Expression and Localization of the Transforming Growth Factor-β Type I receptor and Smads in Preneoplastic Lesions during Chemical Hepatocarcinogenesis in Rats

Little is known about the involvement of Smad-related molecules in the regulation of the Transforming Growth Factor (TGF)-β signaling pathway during hepatocarcinogenesis, particularly with respect to preneoplastic lesions of a rat liver. The aims of this study were to investigate the localizations and temporal expressions of TGF-β Receptor Type 1 (TGR1) and Smads during the promotion stage of chemical hepatocarcinogenesis in rats. We investigated expressions and localizations of TGR1, Smad2, Smad4, and Smad7 by using semi-quantitative RT-PCR and immunohistochemistry in preneoplastic lesions during rat chemical hepatocarcinogenesis induced by Solt and Farber’s method. The down-regulation of TGR1, Smad2, and Smad4 was evident during the later steps of the promotion stage of chemical hepatocarcinogenesis. In contrast with other Smads, increased Smad7 expression was evident during the later steps of the promotion stage. Also immunohistochemistry revealed that the main site of TGR1, Smad2, Smad4, and Smad7 expression was mainly in hepatocytes of the preneoplastic lesions of a rat liver. Dysregulation of the downstream effectors of TGF-β such as TGR1, Smad2, Smad4 and, Smad7 might contribute to the progression of preneoplastic lesions during chemical hepatocarcinogenesis in a rat.

Key Words: Receptors, Transforming Growth Factor Beta; Signal Transduction; Precancerous Conditions; Carcinogens; Liver; Rats

INTRODUCTION

Transforming growth factor-β (TGF-β) family consists of a large number of structurally related polypeptide growth factors that have a crucial role in the regulation of cellular processes, such as cell division, differentiation, lineage determination, motility, adhesion, and apoptosis (1). Smads, small families of structurally related proteins, are signal transducers for the members of the TGF-β superfamily (1). They are molecules of relative molecular mass ranging from 42 kDa to 60 kDa with two regions of homology at the amino and carboxy terminus called the Mad-homology domains, abbreviated to MH1 and MH2, respectively, and connected together by a proline-rich linker sequence (2). Different members of the Smad family have different roles in TGF-β signaling. Smad2 and Smad3 are activated via carboxy-terminal phosphorylation by TGF-β Receptor Type-1 (TGR1) kinases. They form heterotrimeric complexes with Smad4, and thereby act in a pathway-restricted fashion (3). Smad2 and Smad3 are also called receptor-activated Smads. Smad4-receptor-activator Smads complexes then translocates into the nucleus and act as a TGF-β-induced transcriptional activator of target genes (4). So Smad4 is a central mediator of Smad function (5). In contrast, Smad6 and Smad7 function as inhibitors of TGF-β signaling. They bind to TGR1 and interfere with the phosphorylation of the receptor-activated Smads. Consequently, active heterotrimeric Smad complexes are not formed (6, 7). Smad6 preferentially inhibits bone morphogenetic protein (BMP) signaling and Smad7 inhibits TGF-β signaling (8-10). Moreover, because of the importance of the role of TGF-β in the regulation of fundamental cell homeostatic processes, it is not surprising that tumor cells from different origins are either partially or completely resistant to the anti-proliferative effect of TGF-β (11, 12). Although, alterations in the function and expression of TGF-β receptors have been shown to contribute to the resistance to the antiproliferative effects of TGF-β in cancer (13), little is known comparatively about the qualitative or quantitative alterations of Smads within the TGF-β pathway in cancer, especially hepatocarcinogenesis. Experimental hepatocarcinogenesis in rats occurs in distinctly defined stages: initiation, promotion, and progression. The resistant hepatocyte model developed by Solt and Farber has been widely used for studying multistep chemical hepatocarcinogenesis (14, 15). Clonal expansion of genetically altered
preneoplastic hepatocytes occurs during the stage of promotion. This expansion is caused by a selective increase in cell proliferation and a decrease in the apoptosis of genetically altered hepatocytes of initiation stage. However, the exact biological characteristics of preneoplastic lesions of the rat liver are still unclear concerning TGF-β signaling. Also the temporal expression and cellular localization of TGR1 and of Smads in chemical hepatocarcinogenesis are not clearly elucidated, especially in the promotion stage of rat chemical hepatocarcinogenesis. Therefore, we undertook this study to investigate the expressions and localizations of TGR1, Smad2, Smad4, and Smad7 by using semi-quantitative RT-PCR, immunohistochemistry during the promotion stage of chemical hepatocarcinogenesis in the rat.

**MATERIALS AND METHODS**

**Induction of Chemical Hepatocarcinogenesis**

Male Sprague-Dawley rats, weighing 200 g, were maintained on standard pelleted chow and had access to water ad libitum. Solt and Farber’s method was used to induce hepatocarcinogenesis. Briefly, rats were initiated with a single dose (200 mg/kg) of Diethylnitrosamine (DEN), (Sigma Chemical Co. St. Louis, MO, U.S.A.), administered intraperitoneally by injection. Two weeks after the initiation, all rats received a daily oral gavage of 10 mg/kg 2-acetylaminofluorene (2-AAF Sigma), for a period of up to 14 days. A partial hepatectomy was performed 1 week after the 2-AAF treatment. Three animals were sacrificed, as indicated, on 1, 3, 7, 14, 28, 42, and 56 days after the initiation of the partial hepatectomy. A sham operation was performed in the age-matched controls, which did not receive the AAF/DEN treatment. After gross examination of the sample, a portion of each sample was fixed in a 10% buffered formalin solution and routinely processed for hematoxylin and eosin staining and immunohistochemistry during the promotion stage of chemical hepatocarcinogenesis.

**RNA Extraction and Semi-quantitative RT-PCR**

Total RNA was isolated from freshly frozen tissue using an RNeasy Kit (Qiagen, Santa Clarita, CA, U.S.A.). cDNA was synthesized from 5 μg of total RNA using 2 μg of random hexamer (Pharmacia, Uppsala, Sweden), 10 mM dNTP (Boehringer Mannheim, Mannheim, Germany) and 200 U of M-MLV reverse transcriptase (GIBCO BRL) in a final volume of 25 μL. PCR was performed by using 1.25 mM of dNTP, 0.25 U of Taq polymerase (Perkin Elmer, Branchburg, NJ, U.S.A.), 10 pmol of primer pairs compatible with rat TGR1, Smad2, Smad4, Smad7, GST-P, and GAPDH as an internal control, and cDNA using a thermal cycler (Perkin Elmer, Branchburg, NJ, U.S.A.). The sequences, reaction conditions, number of cycles, and the size of product for each of the primer pairs used in this study are summarized in Table 1. The PCR cycling conditions used were: denaturation at 94°C for 1 min, annealing at 55-60°C for 1 min, extension at 72°C for 1 min, and with a final extension at 72°C for 10 min. PCR products were analyzed in a 1.5% agarose gel stained with ethidium bromide. The optical density of each band was measured by using a gelsizing device (Gel Doc 1000, Bio-Rad Laboratories, CA, U.S.A.). Densitometric analysis was used to determine the optical density ratios of TGR1, Smad2, Smad4, Smad7, GST-P versus GAPDH.

**Immunohistochemical Staining**

Sections were dewaxed and rehydrated according to a standard procedure, and washed with PBS. For immunohistochemical staining, sections were heated in a microwave oven at 600W for 2 times of 7 min and for 5 min in 0.01M citrate buffer (pH 6.0). Sections were then immersed in 3% H2O2 to quench endogenous peroxidase activity, and unspecified binding was blocked in 5% normal goat serum (0.1% BSA) was frozen in liquid nitrogen and kept at 70°C prior to RNA isolation. All experiments were conducted in accordance with the guidelines of the institutional review committee at Pusan National University School of Medicine.

**Table 1.** Primer sequences of TGR1, Smad2, Smad4, Smad7, GST-P, and GAPDH used in the present study

| Primers | Sequences | Annealing temp (°C) | No. of cycle | Product size (bp) |
|---------|------------|---------------------|--------------|-------------------|
| TGR1    | 5'-GCAGATTCTTCAGAAAAGCAGTCAGCTG-3' | 57               | 25           | 318               |
|         | 5'-GCCTCAGAACTCTTCCAAACCGACCT-3'  |                   |              |                   |
| Smad2   | 5'-CGATTCAGATGACATGCTCTA-3'       | 60               | 26           | 124               |
|         | 5'-CGCTCTGTTTGTAGCTAGG-3'         |                   |              |                   |
| Smad4   | 5'-GTTCAGATAGTCTCGAGG-3'          | 60               | 26           | 357               |
|         | 5'-GGATCCACAGTATCCATCCAC-3'       |                   |              |                   |
| Smad7   | 5'-CCAAGCTGACACTGCTCCAGA-3'       | 60               | 27           | 106               |
|         | 5'-CGGCTCCAGAAAGAAGTGG-3'         |                   |              |                   |
| GST-P   | 5'-AAATGTTGGAGAAGATGAGAGCT-3'     | 55               | 25           | 167               |
|         | 5'-GATAGTGTAGTATGAGAGG-3'         |                   |              |                   |
| GAPDH   | 5'-ACCACTGTCATGCCCATC-3'          | 57               | 25           | 452               |
|         | 5'-CCACCACCTCTTGTGCTGTA-3'        |                   |              |                   |
in PBS) or 5% normal rabbit serum (0.1% BSA in PBS). Immunohistochemical staining was performed by the avidin-biotin peroxidase complex method with aminoethylcarbazole as a chromogen, using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA, U.S.A.) according to the manufacturer’s instructions. Sections were counterstained with Mayer’s hematoxylin solution. To detect TGR1, Smad2, Smad4, Smad7 and GST-P, we used polyclonal antibodies against TGR1 (rabbit IgG, sc-398, Santa-Cruz, CA, U.S.A.), Smad2 (goat IgG, sc-6200, Santa-Cruz), Smad4 (goat IgG, sc-1909), Smad7 (goat IgG, sc-7004), and GST-P (311, MBL, Nagoya, Japan) were used at a dilution of 1:100, 1:50, 1:50, 1:100, and 1:1,000, respectively. Control experiments included incubation of the sections with normal rabbit or goat IgG or primary antibodies neutralized with an excess of blocking peptide, respectively.

Sequential Quantification of GST-P-positive Lesions

The expression of glutathione S-transferase placental form (GST-P) in focal areas of hepatocytes has been widely used as a marker to identify preneoplastic lesions in the rat liver (17). Areas of GST-P-positive lesions were measured by using a color image processor (Image Pro-plus, Mediacybernetics, Maryland, U.S.A.) and results are expressed in mm² of stained area per cm². Mean and standard deviation of the areas of GST-P-positive lesions per animal, for each area chosen, were used to generate the results shown in Fig. 1.

Detection of Apoptotic Cells

Cells undergoing programmed cell death (apoptosis) were detected in situ by specifically labeling nuclear DNA fragmentation (TUNEL method), using ApopTag in situ detection kit/peroxidase (Intergen, Purchase, NY, U.S.A.), in accordance with the manufacturer’s instructions for the detection of apoptotic cells.

Statistical Analysis

TGR1, Smad2, Smad4, and Smad7 positive cells under the light microscope at 400X were counted and expressed as a percentage of total nuclei counted, involving 1000 nuclei per animal in randomly selected preneoplastic lesions (including altered cellular foci and hyperplastic nodules) of rat liver. The number of TUNEL-positive apoptotic cells was counted in randomly selected preneoplastic lesions and adjacent liver parenchyma, respectively as same method. The results are expressed as means ± standard deviation (SD). The Kruskal-Wallis test was used for the statistical analysis. Significance was de-
Fig. 2. Semi-quantitative RT-PCR analysis shows the early increase of Smad2, Smad4, and TGR1 transcripts and a significant decrease in later steps of promotion (42 days after partial hepatectomy). Transcripts of Smad7 are more strongly expressed throughout the promotion stage than in sham operated control livers. There was no decrease of Smad7 transcripts during the later steps of the promotion stage. A significant increase in the GST-P transcripts is also noted (A). Densitometric analysis of semi-quantitative RT-PCR was used to obtain the relative of optical densities of Smad2 (●), Smad4 (▲), TGR1 (●), and Smad7 (◆) versus GAPDH (B). The figures represent three independent samples. The values shown are mean ± SD. S indicates the sham operated control liver.

Fig. 3. Immunohistochemical staining for TGR1. Some TGR1-positive cells are observed in altered cellular foci (A) 7 days after partial hepatectomy (PH), and an increased number of positive cells are noted in hyperplastic nodules (B) at 28 days after PH. Loss of the expression of TGR1 in hyperplastic nodules (C) at 42 days after PH is observed. TGR1 expression is noted in the cytoplasm of hepatocytes without linear reinforcement (D). Magnification at × 100 for A, B and C, and at × 200 for D.
RESULTS

Sequential Quantification of GST-P-positive Cells in Preneoplastic Lesions of Rat Liver

The histopathological findings of the chemical hepatocarcinogenesis induced by Solt and Farber's method resembled those described previously (14, 15). In brief, 1 month after partial hepatectomy, variable numbers of preneoplastic lesions (clear cell foci, basophilic foci, eosinophilic foci and hyperplastic nodules) were apparent. An increase in the number of hyperplastic nodules was observed with time. Because the expression of GST-P in focal areas of hepatocytes has been widely used as a marker to identify preneoplastic lesions in the rat liver (16), we performed semi-quantitative RT-PCR and immunohistochemistry for GST-P. Fig. 1 and Fig. 2A show that GST-P-positive preneoplastic lesions rapidly increased with time and this was related to the progression of preneoplastic lesions during chemical hepatocarcinogenesis.

Expression of TGR1, and Smads Transcripts in Preneoplastic Lesions of Rat Liver

To verify the temporal expressions of TGR1, Smad2, Smad4, and Smad7 transcripts in the promotion stage of chemical hepatocarcinogenesis, semi-quantitative RT-PCR was performed in each liver sample on 1, 3, 7, 14, 28, 42, and 56 days after partial hepatectomy. Fig. 2 showed that TGR1 transcripts were increased during the early promotion stage and peaked at 28 days after partial hepatectomy. However, TGR1 was decreased with time, especially during the later stage of the promotion (42 days after partial hepatectomy). The transcripts of Smad2 and Smad4 were increased during the early steps of the promotion stage and peaked at 14 and 7 days after partial hepatectomy, respectively. Thereafter, Smad2, and Smad4 were decreased with time (Fig. 2). Sham-operated control liver also showed lower levels of the of TGR1, Smad2, and Smad4 transcripts. In contrast to the other transcripts, Smad7 transcripts were more strongly expressed throughout the promotion stage than in the sham operated control liver, and there was no decrease in the level of Smad7 transcripts during the later steps of the promotion, as occurred for TRG1, Smad2, and Smad4.

Fig. 4. Immunohistochemical staining for Smad2. Smad2-positive cells increases with time, 7 days after partial hepatectomy (PH) (A), and 28 days after PH (B) in preneoplastic lesions. Decreased Smad2 in hyperplastic nodules is seen 56 days after PH (C). Smad2 is localized to the cytoplasm of preneoplastic hepatocytes (D). Magnification at ×100 for A, B and C, and at ×200 for D.
Expression and Localization of TGR1, Smads Proteins in Preneoplastic Lesions of the Rat Liver

To investigate the temporal expression and localization of TGR1, Smad2, Smad4, and Smad7 proteins during the promotion stage of chemical hepatocarcinogenesis, we performed immunohistochemistry in each liver sample taken from 1, 3, 7, 14, 28, 42, and 56 days after partial hepatectomy. Seven Days after partial hepatectomy, some TGR1 positive cells were noted in the preneoplastic lesions, and the adjacent liver parenchyma was either weakly positive or negative (Fig. 3A). With the lapse of time, the number of positive cells increased in the preneoplastic lesions, which presented as altered cellular foci and hyperplastic liver nodules (Fig. 3B). Forty two days after par-
At partial hepatectomy, the numbers of TGR1 positive cells were reduced (Fig. 3C) (Table 2). TGR1 positive cells were mainly hepatocytes, especially in preneoplastic lesions. TGR1 expression was mainly found in the cytoplasm of hepatocytes without linear reinforcement (Fig. 3D). Smad2 and Smad4 proteins showed similar expression patterns (Table 2); during the early

Fig. 6. Immunohistochemical staining for Smad7. Significant increases of Smad7-positive cells are noted with time, 7 (A), 28 (B), and 42 days after PH (C). Smad7 is localized to the cytoplasm of preneoplastic hepatocytes (D). Magnification at × 100 for A, B and C, at × 200 for D.

Fig. 7. Sequential changes in the TUNEL-positive apoptotic cells. TUNEL-positive cells increased with time and were more numerous in the adjacent liver parenchyma than the preneoplastic lesion (A). The values shown are mean ± SD from measurement upon randomly selected separate preneoplastic lesions (open bar) and adjacent liver parenchyma (solid bar). S, P, and L indicates the sham operated control liver, preneoplastic lesions, and adjacent liver parenchyma, respectively.
steps, positive cells increased in altered cellular foci and hyperplastic nodule and with the passage of time their numbers reduced. Scattered positive cells were also observed around the preneoplastic lesions (Fig. 4A, C and Fig. 5A, C). The expressions of Smad2 and Smad4 were mainly located in the cytoplasm of hepatocytes (Fig. 4D and 5D). Interestingly, Smad7 protein was strongly expressed in altered cellular foci and hyperplastic nodules, and positive cells were markedly increased with the increase with time (Fig. 6A, B). There was no decrease of Smad7 protein during the later steps of the promotion stage (Fig. 6C) (Table 2). Some scattered Smad7 positive cells were also observed around the preneoplastic lesions. The expression of Smad7 was mainly found in the cytoplasm of hepatocytes (Fig. 6D).

Sequential Quantification of TUNEL-positive Cells in Preneoplastic Lesions of Rat Liver

Sequential quantification of TUNEL-positive apoptotic cells showed that apoptotic cells increased with time and more numerous in the adjacent liver parenchyma than in the preneoplastic lesions during promotion stage of chemical hepatocarcinogenesis of the rat (p<0.05) (Fig. 7).

**DISCUSSION**

One notable observation made in this study was the decrease of TGR1, Smad2 and Smad4 expressions and the increase of Smad7 expression with time during the promotion stage of rat hepatocarcinogenesis. This finding suggests that the dysregulation of downstream effectors of TGF-β may contribute to the progression of preneoplastic lesions in chemical hepatocarcinogenesis in the rat. The TGF-β signaling pathway is a central component of the mechanisms that control cellular proliferation and apoptosis in the liver (18). The disruption of TGF-β signaling at the pre-receptor, receptor, and post-receptor levels that occurs can cause dysregulation of apoptosis and result in hepatocarcinogenesis in mice (19), rats (20-22), and humans (23, 24). These reports suggest that the resistance to the growth inhibitory and apoptotic effects of TGF-β are largely due to the loss of TGF-β receptors in hepatocellular carcinomas and in preneoplastic lesions of the liver. However, little is known of the involvement of Smads in the regulation of the TGF-β signaling pathway in liver cells and the possible disruption of these genes during hepatocarcinogenesis. Nevertheless, the impact that Smad genes have on both growth inhibition and apoptosis suggests that these genes may play a significant role in hepatocarcinogenesis. In our study, the down-regulations of TGR1, receptor activated Smad2 and common-mediated Smad4 were evident during the late promotion stage of chemical hepatocarcinogenesis. We thought that the down regulation of TGR1, Smad2, and Smad4 might be a means of resisting the anti-proliferative effects of TGF-β. However, the precise mechanisms of the down-regulation of TGR1 and Smads in preneoplastic lesions of rat liver are not known. Some studies have reported that several mutations of signaling molecules, including TGF-β family members, their receptors, or Smad genes might be evidences for disrupted signaling in many cancers, such as human and rat hepatocellular carcinomas (25, 26). Mutations of the Smad2-, and Smad4-encoding gene sequences, but not those of Smad3 or the inhibitory Smad6 or Smad7, have been detected in several carcinomas, but overall, are uncommon (27, 28). These observations suggest that some Smads act as tumor suppressors in carcinogenesis. Tumor-associated mutations in Smad2 and Smad4 occur most frequently in the MH2 domain, which mediates heteromeric complex formation and transcriptional activation (29, 30). Taken together, the down-regulation of TGR1, Smad2, and Smad4 might contribute to the expansion of preneoplastic lesions of rat liver. However, exact molecular mechanisms of such down regulation remain to be elucidated. In our study, increased Smad7 expression was seen during the promotion stage of chemical hepatocarcinogenesis in the rat. The increase of inhibitory smads, such as Smad6 and Smad7 might contribute to the progression of preneoplastic lesions in chemical hepatocarcinogenesis in rats by reducing the ability of TGF-β to inhibit their growth. This contention is in agreement to the finding that the endogenous TGF-β-mediated induction of Smad7 resulted in a higher "threshold" requirement for the anti-proliferative signals mediated by receptor-regulated Smads, and might be involved in reduced responsiveness to cytokines in some human hepatocellular carcinoma cells (29). Also, enhanced Smad7 levels, as observed in pancreatic carcinomas, may decrease Smad responsiveness (30). Increased expressions of Smad6 and Smad7 have been described in human pancreatic and rat prostatic carcinoma (30-32). Therefore, we suggest that inhibitory Smads, such as Smad7, may be involved in the progression of preneoplastic lesions in rat liver by inducing resistance to apoptotic death by TGF-β.

To investigate the role of TGR1 and Smads in hepatocarcinogenesis, the sites of their expressions and temporal nature of their expressions should be determined as a first step. In our study, in the early steps of the promotion stage of chemical hepatocarcinogenesis, TGR1, Smad2, Smad4 and Smad7 positive cells were observed to increase in preneoplastic lesions, and subsequently, TGR1, Smad2 and Smad4 positive cells decreased coincidently with result of RT-PCR. However, Smad7 positive cells did not decrease with time, and immunohistochemistry revealed that hepatocytes of preneoplastic lesions of rat liver were the main site of TGR1, Smad2, Smad4 and Smad7 expression. It is tempting to speculate, therefore, that the expressions of TGR1, Smad2, and Smad4 in hepatocytes of preneoplastic lesions, especially during the early times of the promotion stage, might contribute to the removal of genetically unaltered hepatocytes. In addition, the decreased expressions of TGR1, Smad2, Smad4, and the increased expression of Smad7 during the late times of the promotion stage might
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