EXPRESSON OF SMAD PROTEINS IN HUMAN COLORECTAL CANCER

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Escape from transforming growth factor-β (TGF-β)-induced inhibition of proliferation has been observed in many tumor cells and may contribute to loss of growth control. Smad proteins have been identified as major components in the intracellular signaling of TGF-β family members. In this study, we examined the expression of receptor-activated, common-mediator and inhibitory Smads by immunohistochemistry in human colorectal cancers. We found increased expression of receptor-activated Smads in a fraction of the tumor cells, while no immunostaining for Smad2, Smad3 or Smad5 and only occasional staining for Smad1/8 was found in epithelial mucosa of normal colon. No or only weak staining for receptor-activated Smads, common-mediator Smad4 and inhibitory Smads was observed in the tumor stroma. Common-mediator Smad4 and inhibitory Smads were detected in cells of both tumor and normal tissues. We observed a distinct pattern of Smad4 immunostaining of epithelial cells along colon crypts, with high expression in zones of terminal differentiation. Our data show selective up-regulation of receptor-activated Smad proteins in human colorectal cancers and suggest involvement of Smad4 in differentiation and apoptosis of surface epithelial cells of normal crypts. Int. J. Cancer 82:197–202, 1999. © 1999 Wiley-Liss, Inc.

Transforming growth factor-β (TGF-β) family members are involved in the regulation of cell proliferation, differentiation, motility and apoptosis (Roberts and Sporn, 1990). TGF-β is a potent inhibitor of cell growth, and resistance to TGF-β-induced growth inhibition is often observed in tumor cells (Roberts and Sporn, 1990; Lahm and Odartchenko, 1993). Reduced responsiveness to TGF-β was reported to be an important event in colorectal carcinogenesis (Manning et al., 1991). Loss of cell sensitivity to TGF-β was found to be associated with mutations of specific receptors or deregulation of intracellular signaling pathways (for review: Roberts and Sporn, 1990; Lahm and Odartchenko, 1993; Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998).

Smad proteins play a pivotal role in intracellular TGF-β signaling (for review: Heldin et al., 1997; Massagué, 1998). Eight mammalian Smads have been described and divided into 3 groups based on their functions in signal transduction. The first group consists of receptor-activated Smads, which are specifically phosphorylated by different type I serine/threonine receptors of the TGF-β family. Smad2 and Smad3 are phosphorylated by activated type I receptors for TGF-β and activin; Smad1, Smad5 and Smad8 are phosphorylated by activated type I receptors for bone morphogenetic proteins (BMPs). Another type of Smad molecule, a common-mediator Smad4, forms complexes with receptor-activated Smads. These complexes are translocated to the cell nuclei, where they regulate expression of target genes. The third Smad group consists of Smad6 and Smad7, which play inhibitory roles in TGF-β signaling by blocking phosphorylation of receptor-activated Smads or by competitive inhibition of complex formation of receptor-activated Smads with the common-mediator Smad4 (for review: Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998).

Inactivating mutations of Smad proteins have been found in human cancers. In particular, Smad4 is altered in pancreatic and colorectal cancers. Alterations of receptor-activated Smad2 and Smad3 in tumor cells have also been described (for review: Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998).

Mutations of Smad proteins render them inactive, thus providing resistance of cells to TGF-β-induced growth inhibition. To date, no systematic investigation of the expression of Smad proteins of all groups in the same specimen of human cancer has been reported. We addressed this question by studying expression of all Smads in specimens of human colorectal cancer by immunohistochemistry. Up-regulation of receptor-activated Smad proteins in a fraction of the tumor cells was found, while common-mediator Smad4 and inhibitory Smad6 and Smad7 were detected in tumor cells as well as in cells of normal colon. We found high expression of common-mediator Smad4 in colunar absorptive cells of the surface epithelium but not in the zones of active cell proliferation. This suggests a role for Smad4 in terminal differentiation and apoptosis of epithelial cells of colon crypts.

MATERIAL AND METHODS

Tissue specimens

Specimens of human colorectal tumor tissues and adjacent morphologically normal tissues were collected at the Department of Proctology, Lviv State Medical University, Ukraine. Adjacent morphologically normal tissues were collected 10 to 15 cm from tumor tissues and did not manifest any histological characteristics of transitional mucosa (Filipe, 1984). Immediately after resection, tissues were fixed overnight in 4% formaldehyde and paraffin-embedded according to standard techniques (Hogan et al., 1994). Sections of 4 μm were cut and collected on silane-coated glass slides. The grade of malignancy was histopathologically classified according to the TNM classification (UIICC, 1987). Among the 14 specimens collected, 11 were T3, one was T2 and one was T4 grade. In no case were distal metastases detected.

Antibodies

Rabbit anti-sera raised against peptides derived from linker regions of Smad1 (BSP/TFP) and Smad5 (SSN) have been described by Tamaki et al. (1998). Anti-sera raised against peptides derived from linker regions of Smad2 (DQQ), Smad3 (DHQ) and Smad4 (HPP) have been described by Nakao et al. (1997). Anti-serum against Smad7 (KER) was raised against the synthetic peptide KERQLELLQAVRSRGGTRTA. Each anti-serum to receptor-activated and common-mediator Smad4 has been found to be specific to respective Smad proteins and to not cross-react with other Smads, except for the Smad1 anti-serum, which cross-reacts with Smad8 (Smad1/8 anti-serum), and the Smad7 anti-serum, which cross-reacts with Smad6 (Smad6/7 anti-serum), by Western blotting assays. Anti-sera against Smads were affinity-purified using CNBr-activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden) with immobilized specific peptides according to the manufacturer’s protocol. Affinity-purified rabbit polyclonal anti-

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of 14 specimens, respectively. TGF-β1 antibody, a mouse anti-proliferating cell nuclear antigen (PCNA, C10) monoclonal antibody (MAb) and affinity-purified rabbit polyclonal anti-adenomatous polyposis coli (APC, C20) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A purified mouse anti-human DCC (deleted in colon carcinomas) MAb was purchased from PharMingen (San Diego, CA). A purified mouse anti-human carcino-embryonic antigen (CEA) MAb was obtained from Pierce (Boule Nordic, Huddinge, Sweden).

Immunohistochemistry

Sections were deparaffinized, rehydrated in ascending alcohol dilutions and washed in PBS. Tissues were microwave pre-treated 3 times for 3 min in citrate buffer, pH 6.0. ABC elite peroxidase immunohistochemical staining was performed as suggested by the manufacturer (Vector, Burlingame, CA). The primary antibodies were used in the following concentrations: affinity-purified antibodies against Smads1, -2, -3, -4, -5 and -6/7, 5 µg/ml; antibodies against TGF-β1, 4 µg/ml; against DCC, 12 µg/ml; against APC, 7 µg/ml; against PCNA, 15 µg/ml; and against CEA, 4 µg/ml. Tissues were incubated overnight with a biotinylated goat anti-rabbit IgG IgG (Vector), followed by incubation with Vectastain ABC Elite Complex (Vector). The immunoreaction was visualized using 3-amino-9-ethylcarbazole (Merck, Whitehouse Station, NJ) as a chromogen in the presence of 0.02% hydrogen peroxide (Merck), counterstained with hematoxylin and eosin and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL). In control experiments to exclude non-specific reactions of secondary antibodies or ABC complexes, each specimen was processed without primary antibodies. The specificity of all Smad antibodies was confirmed in experiments with blocking of the immunohistochemical staining by pre-incubation of antibodies with an excess of the corresponding peptide. Immunostaining was scored as negative ( ), positive (+) or strongly positive (+++).

Terminal deoxytransferase-mediated deoxyuridine nick end-labeling (TUNEL) assay

Sections were deparaffinized, rehydrated in ascending alcohol dilutions and washed in PBS. Pepsin or proteinase K treatment was found to be not necessary. Tissues were incubated in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min. TUNEL assay using alkaline phosphatase as a secondary detection system was performed as suggested by the manufacturer (Boehringer-Mannheim, Mannheim, Germany). Sections were counterstained with Mayer’s hematoxylin and mounted with Fluoromount G.

RESULTS

Expression of receptor-activated Smads

Phosphorylation of receptor-activated Smad proteins by specific receptor complexes is the first step in the initiation of TGF-β family signaling. Smad expression levels have been shown to define the intensity of signaling in cultured mammalian cells (for review: Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998). We therefore evaluated the expression of Smad proteins in normal and tumor tissues of the colon. Using receptor-activated Smad-specific antibodies (Fig. 1), we found immunostaining of 5% to 50% of tumor cells (Fig. 2b,d,f,h). This staining was cytoplasmic in most cells. BMP-specific Smad1/8 and Smad5 were detected in 10 and 8 of 14 specimens, respectively. TGF-β1/activin-specific Smad2 and Smad3 were detected in 12 specimens each (Table I). No correlations were found between the expression of receptor-activated Smads in tumor cells and age or sex of patients.

In tumor stroma, no expression of Smad2 or Smad3 and only very weak expression of Smad1/8 and Smad5 was seen. In addition, we were not able to detect Smad2, Smad3 and Smad5 in epithelial mucosae and in lamina propria of adjacent, histologically non-altered colon. This mucosa does not exhibit features of transitional mucosa (Filipe, 1984). However, Smad1/8 was expressed in some lamina propria and columnar absorptive epithelial

| Cell staining | Smad1/8 | Smad2 | Smad3 | Smad4 | Smad5 | Smad6/7 |
|---------------|--------|-------|-------|-------|-------|---------|
| N             | 12     | 4     | 12    | 2     | 12    | 6       |
| T             | 4      | 12    | 13    | 2     | 12    | 6       |
| N.D.          | 2      | 2     | 1     | 1     | 1     | 2       |

Immunostaining of epithelial cells in normal mucosa and tumor cells is shown. The number of samples with different intensities of cell staining is indicated. –, no staining found; +, staining seen in 5% to 30% of cells; ++, staining seen in more than 30% of cells; N.D., not determined; N, normal tissue; T, tumor tissue. Detailed data available upon request.

Expression of Smad4 and Smad6/7

Common-mediator Smad4 was detected in 12 of 14 specimens of carcinoma. Cytoplasmic immunostaining of up to 50% of tumor

FIGURE 1 – Specificity of Smad anti-sera. COS-1 cells were transfected with Flag-tagged Smad1 (lanes 1), Smad2 (lanes 2), Smad4 (lanes 4), Smad6 (lanes 6) and Smad7 (lanes 7); with HA-tagged Smad5 (lanes 5); with myc-tagged Smad3 (lanes 3); or with empty vector (lanes 8). Western blotting assay of whole-cell lysates with indicated antibodies was performed. Arrows show migration of respective Smad proteins.
cells and occasional immunostaining of stromal cells were found (Fig. 3a). Smad4 was detected also in the epithelial mucosa but not in the lamina propria of normal mucosa. Immunostaining of Smad4 along the epithelium of colon crypts showed a very distinct pattern. Strong Smad4 staining was detected in the apical side of columnar absorptive epithelial cells, while weak staining was found in a proliferative zone of crypts (Fig. 3b,c). Strong TGF-β1 immunostaining was found in cells of the lamina propria underlying the columnar absorptive epithelial cells (Fig. 3e,f). A similar pattern was reported for TGF-β1 immunostaining of cells along crypts by Avery et al. (1993). Immunostaining for APC was co-localized with staining of cells for Smad4 (Fig. 3g). The increased expression of APC in columnar absorptive cells on the top of the crypts is characteristic for normal colorectal mucosa (Smith et al., 1993). As measured by TUNEL staining, we observed a higher incidence of apoptosis in this zone (Fig. 3h).
Inhibitory Smad6/7 immunostaining was observed in 12 tumor specimens. The percentage of tumor cells immunostained for inhibitory Smads was higher than that for other Smad proteins. This staining was efficiently blocked in the presence of an excess of specific peptide (data not shown). The staining was cytoplasmic in most cells. Inhibitory Smads were also detected in stromal cells of tumor tissues (Fig. 4b). However, the intensity of staining in tumor stroma was lower than in tumor. Unlike receptor-activated Smads,
Expression of APC, DCC, CEA and TGF-β1

Mutations in the APC gene have been identified as one of the first steps in colorectal carcinogenesis (see references in Kinzler and Vogelstein, 1996; Smyrk and Lynch, 1997). In accordance, we determined in normal tissue specimens, cytoplasmic immunostaining of TGF-β1 was detected in epithelial cells and cells of the lamina propria (Fig. 3e,f). In tumor tissues, staining was detected in tumor and stromal cells (data not shown).

Expression of APC, DCC, CEA and TGF-β1

Mutations in the APC gene have been identified as one of the first steps in colorectal carcinogenesis (see references in Kinzler and Vogelstein, 1996; Smyrk and Lynch, 1997). In accordance, we found a loss of DCC staining in only 2 specimens, in contrast with previous observations that DCC is lost in more than 70% of colorectal cancers (Fearon et al., 1990). However, inactivation of DCC by mutations has also been reported (Fearon et al., 1990). We did not analyze DCC and APC for the presence of inactivating mutations; thus, we were not able to evaluate their functional intactness.

Expression of TGF-β1 was detected in all tested samples. In normal tissue specimens, cytoplasmic immunostaining of TGF-β1 was found in epithelial cells and cells of the lamina propria (Fig. 3e,f). In tumor tissues, staining was detected in tumor and stromal cells (data not shown).

TABLE II – IMMUNOSTAINING FOR APC, DCC, CEA AND TGF-β1

| Cell staining | APC | DCC | CEA | TGF-β1 |
|---------------|-----|-----|-----|--------|
|               | N   | T   | N   | T      |
| –             | 1   | 7   | —   | —      |
| +             | 10  | 6   | 11  | 11     |
| ++            | —   | —   | 13  | 12     |
| N.D.          | 3   | 1   | 3   | 1      |
|               | 1   | 2   | 1   | 2      |

Immunostaining of epithelial cells in normal mucosa and tumor cells is shown. The number of samples with different intensities of cell staining is indicated. –, no staining seen; +, staining seen in 5% to 30% of cells; ++, staining seen in more than 30% of cells; N.D., not determined; N, normal tissue; T, tumor tissue. Detailed data available upon request.

Inhibitory Smads were detected also in epithelial mucosa of normal colon. Stained cells were observed in lamina propria, mucosa muscularis and crypt epithelium (Fig. 4a).

Thus, common-mediator Smad4 and inhibitory Smad6/7 were detected in tumor cells as well as in normal epithelial mucosa. Increased staining for Smad4 was found in one specimen and for Smad6/7 in 2 specimens. However, we did not find immunostaining for Smad4 in 2 samples of tumor tissues or for Smad6/7 in 2 samples of 14. This diversity of immunostaining suggests deregulation of mechanisms controlling expression of these Smads in tumor cells (Table I).

DISCUSSION

Deletion or inactivating mutations of Smads have been found in many human cancers. Mutations of Smad4 have been observed in a high proportion of pancreatic carcinomas and less frequently in colorectal, prostatic, gastric, head, neck, biliary tract and lung cancers (see references in Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998). Germ-line mutations of Smad4 have been found in patients with juvenile polyposis, a condition predisposing to colorectal cancer. A tumor-suppressor role of Smad4 in colorectal carcinogenesis was also demonstrated in experiments with compound APC/Smad4 mice and by knock-out of the Smad4 gene in cells of colorectal cancers. Mutations of Smad2 have been found in colorectal and lung cancers. In 2 cases of colorectal cancer, a loss of heterozygosity of Smad3 also was detected, and targeted disruption of the Smad3 gene in mice led to development of colorectal cancer (see references in Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998). No genetic alterations of Smads involved in BMP signaling or of inhibitory Smads have been reported in human cancers so far. However, the chromosomal localization of these Smads in regions frequently mutated in cancer suggests their involvement in carcinogenesis (Riggins et al., 1996).

In the presented work, we have systematically investigated the expression of receptor-activated, common-mediator and inhibitory Smads in the same specimens of human colorectal cancer. Increased expression of receptor-activated Smads in tumor cells compared with epithelial cells of histologically normal mucosa was found.

Inhibitory Smads, as well as Smad4, were found in both normal colon mucosa and tumor cells. In normal colon mucosa, these Smads were detected in all samples, but in tumors their expression varied: in some specimens no expression was seen, whereas in other specimens expression was high. These observations suggest that expression of inhibitory and common-mediator Smads is perturbed in cancer cells. The variations of inhibitory Smad6/7
staining among the normal colon and tumor cells together with the cotranspecific peptide blocking of the staining suggest that the observed high-intensity staining found for the normal colon is specific. However, as for receptor-activated Smads, the functional relevance of this finding remains to be investigated.

The distinct pattern of Smad4 expression in epithelial cells along colon crypts suggests involvement of this Smad protein in the regulation of cell differentiation and apoptosis. Surface epithelial cells expressing high levels of Smad4 are those undergoing terminal differentiation, followed by apoptosis and shedding of dead cells into the lumen (Cotran et al., 1994). These surface epithelial cells also show increased expression of TGF-β1 (Fig. 3e,f). A similar pattern of TGF-β1 immunostaining was previously found by Avery et al. (1993), who also reported that such a pattern was found only in normal colonic mucosa and not in mucosa affected by an adjacent tumor. These observations and the previously reported immunohistochemical staining for APC (Smith et al., 1993; Morin et al., 1996) agree with our immunohistochemical and histological data on normal colonic mucosa samples.

The importance of APC for early colorectal tumorigenesis is well documented (see references in Kinzler and Vogelstein, 1996; Smyrk and Lynch, 1997). This protein is expressed in normal epithelial cells as they migrate toward the top of crypts (Fig. 3d). We have shown that increased expression of APC in colonic cells also leads to apoptosis. TGF-β induces apoptosis in many types of cell (see references in Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998). Ectopic expression of Smad4 by kidney (MDCK) epithelial cells (Atfi et al., 1997) and of Smad3 by lung epithelial cells (Yanagisawa et al., 1998) also induces apoptosis. Thus, such observations and our data on expression of common-mediator Smad4 and TGF-β1 on the top of crypts suggest the involvement of Smad-dependent TGF-β signaling in the terminal differentiation and apoptosis of surface epithelial cells.

The characteristic of specimens was complemented by investigation of the expression of TGF-β1, DCC, APC and CEA. We found loss of APC in 50% of the tumor specimens and upregulation of CEA in all tumor specimens, which is in agreement with reported changes in expression of APC and CEA at different stages of colorectal tumorigenesis (Kinzler and Vogelstein, 1996; Smyrk and Lynch, 1997). The reported cooperation of genetic alterations in APC and Smad4 genes in the malignant progression of colorectal tumors (Takaku et al., 1998) was not observed in our study. Thus, we did not note any correlation between loss of APC and level of Smad4 protein expression in the same tumor specimens. However, in epithelial cells of normal colon crypt, increased co-expression of APC and Smad4 was found (Fig. 3). Surprisingly, we observed loss of DCC in only 2 of 13 specimens. Others have reported loss of DCC in more than 70% of colorectal cancers (Fearon et al., 1990). However, we were not able to evaluate the functional activity of DCC; thus, the presence of inactivating mutations cannot be excluded.

The up-regulation of receptor-activated Smads in a fraction of the tumor cells compared to normal mucosa supports the notion that there are alterations in TGF-β signaling in colorectal carcinoma. The importance of TGF-β signaling in colorectal tumorigenesis. The reported cooperation of genetic alterations in APC and Smad4 genes and no changes of common-mediator Smad4 and inhibitory Smads suggests that they are regulated by different mechanisms in the course of tumor progression.

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