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Activated Hepatic Stellate Cells Induce Tumor Progression of Neoplastic Hepatocytes in a TGF- β Dependent Fashion

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Abstract

The development of hepatocellular carcinomas from malignant hepatocytes is frequently associated with intra- and peritumoral accumulation of connective tissue arising from activated hepatic stellate cells. For both tumorigenesis and hepatic fibrogenesis, transforming growth factor (TGF)- β signaling executes key roles and therefore is considered as a hallmark of these pathological events. By employing cellular transplantation we show that the interaction of neoplastic MIM-R hepatocytes with the tumor microenvironment, containing either activated hepatic stellate cells (M1-4HSCs) or myofibroblasts derived thereof (M-HTs), induces progression in malignancy. Cotransplantation of MIM-R hepatocytes with M-HTs yielded strongest MIM-R generated tumor formation accompanied by nuclear localization of Smad2/3 as well as of β -catenin. Genetic interference with TGF- β signaling by gain of antagonistic Smad7 in MIM-R hepatocytes diminished epithelial dedifferentiation and tumor progression upon interaction with M1-4HSCs or M-HTs. Further analysis showed that tumors harboring disrupted Smad signaling are devoid of nuclear β -catenin accumulation, indicating a crosstalk between TGF- β and β -catenin signaling. Together, these data demonstrate that activated HSCs and myofibroblasts directly govern hepatocarcinogenesis in a TGF- β dependent fashion by inducing autocrine TGF- β signaling and nuclear β -catenin accumulation in neoplastic hepatocytes. These results indicate that intervention with TGF- β signaling is highly promising in liver cancer therapy.

The interaction between tumor cells and their microenvironment fundamentally affects cancer development by triggering cell proliferation and survival as well as the capability to invade the surrounding tissue for subsequent spreading and colonization (Bissell and Radisky, 2001; Bhowmick et al., 2004). In the majority of tumors, the aberrant networks of cytokines and chemokines including their cognate receptors are critically involved in the progression of primary cancer to fatal metastatic disease (Balkwill, 2004). The key cascades are regulated by paracrine and autocrine mechanisms which are central to the communication between cancer and neighboring cells, the latter collectively known as tumor-stroma (Coussens and Werb, 2002; Bhowmick and Moses, 2005). Depending on the complexity of the tissue context, the stroma includes activated fibroblasts, blood vessel cells and immune cells such as tumor-associated macrophages (TAMs) or leukocytes (Pollard, 2004).

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In the liver, hepatocytes represent the major cell type of the parenchyme, whereas the non-parenchymal compartment is composed of various cell types including hepatic stellate cells (HSCs; also referred to as Ito cells) and liver-specific macrophages, termed Kupffer cells. During liver injury due to viral infection or long-term insult of hepatotoxins, HSCs get activated to myofibroblasts (MFBs) in response to platelet-derived growth factor (PDGF) and transforming growth factor (TGF)- β (Friedman, 1999; Ramadori and Armbrust, 2001). These changes in cell fate of HSCs towards MFBs provide the cellular basis for the establishment of hepatic fibrosis and cirrhosis (Friedman et al., 2000; Pinzani et al., 2005), which are characterized by the vast remodeling of the extracellular matrix (ECM) and altered expression of growth factors (Friedman, 2003). Upon progression of hepatocellular carcinomas (HCCs), which develop from the aberrant growth of hepatocytes (Fausto, 1999; Thorgeirsson and Grisham, 2002), MFBs abundantly localize in fibrotic deposits and surround the parenchyme intra- and peritumorally. At this stage, malignant hepatocytes of human HCCs frequently show autocrine secretion of TGF- β 1 (Shirai et al., 1994; Bedossa et al., 1995; Factor et al., 1997), loss of tumor suppressors such as E-cadherin (Osada et al., 1996) and stabilization of nuclear β -catenin (Buendia, 2000).

We previously established a cellular model of HCC progression which reflects changes in epithelial plasticity leading to a metastatic phenotype of either cytoplasmic Met transgenic (Amicone et al., 1997) or p19^{ARF} null (MIM) hepatocytes (Mikula et al., 2004). The progression to higher malignancy of hepatocytes occurs through the collaboration of hyperactive Ras and TGF- β signaling. While MIM-R cells expressing oncogenic Ha-Ras display epithelial organization and show moderate tumorigenicity, MIM-R hepatocytes treated with TGF- β exhibit a fibroblastoid phenotype accompanied by a dramatic increase in malignancy towards metastatic properties (Gotzmann et al., 2002, 2006; Fischer et al., 2005). This model of hepatocellular cancer progression is particularly associated with downregulation of E-cadherin, nuclear accumulation of β -catenin as well as autocrine secretion of TGF- β , and is thus considered as a significant cellular correlate to human HCC progression (Gotzmann et al., 2004; Lee et al., 2004; Eger and Mikulits, 2005). In addition, we recently established p19^{ARF} deficient immortalized HSCs, referred to as M1-4HSCs, which show expression of characteristic marker proteins of activated HSCs such as alpha-smooth muscle actin (α -SMA), glial fibrillary acidic protein (GFAP), pro-collagen I and desmin (Proell et al., 2005). These non-tumorigenic M1-4HSCs undergo a further transition to MFBs in vitro upon treatment with TGF- β (termed M-HTs), and thus provide a highly suitable cellular tool to analyze the molecular and cellular mechanisms involved in liver fibrogenesis.

Non-parenchymal liver cells have been described as the major source of TGF- β , while hepatocytes of the healthy adult liver fail to express detectable levels of this cytokine (Rossmannith and Schulte-Hermann, 2001). The cell cycle inhibitory and proapoptotic function of TGF- β on healthy hepatocytes might, therefore, depend on paracrine regulation. In contrast, HCCs most frequently show high levels of TGF- β along with malignant progression (Bedossa et al., 1995), indicating a tumor-promoting role of TGF- β in liver tumorigenesis. Moreover, elevated TGF- β concentrations have been observed in sera of HCC patients (Shirai et al., 1994) and previous findings indicate that TGF- β 1 constitutively activates Smad2 in an autocrine fashion (Matsuzaki et al., 2000a). Yet, the ambiguous role of TGF- β in hepatocarcinogenesis as well as the specific contributions of the various non-parenchymal cell types in the paracrine to autocrine TGF- β regulation of hepatocytes in vivo still remains a matter of debate.

Here we employed a cellular transplantation model to analyze the epithelial-mesenchymal interaction by the crosstalk of malignant hepatocytes with the fibrotic stroma. This model of microenvironmental interaction with cancerous cells is based on (i) the TGF- β dependent

tumor progression of MIM-R hepatocytes (Gotzmann et al., 2002; Mikula et al., 2003, 2004; Fischer et al., 2005), (ii) activated M1-4HSCs or myofibroblastoid M-HTs which mimic the major fibrotic cell types (Proell et al., 2005), and (iii) the subcutaneous cotransplantation of these hepatic cell populations which allows to study a defined intercellular communication in vivo. We show that M1-4HSCs, and even more pronounced M-HT cells, strongly enhance tumor progression of epithelial MIM-R hepatocytes as observed by nuclear accumulation of Smad2/3 as well as β -catenin. These data implicate that the pathophysiological relationship between hepatic fibrosis and cancer progression critically relies on TGF- β which might be of particular relevance for the therapy of liver carcinoma.

MATERIALS AND METHODS

Cell culture

Immortalized MIM-1-4 hepatocytes (Mikula et al., 2004) and malignant MIM-R hepatocytes expressing oncogenic Ha-Ras were seeded on rat tail collagen-coated tissue culture plastic, and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics as described recently (Gotzmann et al., 2002; Fischer et al., 2005). MIM-1-4 cells were additionally supplemented with 40 ng/ml TGF- α (Sigma, St. Louis, MO), 30 ng/ml insulin-like growth factor (IGF)-II (Sigma) and 1.4 nM insulin (Sigma). MIM-R-Smad7 hepatocytes (MIM-RS7) were generated by retroviral transmission of MIM-R cells with the vector pBabeSmad7 harboring full length murine Smad7 cDNA (Liu et al., 2003) and subsequent selection with puromycin (Sigma). The pool of MIM-RS7 hepatocytes was kept in RPMI 1640 and 10% FCS as described for MIM-R hepatocytes. The immortalized M1-4HSCs HSC line was cultured in DMEM plus 10% FCS and antibiotics. Myofibroblastoid M-HTs derived from long-term treatment of M1-4HSCs with TGF- β 1 (R&D Systems, Minneapolis, MN) were propagated in DMEM supplemented with 10% FCS, 1 ng/ml TGF- β 1 and antibiotics as described recently (Proell et al., 2005). All cells were kept at 37°C and 5% CO₂ and routinely screened for the absence of mycoplasma.

Enzyme linked immunosorbent assay (ELISA) for TGF- β 1

For determination of TGF- β 1 secreted into the medium, cells were grown in RPMI plus 10% FCS for 24 h. ELISAs were performed in duplicate, using the human TGF- β 1 immunoassay according to the instructions of the manufacturer (Bender MedSystems, Vienna, Austria). All values were normalized to background measurements from respective growth media and calculated on the basis of a TGF- β 1 standard curve.

Tumor formation in vivo

The individual cell types were detached from tissue culture plates, washed with phosphate buffered saline (PBS) and cell numbers were determined using a multichannel cell analyzer (CASY, Schärfe Systems, Reutlingen, Germany). Subsequently, 1×10^6 cells of each cell type resuspended in 50 μ l Ringer solution, were subcutaneously injected each into three individual immunodeficient SCID/BALB/c mice as outlined recently (Mikula et al., 2003). The volumes of tumors were calculated employing the formula: diameter $\times$ diameter $\times$ length/2. Eighteen to twenty days after cell injection, tumors were surgically removed and further processed for immunohistochemistry and confocal immunofluorescence microscopy. Experiments were performed in triplicate and carried out according to the Austrian guidelines for animal care and protection. One representative out of two independent experiments is shown.

Immunohistochemistry

Experimental tumors were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Four micrometers thick sections were stained with hematoxylin and eosin for standard microscopy (Mikula et al., 2004). For immunohistochemistry, anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), anti- β -catenin (Upstate Biotechnology, Lake Placid, NY) and anti-Smad2/3 (Transduction Laboratories, Lexington, UK) antibodies were used each at a dilution of 1:100. Corresponding biotinylated secondary antibodies were employed and visualization was performed with the vectastain ABC kit using diaminobenzidine as substrate (Vector Laboratories, Burlingame, CA).

Confocal immunofluorescence microscopy

Cells were fixed and permeabilized as described recently (Mikula et al., 2003). Primary antibodies were used at following dilutions: anti-E-cadherin and anti- β -catenin (Transduction Laboratories), 1:100; anti-Smad2/3 (Transduction Laboratories), 1:100. After application of cy5-dye conjugated secondary antibodies (Jackson Laboratories, West-Grove, PA), imaging of cells was performed with a TCS-SP confocal microscope (Leica, Heidelberg, Germany).

Proliferation kinetics

Cells (1×10^5) were seeded in triplicate on petri dishes with medium in the absence or presence of TGF- β 1. The number of cells in the corresponding cell populations was determined periodically in a multichannel cell analyzer (CASY; Schärfe Systems). Cumulative cell numbers were calculated from the cell counts plus dilution factors as described recently (Fischer et al., 2005).

Immunoblotting

The preparation of cellular extracts, separation of proteins by SDS-polyacrylamide gel electrophoresis, and Immunoblotting were performed as described recently (Fischer et al., 2005). Immunological detection of proteins was performed with the SuperSignal detection system (Pierce Chemical Company, Rockford, IL). The primary antibodies were used at the dilutions: anti Smad7 (Santa Cruz Biotechnology), 1:1,000; anti-actin (Sigma), 1:2,500. Horseradish peroxidase-conjugated secondary antibodies (Calbiochem, LaJolla, CA) were used at dilutions of 1:10,000.

Transient transfections and reporter gene assays

Cells were plated at a density of 5×10^4 cells per 12-well plate 1 day before transfection. Lipofectamine Plus was used for transient transfections as recommended by the manufacturer (Invitrogen, Carlsbad, CA). To analyze the transcriptional response mediated by Smad3, cells were co-transfected with (CAGA)₁₂-Luc, containing concatemeric Smad3 consensus binding sites linked to the luciferase reporter, and a β -galactosidase reporter as described recently (Gotzmann et al., 2006). After cell lysis, the luciferase activity was determined by a Luminoskan (Labsystems, Farnborough, UK). All assays were performed in triplicate and results represent the average of three independent experiments after normalization to β -galactosidase activities.

Recovery of tumor cells

In order to recover GFP-positive MIM-R or MIM-RS7 hepatocytes from tumors, small slices of tumor tissue were minced with a sterile scalpel, incubated with 1,5 caseinolytic U/ml dispase (Sigma) in RPMI 1640 plus 10% FCS for 15 min at 37°C, and washed twice with PBS. Cells were filtered through a cell strainer (Becton Dickinson, Franklin Lakes, NJ), put

in culture plates and subcultured in RPMI 1640 supplemented with 15% FCS (Gotzmann et al., 2002).

Statistics

All results are expressed as mean $\pm$ standard error of the median (SEM). Comparisons between experimental tumors were performed using Student's *t*-test for paired and unpaired data. The significance was determined at $P < 0.05$.

RESULTS

Increased tumor formation of neoplastic hepatocytes after cotransplantation with hepatic HSCs or MFBs

We employed well-established cellular models mimicking hepatic fibrosis and HCC for subcutaneous cotransplantation into immunocompromized mice in order to accurately study hepatic tumor-stroma interaction in a defined *in vivo* context. Therefore, epithelial malignant MIM-R hepatocytes, derived from parental MIM-1-4 cells harboring hyperactive Ras (Fischer et al., 2005), were coinjected with equal amounts of either non-tumorigenic activated HSCs, termed M1-4HSCs, or non-tumorigenic myofibroblastoid derivatives, referred to as M-HTs (Proell et al., 2005). Both, non-parenchymal M1-4HSC and M-HT cells have recently been demonstrated to proliferate with very comparable kinetics (Proell et al., 2005). In addition, cotransplantation of malignant MIM-R cells with parental, non-tumorigenic MIM-1-4 hepatocytes was performed as control, showing tumor formation comparable to that of MIM-R hepatocytes alone (data not shown). Sixteen days after cell injections, the tumors generated by MIM-R hepatocytes cotransplanted with myofibroblastoid M-HT cells were twofold larger than control tumors from coinjections of MIM-R cells with parental MIM-1-4 hepatocytes (Fig. 1). On day 20 after cotransplantation, MIM-R cells plus M-HT derived tumors displayed an even threefold enhanced cancer formation as compared to control. Notably, cotransplantation of MIM-R plus M1-4HSCs showed also enforced tumor formation, although to a lower extent than those generated by interaction with M-HTs (Fig. 1). These observations indicated that both M1-4HSCs and myofibroblastoid M-HT cells, the latter to an increasing degree, are capable to positively affect tumor formation of malignant hepatocytes.

Fibrotic tumor-stroma causes nuclear accumulation of Smad2/3 as well as β -catenin in malignant hepatocytes

Next, we addressed the question whether the augmented tumor formation correlates with the progression in tumorigenesis. Therefore, MIM-R derived tumors generated either by cotransplantation with parental MIM-1-4 hepatocytes (control), M1-4HSCs or myofibroblastoid M-HT cells were subjected to histological analysis. H&E staining revealed differentiated control tumors containing MIM-R hepatocytes with an epithelial morphology (Fig. 2). In contrast, coinjection with the non-parenchymal cell types M1-4HSC and M-HT resulted in less differentiated tumors, as observed by the loss of the epithelial organization of MIM-R hepatocytes and their spindle-shaped morphology after interaction with M-HT cells (Fig. 2). Most remarkably, immunohistochemical analysis further showed nuclear accumulation of Smad2/3 upon interaction with M1-4HSC, and more pronounced, through the crosstalk with myofibroblastoid M-HTs (Fig. 2). These findings are comparable to recent data demonstrating that (i) the collaboration of hyperactive Ras with constitutive TGF- β signaling leads to the progression in tumorigenesis by the transition of epithelial MIM-R cells to a fibroblastoid and invasive phenotype, and that (ii) Smad2/3 and Smad4 accumulate in nuclei of MIM-R hepatocytes after TGF- β activation to establish and stabilize an autocrine regulation of TGF- β signaling which confers tumor-progressive functions of TGF- β (Gotzmann et al., 2002; Fischer et al., 2005). Interestingly, M1-4HSC cells have been

found to secrete significant amounts of TGF- β 1 in vitro. Moreover, M-HTs exceeded this level (Fig. S1, Supplementary Material), which might explain the malignant progression of MIM-R hepatocytes (Gotzmann et al., 2006). Similar to nuclear Smad2/3 stabilization after coinjection of neoplastic hepatocytes and non-parenchymal cells, β -catenin was found to delocalize from cell boundaries to nuclei in a large proportion of malignant MIM-R hepatocytes, particularly after cotransplantation with M-HT cells (Fig. 2). Together, these results indicate that the enhanced tumor formation of MIM-R hepatocytes after cotransplantation with M1-4HSCs or M-HTs correlates with an increased cancer progression which might depend on active TGF- β signaling induced in a paracrine fashion.

Genetic interference with TGF- β signaling impairs tumor formation and progression of neoplastic hepatocytes after cotransplantation

In order to analyze paracrine TGF- β regulation between neoplastic hepatocytes and non-parenchymal cells, we aimed at interfering with TGF- β signaling. Overexpression of antagonistic Smad7 has already been demonstrated to be effective in the disruption of TGF- β signaling due to inhibition of Smad phosphorylation in HCC and HSC cells (Matsuzaki et al., 2000b; Dooley et al., 2003). As expected, MIM-R hepatocytes expressing exogenous Smad7 (Liu et al., 2003), referred to as MIM-RS7, showed an about 1.5-fold overexpression of Smad7 (Fig. S2A, Supplementary Material) which was accompanied by a strong reduction of nuclear Smad2/3 accumulation after TGF- β 1 treatment in vitro (Fig. 3A). In line with these data, MIM-RS7 hepatocytes treated with TGF- β 1 showed impaired transactivation of a Smad3 responsive luciferase reporter when compared to MIM-R cells under these conditions (Fig. S2B, Supplementary Material). Exogenous expression of Smad7 prevented TGF- β mediated cell death in parental MIM-1-4 hepatocytes and resulted in an even higher proliferation rate of MIM-RS7 cells in the presence of TGF- β 1 as compared to MIM-R hepatocytes under these conditions (Fig. S3, Supplementary Material). Furthermore, MIM-RS7 hepatocytes generated subcutaneous tumors in SCID mice comparable to those derived from MIM-R cells by cotransplantation with parental MIM-1-4 cells (Fig. 3B). Interestingly, coinjection of MIM-RS7 with M-HT cells revealed that MIM-RS7 hepatocytes were efficiently blocked in malignant progression compared to tumors derived from MIM-R cells plus M-HTs (Fig. 3B). At day 14 and day 18 after cotransplantation, the MIM-R plus M-HT derived tumors showed again twofold and threefold increased tumors, respectively, whereas MIM-RS7 plus M-HT generated tumors were comparable to control levels obtained by coinjection of MIM-R and parental MIM-1-4 (Fig. 3B). In accordance with these data, MIM-RS7 cells were also devoid of enhanced tumor formation after cotransplantation with M1-4HSC cells (data not shown).

In fact, MIM-RS7 derived tumors at day 18 after coinjection with either M1-4HSCs (data not shown) or M-HTs showed hepatocytes with a differentiated morphology along with a poor frequency of Smad2/3 accumulation in nuclei (Fig. 4, upper and middle part). These observations were comparable to those differentiated tumors resulting from coinjections of both MIM-RS7 or MIM-R with parental MIM-1-4 hepatocytes (Figs. 2 and 4). Most remarkably, β -catenin was mainly distributed in the cytoplasm rather than localized in nuclei of MIM-RS7 hepatocytes cotransplanted with either M1-4HSCs (data not shown) or M-HTs (Fig. 4, lower part). In conclusion, these data indicate that the secretion of TGF- β by M1-4HSCs, or even more pronounced by M-HT cells, is responsible for the cancer progression of neoplastic MIM-R hepatocytes in vivo. Furthermore, these experiments provide genetic evidence that the paracrine regulation of TGF- β signaling is essential to induce the malignant progression of hepatocytes, which is indicated by active Smad signaling and by the nuclear accumulation of β -catenin.

The crosstalk of non-parenchymal cell types with neoplastic hepatocytes disintegrates cell adhesion in a TGF- β dependent fashion

GFP-positive malignant MIM-R and MIM-RS7 hepatocytes were recultivated from isolated tumor tissues and further analyzed for epithelial characteristics such as the adherens junction component E-cadherin, which crucially participates in intercellular communication, as well as β -catenin, which physically interacts with E-cadherin in healthy adult hepatocytes. As observed in vivo, MIM-R cells recovered from cotransplantation with parental epithelial MIM-1-4 hepatocytes showed localization of both E-cadherin and β -catenin at cell-to-cell boundaries indicating intact cell adhesion, whereas MIM-R hepatocytes after recultivation of coinjections with non-parenchymal M1-4HSCs displayed loss of E-cadherin and strongly reduced β -catenin levels at cell borders (Fig. 5, left part). Indeed, the abrogation of intact epithelial cell–cell contacts was even more pronounced in those MIM-R hepatocytes cotransplanted with M-HT cells, since β -catenin levels also declined below the detection limit of immunofluorescence microscopy (Fig. 5, left part). In sharp contrast, recovered GFP-positive MIM-RS7 hepatocytes showed clear staining of E-cadherin as well as β -catenin at cell–cell interactions irrespective of prior cotransplantation with either parenchymal MIM-1-4 or non-parenchymal M1-4HSC cells (Fig. 5, right part). Isolated MIM-RS7 hepatocytes after coinjection with M-HT cells also displayed E-cadherin and β -catenin at cell borders, although in more faint amounts, suggesting intact cell adhesion complexes and the ability of epithelial organization. These data indicate that activation of TGF- β signaling in malignant hepatocytes, induced by HSCs or MFBs in a paracrine manner, provides progression in tumorigenesis. The inhibition of TGF- β signaling through expression of antagonistic Smad7, however, is efficiently capable to counteract this event.

DISCUSSION

The interaction of tumor cells with the microenvironment has been recognized to be central for cancer progression and metastatic colonization (Witz and Levy-Nissenbaum, 2006). In particular, the determination of paracrine regulatory loops between cancerous cells and the host as well as the evaluation of their contribution to tumor cell homeostasis are of particular relevance. Yet, the molecular framework of the tumor-host crosstalk in the specific tissue context and its consequences on carcinogenesis are largely unknown and rather based on observations from individual cell types. In this study we tackled the issue on the progression of malignant hepatocytes and its modulation by activated HSCs and MFBs, the latter representing the major constituents of the peri- and intratumoral connective tissue (Friedman et al., 2000; Pinzani et al., 2005). The simultaneous xenografting of well-characterized hepatic cellular models rather than cocultivation in simple tissue culture or in three-dimensional gel matrices was considered to particularly accomplish this task. In vivo, we show evidence that paracrine feedback mechanisms governed by activated HSCs and MFBs strongly affect the malignant progression of neoplastic hepatocytes by (i) induction of active TGF- β signaling, (ii) nuclear accumulation of β -catenin, and (iii) abrogation of E-cadherin mediated cell-to-cell contacts. Genetic intervention with TGF- β signaling leading to loss of paracrine TGF- β regulation in neoplastic hepatocytes confirmed this finding. Hence, these data indicate that the tumor-progressive TGF- β signaling is induced by paracrine regulation in hepatocytes and furthermore, is molecularly linked to activation of β -catenin.

We recently showed that tumor progression in response to TGF- β depends on the synergism with Ras-Erk/MAPK signaling in MIM-R hepatocytes which provides cell cycle progression and resistance to apoptosis (Fischer et al., 2005). In this respect, TGF- β secreted by M1-4HSCs and to a larger extent by M-HTs is therefore predominantly responsible for the enforced tumor formation of neoplastic MIM-R hepatocytes. This cooperation of TGF- β and MAPK pathways is accompanied by cytoplasmic redistribution or even loss of the tumor-suppressor E-cadherin and the disruption of E-cadherin/ β -catenin complexes at cell

boundaries (Gotzmann et al., 2002, 2006), as observed in recultivated MIM-R hepatocytes cotransplanted with either M1-4HSCs or M-HTs (Fig. 5). Disintegration of adherens junction components has been described during the epithelial to mesenchymal transition (EMT) of MIM-R hepatocytes, which results in autocrine TGF- β secretion and gain in malignancy by acquisition of an invasive phenotype (Gotzmann et al., 2002). As reported recently, the transcriptional repression of E-cadherin by the TGF- β mediated induction of snail and delta-EF-1 might be responsible for the disruption of adherens junctions (Gotzmann et al., 2006). Hence, the crosstalk of neoplastic epithelial hepatocytes with HSCs/MFBs induces an EMT-like cellular signature depending on TGF- β , since interference with TGF- β signaling by employing cells expressing inhibitory Smad7 were capable to retain intact cell adhesion complexes.

Unexpectedly, β -catenin was found to localize in a subset of cell nuclei of MIM-R derived tumors cotransplanted with either M1-4HSCs or M-HTs (Fig. 2). This observation is of particular interest, since about 50% of human HCCs show nuclear β -catenin accumulation, thus representing a hallmark of hepatocarcinogenesis (de La Coste et al., 1998; Buendia, 2000). Conflictingly, various studies on nuclear β -catenin localization in human HCCs reported the correlation with differentiated tumors and good prognosis (Hsu et al., 2000; Mao et al., 2001), whereas others showed nuclear β -catenin linked to aggressive cancer entities and poor survival (Polakis, 2000; Wong et al., 2001). Our very recent study revealed that TGF- β causes the induction of PDGF signaling which subsequently activates β -catenin to nuclear localization (Fischer et al., manuscript submitted; Gotzmann et al., 2006). Most remarkably, active β -catenin has been found to associate with growth arrest of malignant MIM-R hepatocytes through expression of p16^{INK4A} and resistance to anoikis, the latter event being crucial for disseminating cancer cells to survive in the blood stream. However, it remains to be elucidated whether receptor regulated Smad2/3 and common Smad4 can interact with LEF upon hepatocellular carcinoma progression, as found during early embryogenesis of *Xenopus* (Attisano and Labbe, 2004).

The contribution of non-parenchymal liver cells such as Kupffer cells, activated HSCs and MFBs to the progression of HCCs is still an open question (De Bleser et al., 1997; Roth et al., 1998; Proell et al., 2005). The data obtained in this study show compelling evidence for the increased formation and progressively disordered architecture of tumors cotransplanted with either activated HSCs or MFBs (Figs. 1 and 2, middle and right part). Extrapolation of these findings with recent data supports clarification of the scenario, how paracrine and autocrine TGF- β regulation of hepatocytes and non-parenchymal liver cells are executed. As depicted in Figure 6, tumor associated macrophages (TAMs) comparable to activated Kupffer cells, most probably induced by stress conditions such as, for example, hypoxia (Tsukamoto et al., 1990), produce TGF- β which further stimulates activated HSCs to complete the transition to MFBs. TAMs, activated HSCs and MFBs each on its own secrete TGF- β , and positively regulate the progression of neoplastic hepatocytes in a paracrine fashion. Hepatocytes proceeding in tumorigenesis and displaying an EMT-like, invasive phenotype show active TGF- β signaling and produce TGF- β themselves by conducting autocrine TGF- β regulation. The reciprocal relationship between the various non-parenchymal liver cell types of the hepatic tumor-stroma and the malignant hepatocytes by paracrine and autocrine regulation of TGF- β signaling might explain the progression of HCCs at the molecular and cellular level. Since HCC develops from the chronically injured liver, which involves stimulation of Kupffer cells and activation of HSCs to MFBs, these non-parenchymal liver cells are the major source of TGF- β production, and thus being predominantly responsible for the progression of initiated tumor nodes.

Most of the recent studies dealing with cell–cell interactions in vitro commonly suffer from drawbacks on cell culture media or supplemented growth factor cocktails and frequently do

not properly reflect the conditions in vivo. In this regard, the cellular transplantation model far more mimics the pathological tumor situation at physiological levels. By employing these in vivo conditions, we were able to provide evidence that activated HSCs and MFBs induce TGF- β driven cancer progression of hepatocytes. These results suggest that pharmacological targeting of TGF- β signaling in late stage hepatocarcinogenesis is effective in interfering with both the malignant progression of hepatocytes as well as with the tumor promoting consequences of the hepatic stroma. On the one hand, intervention with TGF- β signaling using soluble TGF- β antagonists might inhibit the transition of activated HSCs to MFBs as well as the secretion of TGF- β and its subsequent paracrine regulation on malignant hepatocytes. On the other hand, the same antagonists destabilize autocrine regulation of TGF- β signaling of hepatocytes at advanced malignant stages. These synergistic aspects provide the clear cut rationale for the development and application of efficacious anti-TGF- β drugs in HCC therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

α-SMA	alpha smooth muscle actin
ECM	extracellular matrix
GFAP	glial fibrillary acidic protein
HSC	hepatic stellate cell
MFB	myofibroblast
PDGF	platelet-derived growth factor
SCID	severe combined immunodeficiency
TAM	tumor associated macrophage
TGF	transforming growth factor

LITERATURE CITED

- Amicone L, Spagnoli FM, Spath G, Giordano S, Tommasini C, Bernardini S, De Luca V, Della Rocca C, Weiss MC, Comoglio PM, Tripodi M. Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes. *EMBO J.* 1997; 16:495–503. [PubMed: 9034332]
- Attisano L, Labbe E. TGFbeta and Wnt pathway cross-talk. *Cancer Metastasis Rev.* 2004; 23:53–61. [PubMed: 15000149]
- Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer.* 2004; 4:540–550. [PubMed: 15229479]

- Bedossa P, Peltier E, Terris B, Franco D, Poynard T. Transforming growth factor-beta 1 (TGF-beta 1) and TGF-beta 1 receptors in normal, cirrhotic, and neoplastic human livers. *Hepatology*. 1995; 21:760–766. [PubMed: 7875675]
- Bhowmick NA, Moses HL. Tumor-stroma interactions. *Curr Opin Genet Dev*. 2005; 15:97–101. [PubMed: 15661539]
- Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004; 432:332–337. [PubMed: 15549095]
- Bissell MJ, Radisky D. Putting tumours in context. *Nature Rev Cancer*. 2001; 1:46–54. [PubMed: 11900251]
- Buendia MA. Genetics of hepatocellular carcinoma. *Semin Cancer Biol*. 2000; 10:185–200. [PubMed: 10936068]
- Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002; 420:860–867. [PubMed: 12490959]
- De Bleser PJ, Niki T, Rogiers V, Geerts A. Transforming growth factor-beta gene expression in normal and fibrotic rat liver. *J Hepatol*. 1997; 26:886–893. [PubMed: 9126804]
- de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA*. 1998; 95:8847–8851. [PubMed: 9671767]
- Dooley S, Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, Ten Dijke P, Gressner AM. Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology*. 2003; 125:178–191. [PubMed: 12851882]
- Eger A, Mikulits W. Models of epithelial to mesenchymal transition. *Drug Discov Today: Dis Models*. 2005; 2:57–63.
- Factor VM, Kao CY, Santoni-Rugiu E, Voitach JT, Jensen MR, Thorgeirsson SS. Constitutive expression of mature transforming growth factor beta1 in the liver accelerates hepatocarcinogenesis in transgenic mice. *Cancer Res*. 1997; 57:2089–2095. [PubMed: 9187100]
- Fausto N. Mouse liver tumorigenesis: Models, mechanisms, and relevance to human disease. *Semin Liver Dis*. 1999; 19:243–252. [PubMed: 10518304]
- Fischer AN, Herrera B, Mikula M, Proell V, Fuchs E, Gotzmann J, Schulte-Hermann R, Beug H, Mikulits W. Integration of Ras subeffector signaling in TGF-beta mediated late stage hepatocarcinogenesis. *Carcinogenesis*. 2005; 26:931–942. [PubMed: 15705598]
- Friedman SL. Cytokines and fibrogenesis. *Semin Liver Dis*. 1999; 19:129–140. [PubMed: 10422196]
- Friedman SL. Liver fibrosis—from bench to bedside. *J Hepatol*. 2003; 38(Suppl 1):S38–S53. [PubMed: 12591185]
- Friedman SL, Maher JJ, Bissell DM. Mechanisms and therapy of hepatic fibrosis: Report of the AASLD Single Topic Basic Research Conference. *Hepatology*. 2000; 32:1403–1408. [PubMed: 11093750]
- Gotzmann J, Huber H, Wolschek M, Jansen B, Schulte-Hermann R, Beug H, Mikulits W. Hepatocytes convert to a fibroblastoid-like phenotype through the cooperation of TGF-b1 and Ha-Ras: Steps towards invasiveness. *J Cell Sci*. 2002; 115:1189–1202. [PubMed: 11884518]
- Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, Mikulits W. Molecular aspects of epithelial cell plasticity: Implications for local tumor invasion and metastasis. *Mutat Res*. 2004; 566:9–20. [PubMed: 14706509]
- Gotzmann J, Zojer M, Fischer ANM, Mikula M, Proell V, Huber H, Jechlinger M, Waerner T, Weith A, Beug H, Mikulits W. A crucial function of PDGF in TGF-beta mediated cancer progression of hepatocytes. *Oncogene*. 2006 [Epub ahead of print].
- Hsu HC, Jeng YM, Mao TL, Chu JS, Lai PL, Peng SY. Beta-catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. *Am J Pathol*. 2000; 157:763–770. [PubMed: 10980116]
- Lee JS, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, Thorgeirsson SS. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. *Nat Genet*. 2004; 36:1306–1311. [PubMed: 15565109]

- Liu X, Lee J, Cooley M, Bhogte E, Hartley S, Glick A. Smad7 but not Smad6 cooperates with oncogenic ras to cause malignant conversion in a mouse model for squamous cell carcinoma. *Cancer Res.* 2003; 63:7760–7768. [PubMed: 14633701]
- Mao TL, Chu JS, Jeng YM, Lai PL, Hsu HC. Expression of mutant nuclear beta-catenin correlates with non-invasive hepatocellular carcinoma, absence of portal vein spread, and good prognosis. *J Pathol.* 2001; 193:95–101. [PubMed: 11169521]
- Matsuzaki K, Date M, Furukawa F, Tahashi Y, Matsushita M, Sakitani K, Yamashiki N, Seki T, Saito H, Nishizawa M, Fujisawa J, Inoue K. Autocrine stimulatory mechanism by transforming growth factor beta in human hepatocellular carcinoma. *Cancer Res.* 2000a; 60:1394–1402. [PubMed: 10728705]
- Matsuzaki K, Date M, Furukawa F, Tahashi Y, Matsushita M, Sugano Y, Yamashiki N, Nakagawa T, Seki T, Nishizawa M, Fujisawa J, Inoue K. Regulatory mechanisms for transforming growth factor beta as an autocrine inhibitor in human hepatocellular carcinoma: Implications for roles of smads in its growth. *Hepatology.* 2000b; 32:218–227. [PubMed: 10915727]
- Mikula M, Gotzmann J, Fischer AN, Wolschek MF, Thallinger C, Schulte-Hermann R, Beug H, Mikulits W. The proto-oncoprotein c-Fos negatively regulates hepatocellular tumorigenesis. *Oncogene.* 2003; 22:6725–6738. [PubMed: 14555986]
- Mikula M, Fuchs E, Huber H, Beug H, Schulte-Hermann R, Mikulits W. Immortalized p19ARF null hepatocytes restore liver injury and generate hepatic progenitors after transplantation. *Hepatology.* 2004; 39:628–634. [PubMed: 14999681]
- Osada T, Sakamoto M, Ino Y, Iwamatsu A, Matsuno Y, Muto T, Hirohashi S. E-cadherin is involved in the intrahepatic metastasis of hepatocellular carcinoma. *Hepatology.* 1996; 24:1460–1467. [PubMed: 8938181]
- Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver diseases: Diagnosis and management. *J Hepatol.* 2005; 42(Suppl(1)):S22–S36. [PubMed: 15777570]
- Polakis P. Wnt signaling and cancer. *Genes Dev.* 2000; 14:1837–1851. [PubMed: 10921899]
- Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer.* 2004; 4:71–78. [PubMed: 14708027]
- Proell V, Mikula M, Fuchs E, Mikulits W. The plasticity of p19(ARF) null hepatic stellate cells and the dynamics of activation. *Biochim Biophys Acta.* 2005; 1744:76–87. [PubMed: 15878400]
- Ramadori G, Armbrust T. Cytokines in the liver. *Eur J Gastroenterol Hepatol.* 2001; 13:777–784. [PubMed: 11474306]
- Rossmann W, Schulte-Hermann R. Biology of transforming growth factor beta in hepatocarcinogenesis. *Microsc Res Tech.* 2001; 52:430–436. [PubMed: 11170302]
- Roth S, Gong W, Gressner AM. Expression of different isoforms of TGF-beta and the latent TGF-beta binding protein (LTBP) by rat Kupffer cells. *J Hepatol.* 1998; 29:915–922. [PubMed: 9875638]
- Shirai Y, Kawata S, Tamura S, Ito N, Tsushima H, Takaishi K, Kiso S, Matsuzawa Y. Plasma transforming growth factor-beta 1 in patients with hepatocellular carcinoma. Comparison with chronic liver diseases. *Cancer.* 1994; 73:2275–2279. [PubMed: 7513247]
- Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet.* 2002; 31:339–346. [PubMed: 12149612]
- Tsukamoto H, Gaal K, French SW. Insights into the pathogenesis of alcoholic liver necrosis and fibrosis: Status report. *Hepatology.* 1990; 12:599–608. [PubMed: 2205558]
- Witz IP, Levy-Nissenbaum O. The tumor microenvironment in the post-PAGET era. *Cancer Lett.* 2006 [Epub ahead of print].
- Wong CM, Fan ST, Ng IO. beta-Catenin mutation and overexpression in hepatocellular carcinoma: Clinicopathologic and prognostic significance. *Cancer.* 2001; 92:136–145. [PubMed: 11443619]

Fig. 1.

Cotransplantation of non-tumorigenic M1-4HSCs or M-HTs increases tumor formation of malignant MIM-R hepatocytes. The tumors were generated from MIM-R hepatocytes subcutaneously coinjected with either parental MIM-1-4 hepatocytes (white bars), immortalized M1-4HSCs (gray bars) or myofibroblastoid M-HTs (black bars) into immunocompromized recipient SCID mice. Error bars denote SEM. The star indicates $P < 0.05$.

Fig. 2.

Increased tumor formation is associated with nuclear localization of Smad2/3 and β -catenin. Tumor tissues were collected 20 days after cotransplantation. Morphology of tumor cells as shown by standard histology with hematoxylin/eosin staining (H&E). To visualize exogenous MIM-R hepatocytes, tumor sections were stained with anti-GFP antibody. Cotransplanted MIM-R hepatocytes show nuclear accumulation of Smad2/3 and β -catenin in the context of M1-4HSCs, and to a larger extent, when interacting with myofibroblastoid M-HTs. Insets show stainings of tumor sections at higher magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3.

Smad7 overexpression disrupts TGF- β signaling in MIM-RS7 cells and diminishes M-HT driven tumor formation in vivo. **A:** As visualized by confocal immunofluorescence analysis, MIM-R cells show nuclear translocation of Smad2/3 after 4 h of TGF- β 1 treatment (2.5 ng/ml) in culture, whereas MIM-RS7 cells display rare to absent nuclear Smad2/3 localization under these conditions. **B:** MIM-R (white bars) or MIM-RS7 hepatocytes (light gray bars) were subcutaneously coinjected with MIM-1-4 cells. In addition, MIM-R (dark gray bars) or MIM-RS7 cells (black bars) were each cotransplanted with M-HTs. Tumor formation is denoted by tumor volumes after cell transplantation into SCID mice and is expressed in percent of the highest level (100%). Error bars denote SEM. The star indicates $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 4.

Disruption of TGF- β signaling by overexpression of antagonistic Smad7 suppresses the tumor progression induced by M-HTs in vivo. Tumor tissues were collected 18 days after cotransplantation. Immunohistochemistry employing standard histology (H&E, upper part) as well as anti-Smad2/3 (middle part) and anti- β -catenin (lower part) antibodies. Insets represent staining of tumor sections at higher magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5.

Disintegration of cell adhesion by paracrine TGF- β regulation. Recovery of MIM-RS7 hepatocytes cotransplanted with M1-4HSCs or M-HT cells reveals epithelial organization (right part), whereas MIM-R hepatocytes coinjected with M1-4HSCs or M-HTs show loss of cell–cell adhesion (left part). GFP-positive hepatocytes were recultivated from tumors generated by MIM-R and MIM-RS7 hepatocytes which were each cotransplanted with either MIM-1-4, M1-4HSCs or M-HTs. Recovered cells were subjected to immunofluorescence analysis by staining with anti-E-cadherin and anti- β -catenin antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6.

Simplified model depicting TGF- β driven hepatocellular tumor-stroma interactions. The crosstalk of tumor-associated macrophages (TAMs) with activated HSCs and neoplastic hepatocytes induces TGF- β dependent activation to myofibroblasts (MFBs) and a gain in malignancy of hepatocytes, respectively. Activated HSCs and MFBs as well as TAMs secrete TGF- β and positively regulate the progression of malignant hepatocytes in a paracrine fashion, which finally leads to tumor-progressive and autocrine TGF- β signaling. Red arrows, paracrine TGF- β regulation; winded red arrows, autocrine TGF- β secretion; dotted red arrows, maintenance of autocrine TGF- β regulation by paracrine regulation. Yellow to green shaded arrows; activation of HSCs to MFBs; blue to purple shaded arrows, malignant progression of hepatocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]