Epithelial to Mesenchymal Transition in Madin-Darby Canine Kidney Cells Is Accompanied by Down-regulation of Smad3 Expression, Leading to Resistance to Transforming Growth Factor-β-induced Growth Arrest*

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In normal epithelial cells, transforming growth factor-β (TGF-β) typically causes growth arrest in the G1 phase of the cell cycle and may eventually lead to apoptosis. However, transformed cells lose these inhibitory responses and often instead show an increase in malignant character following TGF-β treatment. In the canine kidney-derived epithelial cell line, MDCK, synergism between activation of the Raf/MAPK pathway and the resulting autocrine production of TGF-β triggers transition from an epithelial to a mesenchymal phenotype. During this process, these cells become refractive to TGF-β-induced cell cycle arrest and apoptosis. TGF-β signals are primarily transduced to the nucleus through complexes of receptor-regulated Smads, Smad2 and Smad3 with the common mediator Smad4. Here we show that the transition from an epithelial to mesenchymal phenotype is accompanied by gradual down-regulation of expression of Smad3. Restoration of Smad3 to previous levels of expression restores the cell cycle arrest induced by TGF-β without reverting the cells to an epithelial phenotype or impacting on the MAPK pathway. Regulation of apoptosis is not affected by Smad3 levels. These data attribute to Smad3 a critical role in the control of cell proliferation by TGF-β, which is lost following an epithelial to mesenchymal transition.

Transforming growth factor-β (TGF-β) plays two rather paradoxical roles in cancer (1–3). At early stages, it acts as a tumor suppressor primarily through its ability to induce growth arrest and apoptosis in epithelial cells from which the majority of human tumors are derived. However, as tumors progress, they frequently become resistant to the growth inhibitory and pro-apoptotic effects of TGF-β, and at late stages, TGF-β acts as a tumor promoter. It acts directly on tumor cells to enhance epithelial to mesenchymal transition (EMT), increase motility, invasiveness, and metastasis and acts indirectly on the surrounding stroma to enhance angiogenesis and decrease immune surveillance (1–3).

We previously described a model tissue culture system that exhibits certain parallels to the process of tumorigenesis with respect to the role of TGF-β signaling (4). Madin-Darby canine kidney (MDCK) cells are an untransformed immortalized dog kidney epithelial cell line that are characterized by the formation of a simple monolayer of cells with high electrical resistance across it and the expression of epithelial markers such as E-cadherin and ZO-1, which are components of adherens and tight junctions (5). The activation in these cells of the Raf/ERK MAPK pathway by an inducible Raf construct, ΔRaf-ER, causes autocrine expression of TGF-β (4). This synergizes with ERK MAPK to induce a gradual change in phenotype from epithelial to mesenchymal, a process during which epithelial markers are lost, adherens and tight junctions degrade, and the cells express mesenchymal markers such as vimentin and become more motile (4). The activation of Raf in MDCK cells leads to rapid induction of protection from apoptosis induced by exogenous TGF-β and other death stimuli (within 1 day) followed by much slower protection from the specific growth inhibitory effects of TGF-β. Cells remain responsive to TGF-β even after prolonged Raf activation, showing increased invasive behavior.

The signaling pathways downstream of the TGF-β receptors are now known in some detail. The major signal transducers are the Smads. Receptor activation leads to phosphorylation and activation of the receptor-regulated Smads, Smad2 and Smad3. They form complexes with the common mediator Smad4, that accumulate in the nucleus and are directly involved in transcriptional activation of target genes, usually in conjunction with other transcription factors (6).

Here we have investigated how the TGF-β signaling pathway is perturbed in these Raf-expressing MDCK cells to allow them to become specifically resistant to the anti-proliferative effects of TGF-β while maintaining other TGF-β responses. We demonstrate that a gradual down-regulation of Smad3 during the process of EMT is sufficient for the cells to lose their ability to undergo growth arrest in response to TGF-β. Responsiveness is restored upon re-expression of Smad3 to previous levels of expression. These data suggest that Smad3 plays a critical role in the control of cell proliferation by TGF-β, which is lost following EMT.
MATERIALS AND METHODS

P19Aces—pBabe-puro EGFP-tagged ΔRaf-1:hbER DD (referred to as ΔRaf-ER) was kindly provided by M. McMahon (7). Human Smad3 cDNA was subcloned into the EcoRI/SacI cloning sites of the pBabe-hygromycin and pBabe-bleomycin vectors (8). The CAGA-lucerase reporter was described previously (9).

Cell Culture and Retroviral Infection—MDCK cell lines stably expressing ΔRaf-ER and their derivative RafT cells, which had been grown continuously in 100 nM 4-OHT tamoxifen (4HT), have been described previously (4). They express the ectopic retrovirus receptor. MDCK RafT cells stably expressing human Smad3 were generated by retroviral infection. GP2 packaging cells were transfected with LipofectAMINE (Invitrogen) with either Smad3-pBabe-hygromycin, or empty vector and then selected with the appropriate antibiotic (100 μg/ml hygromycin or 25 μg/ml bleomycin) for 8 days. MDCK RafT cells were then incubated with retrovirus-containing supernatants and then cultured in medium containing the appropriate antibiotic as described above to select for virus-infected cells. TGF-β (PeproTech) was used at the concentrations indicated in the figure legends. The TGF-β type I receptor inhibitor SB-431542 was used as described previously (10).

Whole Cell and Nuclear Extracts—Nuclear extracts were prepared as described previously (11). Whole cell extracts were prepared in buffer containing 1% Triton X-100 as described previously (12) with the exception of the experiment shown in Fig. 4A where cells were lysed directly into SDS-PAGE gel sample buffer and the experiment shown in Fig. 6A. Whole cell extracts were prepared in RIPA buffer.

Antibodies, Western blotting, Electrophoretic Mobility Shift Assay (EMSA), and Indirect Immunofluorescence—The following antibodies were used: ERK2/p42 MAPK (raised against the peptide CEETARFQP-RGYS); cyclin A (H432, Santa Cruz Biotechnology); Smad4 (B8, Santa Cruz Biotechnology); Smad2, Smad3, and Smad4 (Transduction Laboratories); Smad3 (Zymed Laboratories Inc.); E-cadherin (Transduction Laboratories); p21WAF1/CIP1 (Santa Cruz Biotechnology); pBb (BD Biosciences); and GRB2 (Transduction Laboratories). The anti-PCNA antibody (PC10) was obtained from the Imperial Cancer Research Fund hybridoma unit. For Western blotting, proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore), and immunoactive proteins were visualized by ECL (Amersham Biosciences). EMSAs and indirect immunofluorescence microscopy were performed as described previously (4).

Cell Cycle Analysis—To examine cell cycle distribution, cells were fixed in 70% ethanol, treated with ribonuclease (100 μg/ml) for 5 min at room temperature, stained with 50 μg/ml propidium iodide (BD Biosciences) for 5 min, and analyzed by flow cytometry using excitation at 488 nm.

RNase Protection Assays—All of the probes with the exception of that for E-cadherin used for RNase protection were designed against the human sequences but cross-reacted with the dog sequences. The Smad2 probe recognized the region encoding amino acids 9–86 of the human sequence; Smad3, the region encoding amino acids 107–208; Smad4, the region encoding amino acids 94–227; and GAPDH, the region encoding amino acids 18–77. The γ-actin probe was as described previously (13). For the E-cadherin probe, an E-cadherin fragment was prepared by RT-PCR from total RNA from MDCK cells using the primers (forward 5′-TGACAGGAGCTGATGAG-3′ and reverse 5′-CTCGTCTCT-CAGGACCTGTC-3′) and subcloned into pGEM-T vector (Promega) for probe preparation. All of the probe preparation and RNase protection assays were as described previously (13, 14).

RESULTS AND DISCUSSION

Long Term Activation of Raf Leads to Down-regulation of Smad3 Expression—To understand how MDCK cells alter their response to exogenous TGF-β during the process of Raf-induced EMT, we examined the functions of the Smads in cells expressing ΔRaf-ER but in its inactive form and in the same cells treated with 4HT for different times to activate the ΔRaf-ER. Initially, the translocation of the three TGF-β-regulated Smads (the receptor-regulated Smads, Smad2 and Smad3, and the common mediator Smad, Smad4) to the nucleus upon TGF-β treatment was studied (Fig. 1A). TGF-β-induced translocation of Smad4 to the nucleus was observed in MDCK cells expressing ΔRaf-ER that were untreated or cells that had been induced with 4HT for a short (24 h) or long (14 days) period. Cells growing continuously in 4HT (RafT) also showed this translocation. Similar results were seen for the translocation of Smad2. However, we noted that in cells in which Raf had been activated for longer periods and in which EMT had occurred (see below), Smad3 failed to translocate to the nucleus in response to TGF-β treatment. As expected, in all cases, 4HT treatment induced the phosphorylation of ERK2 as visualized by a shift in mobility in an ERK2 Western blot.

Overall, the expression of Smad proteins in these cells was analyzed in whole cell extracts (Fig. 1B). Short or long term activation of Raf did not affect expression levels of Smad2 and Smad4. However, the activation of Raf for long periods caused a marked decrease in the level of Smad3. After 14 days of Raf activation, Smad3 expression was markedly reduced, and moreover, in cells grown continuously in 4HT (RafT cells), Smad3 could not be detected. Both of these conditions caused EMT as demonstrated by the loss of the epithelial marker E-cadherin. To determine whether the expression of Smad3 was altered at the transcriptional level, the mRNA levels of Smad2, Smad3, and Smad4 were directly measured by RNase protection assay (Fig. 1C). As a control for EMT, the levels of E-cadherin mRNA were also monitored. The level of Smad3 mRNA was very strongly reduced in RafT cells and also in MDCK ΔRaf-ER cells treated with 4HT for 14 days. The E-cadherin probe detected no E-cadherin mRNA in RafT cells and minimal amounts in MDCK ΔRaf-ER cells treated with 4HT for 12 or 14 days. Smad2 and Smad4 mRNA levels were not altered during the process of EMT. Taken together, these results demonstrate that Smad3 expression is down-regulated in MDCK cells that have undergone EMT. The time course of loss of Smad3 expression may lag marginally behind that of E-cadherin loss. Because the reduction in expression of E-cadherin during EMT is thought to involve methylation of the regulatory region of its gene (15), it is possible that methylation may also be involved in the loss of Smad3 expression.

To address the question of whether long term activation of Raf is sufficient to induce loss of Smad3 in the absence of EMT, the function of the type I TGF-β receptor ALK5 was inhibited using the potent and specific drug SB-431542 (10). This blocks the operation of the Raf-induced TGF-β autocrine loop that is essential for EMT (4) and allows us to assay the effects of Raf signaling in the absence of TGF-β signaling. In the presence of SB-431542, prolonged Raf activation failed to induce EMT as assessed by E-cadherin expression levels in immunoblot (Fig. 1D) and immunofluorescence (data not shown). As well as preventing Raf-induced E-cadherin loss, SB-431542 also blocked down-regulation of Smad3 expression (Fig. 1D), indicating that prolonged Raf activation is not sufficient to cause loss of Smad3 expression in the absence of EMT.

A related issue is whether Smad3 down-regulation could occur in response to prolonged TGF-β signaling in the absence of Raf stimulation. However, in the absence of Raf activation by 4HT treatment, TGF-β induces apoptosis in MDCK ΔRaf-ER cells (data not shown) (4), so this cannot be addressed directly. It cannot be ruled out that prolonged TGF-β signaling under conditions where apoptosis or growth arrest are blocked by means other than Raf activation would lead to reduction in Smad3 expression.

Re-expression of Smad3 in MDCK RafT Cells—To determine whether the loss of Smad3 expression seen during EMT was responsible for the altered responsiveness to TGF-β, we restored the levels of Smad3 expression in RafT cells by introducing human Smad3 cDNA in retroviral expression vectors. Two different vectors for Smad3 were used, one being selectable in hygromycin and the other in bleomycin. After drug selection, individual cell clones were grown up from pools of resistant cells. Three clones were chosen for further study, two
of which showed fairly similar levels of expression of Smad3 to wild type MDCK and uninduced MDCK ΔRaf-ER cells (H2, H3) while a third clone, B4b, had higher levels (Fig. 2A). These clones along with parental MDCK RafT cells were maintained in 4HT to maintain the activation of ΔRaf-ER. To test the functionality of Smad3, its translocation to the nucleus in response to TGF-β was assessed (Fig. 2A). As noted previously, Smad3 translocates to the nucleus in response to TGF-β in MDCK ΔRaf-ER cells not treated with 4HT but is absent from RafT cells. The stimulation of RafT clones H2 and H3, which re-express Smad3, with TGF-β induced its translocation into the nucleus at similar levels as in ΔRaf-ER cells. In the case of clone B4b, high levels of Smad3 protein were found in the nucleus before stimulation with TGF-β, most probably because of the high overexpression of Smad3 in this clone. In all of the cases with the exception of B4b, the amount of nuclear phosphoSmad2 upon TGF-β induction was close to normal levels. In the case of B4b, very low levels of phosphorylated Smad2 were detected, suggesting that the high levels of expression of Smad3 may interfere with Smad2 phosphorylation.

The ability of exogenous Smad3 to form DNA-binding complexes with Smad4 on the Smad-binding element of c-Jun (11) was also tested by an EMSA. In MDCK ΔRaf-ER cells untreated or treated with 4HT for only 24 h, TGF-β induced the formation of a nuclear Smad3/Smad4 DNA-binding complex (Fig. 2B). This complex is greatly decreased in the MDCK RafT cells because of the very low expression of Smad3. In all of the MDCK RafT Smad3 re-expressing clones, a DNA-binding complex was formed exclusively upon induction with TGF-β. Even in the case of B4b cells where the high levels of expression of Smad3 caused its localization to the nucleus without TGF-β treatment, virtually no DNA-binding complex was detected in unstimulated cells. In all of the cases, supershift analysis showed that the TGF-β-induced complex contained Smad3 and Smad4 (data not shown). Note that induction of the PAI-1-derived reporter gene CAGA12-luciferase (9) was dependent on TGF-β in all of the Smad3 re-expressing clones, suggesting that high nuclear levels of Smad3 alone were insufficient for the induction of TGF-β-dependent transcriptional responses (data not shown).

The EMT process requires continued synergism between Raf and TGF-β (Fig. 1D) (4, 16). To confirm that the clones used here had not lost Raf expression, the level of phosphorylation of MAPK in cultures containing 4HT was determined. All of the cells treated with 4HT showed high levels of activation of ERK2 (Fig. 2C). In addition, we checked the clones for expression of the ΔRaf-ER construct, which is fused to EGFP (7). The clones all continue to express ΔRaf-ER (Fig. 2D). Moreover, staining for E-cadherin showed that the re-expression of Smad3 had not led to a reversal of EMT and that the cells still showed a mesenchymal phenotype. An RNase protection assay on RNA extracted from unstimulated and 4HT-stimulated MDCK ΔRaf-ER cells, RafT, and RafT clone H3 cells also showed that re-expression of Smad3 did not restore E-cadherin expression (Fig. 2E). The expression of Smad3 mRNA was strongly suppressed in RafT cells but restored to at least wild type levels in H3 cells.
Re-expression of Smad3 in MDCK RafT cells restores sensitivity to the growth inhibitory effects of TGF-β. RafT cells in which continuous activation of Raf-ER has led to acquisition of a mesenchymal phenotype are resistant to cell cycle inhibition in response to TGF-β (4). To analyze whether re-expression of Smad3 in RafT cells restores sensitivity to the growth inhibitory effects of TGF-β, cell cycle distribution was examined in MDCK RafT cells and MDCK RafT cells re-expressing Smad3. Cells were analyzed 24 h after treatment with TGF-β for 4HT for 24 h and analyzed by EMSA. The Smad3-Smad4 DNA-binding complex is indicated. C, ERK2 MAPK activation. Cells as in B were grown sparsely and were left untreated or treated with 7.5 ng/ml TGF-β1 for 24 h. Whole cell extracts (40 μg) were resolved by SDS-PAGE, blotted, and probed with an antibody against ERK2, which recognizes both unphosphorylated and phosphorylated forms. D, restoration of Smad3 expression does not reverse EMT or affect Raf-ER expression. MDCK cells expressing Raf-ER not induced with 4HT, MDCK RafT cells re-expressing Smad3, or not were grown on nitrocellulose filters and methanol:acetone-fixed. Cells were stained with an antibody recognizing E-cadherin and examined by confocal laser scanning microscopy for E-cadherin and EGFP:Raf-ER expression. In the uninduced Raf-ER cells, basal levels of EGFP:Raf-ER were too low to be detected (4). E, expression of Smad3 and E-cadherin was detected by RNase protection. 20 μg of total RNA extracted from MDCK Raf-ER cells, RafT cells, and RafT cells where Smad3 had been re-expressed (clone H3) were analyzed by RNase protection with probes for canine E-cadherin or human Smad3. Pretreatment with 4HT was for 24 h in the case of MDCK Raf-ER cells as indicated and continuous for RafT and H3 cells. The protected fragments (E-cadherin or Smad3) are indicated. γ-Actin probe was used as a loading control. Data are representative of at least three independent experiments.
the TGF-β-induced cell cycle regulation. Re-expression of Smad3 did not resensitize RafT cells to the pro-apoptotic effects of TGF-β (data not shown).

When epithelial cells are stimulated with TGF-β, the retinoblastoma susceptibility product (pRb) is dephosphorylated, leading to inhibition of E2F, a crucial event for cell cycle arrest (17). To understand the molecular mechanisms involved in the rescue of TGF-β-induced cell cycle arrest upon Smad3 re-expression in RafT cells, we looked at the levels of phosphorylation of pRb. MDCK ∆Raf-ER, RafT, and RafT-Smad3 cells growing in serum were treated with TGF-β for 8 h. MDCK ∆Raf-ER cells not induced with 4HT or those that had been induced for 24 h with 4HT showed a shift in mobility of pRb protein toward the dephosphorylated state upon TGF-β stimulation independently of the induction of the Ras/MAPK pathway (Fig. 4A). In contrast, in MDCK RafT cells, very little pRb was dephosphorylated in response to TGF-β. When the MDCK RafT-Smad3 clones were examined, all of them showed clear dephosphorylation of pRb in response to TGF-β. Thus, Smad3 was responsible for mediating TGF-β-induced pRb dephosphorylation.

Another event implicated in cell cycle arrest by TGF-β is the induction of expression of p21WAF1/Cip1 (hereafter referred to as p21), an inhibitor of activated cyclin-dependent kinases (18, 19). Its expression is thought to contribute to the inhibition of CDK4/6-cyclin D and CDK2/cyclin E complexes, leading to hypophosphorylation of pRb and thus preventing the progression of the cell cycle (18). MDCK ∆Raf-ER cells responded to TGF-β by increasing the levels of p21 mRNA (data not shown) and protein (Fig. 4B). However, in MDCK RafT cells, TGF-β did not induce expression of p21 (Fig. 4B). By contrast, MDCK RafT-Smad3 cells showed low p21 levels, which increased when stimulated with TGF-β (Fig. 4B). Thus, the re-expression of Smad3 in these cells restores TGF-β-induced expression of p21.

Cyclin A expression is essential for the progression of cell cycle (20). MDCK ∆Raf-ER cells not induced with 4HT or those that had been induced for 24 h with 4HT down-regulate cyclin A expression in response to TGF-β, whereas MDCK RafT cells are resistant to the TGF-β-induced down-regulation of cyclin A (Fig. 4C) (4). In the MDCK RafT-Smad3 clones, this function was restored, because all of them showed a clear down-regulation of cyclin A in response to TGF-β (Fig. 4C).

Loss of Smad3 during EMT Leads to Resistance to the Anti-proliferative Effects of TGF-β—The data presented here provide an explanation of how cells can become refractory to the growth inhibitory effects of TGF-β during the process of epithelial to mesenchymal transition driven by constitutive activation of the Raf/MAPK pathway that is accompanied by au-
tocrine TGF-β production. Smad3 expression is lost with a time course similar to the loss of E-cadherin expression, while Smad2 and Smad4 are maintained and appear to function normally. Exogenous re-expression of Smad3 restores TGF-β-induced growth arrest.

Previous studies on keratinocytes and mouse embryo fibroblasts in which Smad3 had been deleted have shown that this also leads to a loss of TGF-β-induced growth arrest (21–23). Mouse embryo fibroblasts deleted in Smad2 were likewise resistant to the antiproliferative effects of TGF-β (22), suggesting that Smad2 is also required although not sufficient for TGF-β-induced growth arrest. The loss of Smad3 during EMT driven by oncogenic Ras together with TGF-β-induced growth arrest.

The loss of Smad3 during EMT driven by oncogenic Ras together with TGF-β in cells that normally express a high Smad3 to Smad2 ratio could be critical in releasing them from the growth inhibitory effects of TGF-β. Other TGF-β responses that may provide a competitive advantage for the tumor cells could then be maintained via Smad2 (21), whereas effects on host cells, especially angiogenesis and evasion of immune response, may also make TGF-β production advantageous to the tumor.

An analysis of Smad3 expression levels in tumor cell lines shows that its loss is not uncommon. In a panel of five colorectal tumor cell lines (Colo741, Colo205, HCT116, CaCo2, and HT29), is obviously mesenchymal; therefore, it is resistant to the antiproliferative effects of TGF-β, whereas effects on host cells, especially angiogenesis and evasion of immune response, may also make TGF-β production advantageous to the tumor.

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