDNA as a probe. c-Fos was constitutively expressed in cultured mouse hepatocytes, but it was induced mainly in HepG2 cells by IL-6, PMA, and PDBu. Each panel shows representative data from two individual experiments. C, control; ATP syn, ATP synthase.

**DISCUSSION**

Liver regeneration is a widely studied model of cell proliferation (11–13). Although many growth factors have been proposed as initiators of the onset of liver regeneration, TGF-β has been proposed as a major candidate to arrest proliferation once functional mass has been regained. Although it was known that TGF-β mRNA and protein levels increase a few hours after partial hepatectomy or toxic injury, it was not clear if TGF-β is active or latent (8, 9, 50, 51). Activin A, another member of the TGF-β superfamily, is also induced during liver regeneration and is able to inhibit hepatocyte proliferation and to induce cell death, like TGF-β (52, 53). Intriguingly, data suggest a role for activin A and TGF-β in retaining hepatic cells in the G₀ phase in quiescent liver. Moreover, it has been suggested that they may also participate in remodeling liver architecture through regulation of ECM proteins and in terminating the process of regeneration.

In this study, we examined the activity of the Smad pathway in regenerating liver because this would provide an insight into the state of TGF-β activation during regeneration. We showed that Smad proteins are already activated in quiescent liver, supporting a role for activin A and TGF-β signals in maintaining hepatic cells in the G₀ phase of the cell cycle. Smad proteins are further activated during the early hours of regeneration. Because TGF-β suppresses or opposes hepatocyte proliferation, we hypothesized that some mechanism to block TGF-β signals in hepatic cells would be induced in regenerating liver to allow proliferation to occur. Up-regulation of SnoN and Ski may function as one of the mechanisms that inhibit the TGF-β/Smad pathway in hepatocytes during the course of hepatic regeneration.

TGF-β causes many cells to accumulate in the mid-to-late G₁ phase of the cell cycle by blocking the transition from G₁ to S, directly regulating transcription of cell cycle inhibitors and activators (54–56). However, sometimes cells become refractory to TGF-β signals and can proliferate even in the presence of this cytokine. This frequently occurs in cancer cells, but it has been also observed in proliferating hepatocytes during liver regeneration (27, 60). Several groups have described a down-regulation of the TGF-β receptors during liver regeneration (25, 35, 50, 61). This process occurs between 24 and 48 h after partial hepatectomy or CCl₄ injury, and it has been associated...
with the cell insensitivity to TGF-β during this period of time. On the other hand, several mechanisms may arise in tumor cells to overcome the TGF-β restriction, such as oncogenic activation through cross-talk between various signaling pathways that antagonize TGF-β signaling and that allow the cells to escape growth inhibition, although the loss of TGF-β growth inhibition occasionally occurs with maintenance of other aspects of TGF-β responses, such as transcriptional induction of certain genes, e.g. ECM genes. The induction of TGF-β pathway inhibitors is another mechanism used to inhibit TGF-β signals. For example, Smad6 and Smad7 are inducible proteins that block TGF-β signals by binding type I receptors (26, 27). However, our attempts to examine Smad7 expression during liver regeneration have been inconclusive thus far; therefore, we cannot rule out a role for this factor. On the other hand, induction of other inhibitors such as the transcriptional repressors TGIF, Ski, and SnoN allows cell proliferation through blocking Smad activity (28).

Microarray analysis carried out in our laboratory showed that sno was induced as an immediate-early gene after PH (43). Up-regulation of this kind of Smad inhibitor is a potential mechanism by which hepatocytes could resist TGF-β signals in the course of regeneration. SnoN and Ski are two closely related members of the Ski family of nuclear oncoproteins. They are transcriptional repressors and repress transactivation of Smad proteins. Because TGF-β and activin inhibit the growth of many cell types, it has been suggested that overexpression of SnoN and Ski may render some cells resistant to TGF-β-induced growth arrest. In many tumor cells in which the levels of Ski or SnoN are elevated, it is likely that excess Ski or SnoN can reduce the antiproliferative response to TGF-β. Surprisingly, we found that SnoN and Ski are up-regulated during liver regeneration with different kinetics; sno is induced as an immediate-early gene, whereas ski is a delayed-early gene. Intriguingly, we also found that SnoN and Ski recruit activated Smad proteins during the course of regeneration. SnoN is up-regulated mainly in hepatocytes and binds Smad proteins precisely when the cells are proliferating. Notably, although induction of such repressors has been described previously in some cell lines, this is the first report showing SnoN and Ski up-regulation in an in vivo model of proliferation distinct from cancer.

On the other hand, Smad proteins form a large number of different complexes between each other and with the inhibitors SnoN and Ski during the course of regeneration. This is consistent with the observation that Smad proteins are present in many different protein complexes of various sizes before and after TGF-β signaling depending on the cellular context (57). It is plausible that the different Smad complexes found in regenerating liver could have different functions. However, Smad binding to SnoN and Ski would block only Smad-dependent TGF-β signals such as growth inhibition. Because SnoN induction occurred mainly in hepatocytes, but not in non-parenchymal cells, we suggest that whereas hepatocytes become resistant to TGF-β, non-parenchymal cells may still respond to it. It has been suggested that TGF-β may have Smad-independent functions, mainly related to ECM gene regulation (59). For instance, Smad-independent TGF-β1 signals seem to be the main regulators of ECM genes because fibroblasts from Smad3 or Smad4 knockout mice still increase ECM molecule production upon TGF-β1 treatment; however, TGF-β growth inhibition is blocked in fibroblasts from Smad4 knockout mice (58, 59). Therefore, although SnoN and Ski may be blocking the Smad pathway, Smad-independent TGF-β signals may be working normally during regeneration. Unfortunately, Smad2 and Smad4 as well as SnoN and Ski knockout mice are lethal, so they cannot be used to study the role of these proteins during liver regeneration.

Interestingly, our data show that around 120 h post-hepatectomy, when the TGF-β signals may be required to end regeneration, the Smad-SnoN-Ski complexes still remain. We believe that such complexes may allow Smad proteins to target SnoN and Ski to proteasomes for their degradation at that time. Several groups have shown that TGF-β induces degradation of Ski and SnoN to limit their threshold levels. It has also been shown that such a process is triggered by TGF-β signals (36, 62). However, it remains to be determined if this process occurs during the course of liver regeneration.

From current knowledge, SnoN seems to be involved mainly in the regulation of the TGF-β pathway, whereas Ski has effects independent of the Smad pathway. Ski is also required for transcriptional repression induced by Mad and the thyroid hormone receptor-β as well as by Rb (33). Identification of the genes regulated by the proto-oncogenes sno and ski awaits resolution; thus, at this time, it could be hard to predict what kind of genes are regulated by these repressors during the process of regeneration. Our data lend support to a TGF-β/activin role in arresting liver regeneration after the liver has regained appropriate mass and in maintaining hepatocytes in the quiescent state. Our data indicated that Smad proteins were already active in quiescent liver, but that they were further activated during early hours of regeneration. We also observed that SnoN primarily bound Smad proteins at the early stages of regeneration; however, later on when SnoN levels decreased, Smad binding also decreased. At this point, Smad proteins still showed some activation, and indeed they could be working to arrest liver growth at the point of mass restitution, although it is possible that additional factors (mechanisms) may also contribute. We also believe that the early increase in the TGF-β signals during regeneration may explain the later down-regulation of TGF-β receptors, i.e. the down-regulation may be a consequence of the previous receptor activation. It is possible that SnoN is induced in hepatocytes at early hours after PH to allow cell proliferation, and such an effect would be supported by the later decrease in TGF-β receptor levels. It is possible that the receptor down-regulation is also involved in diminishing the Smad-independent TGF-β signals usually not related to growth inhibition, such as ECM production.

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REFERENCES

1. Massagué, J. (1998) Annu. Rev. Biochem. 67, 753–791
2. Wrana, J. L. (2000) Cell 100, 189–192
3. Rossmanith, W., and Schulte-Hermann, R. (2001) Microsc. Res. Tech. 52, 430–436
4. Wrana, J. L. (1999) Hepatology 29, 1909–1910
5. Carr, B. I., Hayashi, I., Branum, E. L., and Moses, H. L. (1996) Cancer Res. 46, 2330–2334
6. Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K., and Ichihara, A. (1985) Biochem. Biophys. Res. Commun. 133, 1042–1050
7. Russell, W. E., Coffey, R. J., Jr., Ouellette, A. J., and Moses, H. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5126–5130
8. Braun, L., Mead, J. E., Panaza, M., Mukimo, R., Bell, G. I., and Fausto, N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1539–1543
9. Braun, L., Gruppuso, P., Mukimo, R., and Fausto, N. (1990) Cell Growth Differ. 1, 103–111
10. Strain, A. J., Frazer, A., Hill, D. J., and Milner, R. D. (1987) Biochem. Biophys. Res. Commun. 145, 436–442
11. Taub, R. (1998) Clin. Lab. Med. 16, 341–360
12. Fausto, N. (2000) J. Hepatol. (Amst.) 32, 19–31
13. Michalopoulos, G. K., and DeFrances, M. C. (1997) Science 276, 60–66
14. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994) Nature 370, 341–347
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15. Attisano, L., and Lee-Hoeflich, S. T. (2001) Genomic Biol. 2, 3010.1–3010.8
16. Macias-Silva, M., Abdollah, S., Hoodless, P., Pirrone, R., Attisano, L., and Wiman, K. J. (1996) Cell 87, 1215–1224
17. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10669–10674
18. Kretzschmar, M., Liu, F., Hata, A., Doody, J., and Massague, J. (1997) Genes Dev. 11, 894–905
19. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) Cell 95, 779–791
20. Nakao, A., Iwamoto, T., Souchelnyskyy, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) EMBO J. 16, 5533–5536
21. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996) Nature 383, 832–836
22. Massague, J. (2000) Nat. Rev. 1, 169–178
23. Massague, J., and Chen, Y. G. (2000) Genes Dev. 14, 627–644
24. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 386, 465–471
25. Date, M., Matsuzaki, K., Saito, S., Saito, S., Shinozuka, K., Shih, H., Kajita, T., Yamamoto, T., Takata, T., Kato, M., and McKeehan, W. L. (1998) J. Hepatol. 28, 572–583
26. Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimpone, M. A., Jr., Wrana, J. L., and Falb, D. (1997) Cell 88, 1165–1173
27. Nakao, A., Afrakhté, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. (1997) Nature 389, 631–635
28. Liu, X., Sun, Y., Weinberg, R. A., and Lodish, H. F. (2001) Cytokine Growth Factor Rev. 12, 1–8
29. Stavnezer, E., Barkas, A. E., Brennan, L. A., Brodeur, D., and Li, Y. (1986) Nature 323, 103–1038
30. Pearson-White, S., and Crum, R. (1997) Nucleic Acids Res. 25, 2930–2937
31. Pearson-White, S. (1993) Nucleic Acids Res. 21, 4632–4638
32. Colmenares, C., and Stavnezer, E. (1989) Cell 59, 293–303
33. Nomura, T., Khan, M. M., Kaul, S. C., Dong, H. D., Wadhwa, R., Colmenares, C., Kohno, I., and Ishii, S. (1999) Genes Dev. 13, 412–423
34. Fumagalli, S., Doneda, L., Nomura, N., and Larizza, L. (1993) Melanoma Res. 3, 235–240
35. Chari, R. S., Price, D. T., Sue, S. R., Meyers, W. C., and Jirtle, R. L. (1995) Am. J. Surg. 169, 126–132
36. Stroschein, S. L., Bonni, S., Wrana, J. L., and Luo, K. (2001) Genes Dev. 15, 2822–2836
37. Sun, Y., Liu, X., Eaton, W. N., Lane, W. S., Lodish, H. F., and Weinberg, R. A. (1999) Mol. Cell. 4, 1–20
38. Stroschein, S. L., Wang, W., Zhou, S., Zhou, Q., and Lou, K. (1999) Science 286, 771–774
39. Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) Genes Dev. 13, 2196–2206
40. Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K., and Kawabata, M. (1999) J. Biol. Chem. 274, 35269–35277
41. Xu, W., Angelis, K., Danielpour, D., Haddad, M. M., Bischof, O., Campisi, J., Stavnezer, E., and Medrano, E. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5924–5929
42. Gleizes, P. E., Munger, J. S., Nunes, I., Harpel, J. G., Mazzieri, R., Nogueira, I., and Riffkin, D. B. (1997) Stem Cells 15, 190–197
43. Li, W., Li, Y., Leu, J. I., Kovalovich, K., Li, C., and Taub, R. (2001) Hepatology 33, 1377–1386
44. Kovalovich, K., DeAngelis, R. A., Li, W., Furth, E. E., Ciliberto, G., and Taub, R. (2000) Hepatology 31, 149–159
45. Kovalovich, K., Li, W., DeAngelis, R., Greenbaum, L. E., Ciliberto, G., and Taub, R. (2001) J. Biol. Chem. 276, 26605–26613
46. Macias-Silva, M., Hoodless, P. A., Tang, S. J., Buchwald, M., Wrana, J. L. (1998) J. Biol. Chem. 273, 25626–25636
47. Cressman, D. E., Greenbaum, L. E., DeAngelis, R. A., Ciliberto, G., Furth, E. E., Poli, V., and Taub, R. (1996) Science 274, 1379–1383
48. Taub, R. (1996) PASEB J. 10, 413–427
49. Taub, R., Greenbaum, L. E., and Peng, Y. (1999) Semin. Liver Dis. 19, 117–127
50. Grasl-Kraupp, B., Rossmanith, W., Ruttkay-Nedecky, B., Mullauer, L., Kammerer, B., Bursch, W., and Schulte-Hermann, R. (1998) Hepatology 28, 717–726
51. Jakowlew, S. B., Mead, J. E., Daniels, P., Wu, J., Roberts, A. B., and Fausto, N. (1991) Cell Regul. 2, 535–548
52. Schwall, R. H., Robbins, K., Jardieu, P., Chang, L., Lai, C., and Terrell, T. G. (1993) Hepatology 18, 347–356
53. Yasuda, H., Mine, T., Shihata, H., Tsuchiya, T., Miyata, S., and Kojima, I. (1993) J. Clin. Invest. 92, 1491–1496
54. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massague, J. (1990) Cell 62, 175–185
55. Reynolds, T., Poljak, Y., Iavarone, A., and Massague, J. (1995) Genes Dev. 9, 1831–1845
56. Sugiyama, A., Nakagi, M., Shiido, Y., Moriwaki, H., and Muto, Y. (1997) Biochem. Biophys. Res. Commun. 238, 539–543
57. Jayaraman, L., and Massague, J. (2000) J. Biol. Chem. 275, 40710–40717
58. Sirard, C., Kim, S., Mirtos, C., Tachik, P., Hoodless, P. A., Rie, A., Masson, R., Wrana, J. L., and Mak, T. W. (2000) J. Biol. Chem. 275, 2063–2070
59. Ashcroft, G. S., Yang, X., Glick, A. B., Weinstein, M., Letterio, J. J., Mizel, D. E., Anzano, M., Dong-Well, T., Wahl, S. M., Deng, C., and Roberts, A. B. (1999) Nat. Cell Biol. 1, 126–129
60. Bouzahzah, B., Fu, M., Iavarone, A., Factor, V. M., Thorpe, R. S., and Pestell, R. G. (2000) Cancer Res. 60, 4531–4537
61. Date, M., Matsuzaki, K., Matsushita, M., Tabashi, Y., Furukawa, F., and Inoue, K. (2000) Gut 46, 719–724
62. Bonni, S., Wang, H. R., Causin, C. G., Kavask, P., Stroschein, S. L., Luo, K., and Wrana, J. L. (2001) Nat. Cell Biol. 6, 587–595