Thrombospondin 1—a regulator of adenoma growth and carcinoma progression in the APC^{Min/+} mouse model

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Introduction

Recognition of the role of angiogenic regulators during neoplastic development is important for a more complete understanding of the mechanisms involved in tumor growth and metastasis. One class of such regulators is represented by the thrombospondins. Thrombospondin (TSP)-1 and TSP-2 are large multifunctional structurally similar proteins secreted by activated platelets and other types of cells (1, 2). TSP-1 and TSP-2 in neoplasia remain controversial. On one hand, higher levels of TSP-1 have been detected in plasmas of patients with a variety of cancers (17), including breast cancer (18). Additionally, in a murine metastasis model, TSP has been shown to promote metastasis of fibrosarcoma tumor cells to the lung (19). However, other in vitro and in vivo studies have demonstrated inhibitory functions for TSP-1 and TSP-2 (20–27), as well as the TSP-1 receptor (28), in vessel formation, tumor angiogenesis and metastatic potential; properties that should attenuate progression of the tumor. In addition, it has been found that TSP-1 expression is reduced in carcinomas of the colon (29), breast (30) and bladder (31). Moreover, evidence has been presented indicating that activation of the proangiogenic factor, VEGF (vascular endothelial growth factor), occurs in the pre-malignant phase of colorectal tumor development (25), and the switch to an angiogenic phenotype may in part be the result of down regulation of inhibitors, such as TSP-1 (27).

In the current investigation, the relationships between TSP-1 expression and colorectal tumor progression have been assessed using the APCh Min/+ mouse model (a mouse heterozygous for a chain-termination mutation in the 15th exon of the APC gene) that develop multiple intestinal adenomas that undergo early in situ transformation into colon carcinomas (26). These adenomas undergo early in situ transformation into colon carcinomas (26). These adenomas undergo early in situ transformation into colon carcinomas (26). These adenomas undergo early in situ transformation into colon carcinomas (26).

Materials and methods

Mice and tissue preparation

APCMin/+ male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TSP-1+/− mice were generated as described (33). Mice were maintained on an 11% fat diet, as were their littermate controls. After 90 or 120 days, the animals were killed using CO₂ asphyxiation. Immediately
Counts) was obtained by counting adenoma vessels in percentages of cells staining at each category. The PCNA indices were calculated.

Histology and immunohistochemistry
Colon samples and small intestine segments were paraffin-embedded and sectioned. Stained sections were viewed without knowledge of genotypes and evaluated by an independent pathologist (Dr Luis Galup, South Bend Medical Foundation, South Bend, IN). All adenomas were checked by H&E and changes in intestinal structure and cellular architecture were considered carcinomas in situ. However, only the lesions showing invasion through the muscularis mucosa were identified as carcinomas. Sections for immunohistochemistry were cleared with xylene and rehydrated by ethanol. Endogenous peroxidase was blocked with peroxidase block (Zymed, San Francisco, CA). Sections designated for TSP-1 and VEGF (von Willebrand Factor) immunostaining were treated with 0.2% trypsin/PBS at 37°C for 10 min. Slides for PCNA (proliferant cell nuclear antigen) were boiled in 0.01 M citrate buffer solution, pH 6, for 10 min. The sections were incubated overnight at room temperature with monoclonal antibodies against TSP (Ab4, NeoMarkers, Fremont, CA), PCNA (Biogenex, San Ramon, CA) and VEGF (Oncogene Research Products, Boston, MA). The samples were then incubated with biotinylated rabbit anti-mouse antibody (Vector, Burlingame, CA) for 30 min. After incubation with peroxidase (ABC Kit), the stains were visualized with the chromogen, DAB (3’3-diaminobenzidine; Pierce, Rockford, IL) or AEC (3-amin-9-ethyl carbazole; Vector). For VWF immunohistochemistry, the DAB step was performed immediately after incubation with horseradish peroxidase-complexed anti-VWF antibody (DAKO EPOS, Carpinteria, CA).

TUNEL
The TUNEL assay was performed according to a published method (34). Tissue sections were incubated with 5 μg/ml of protease K for 15 min at room temperature in order to digest proteins. Sections were then covered with a buffer containing 30 mM Tris-HCl, pH 7.5, and 2/140 mM sodium cacodylate/1 mM CoCl₂. An aliquot of 0.2 μl of terminal deoxynucleotidyl-transferase (Boehringer Mannheim, Gaithersburg, MD) and 10 μM biotinylated dUTP (Boehringer Mannheim) were added to the sections. The slides were incubated in a humidified chamber at 37°C for 60 min, washed with 50 mM Tris-HCl, pH 7, and finally with PBS. The sections were then incubated for 30 min with the ABC system and DAB was used as the chromogen.

Statistical methods
Non-parametric ANOVA and Fisher’s tests were used. P values <0.05 were considered significant. The correlations between Hscore of TSP-1, MVC and PCNA indices were calculated using the Fisher t-test for paired comparisons.
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Fig. 1. Immunohistochemical analysis of normal and adenomatous mouse intestine from mice. TSP-1 expression in normal intestine (A and B). Staining for TSP-1 was always present in the lamina propria (LP) and submucosa (SM). Scattered epithelial cells (EC) of the surface were weakly positive. (A) TSP-1 is expressed in some lymphocytic populations in a lymph nodule (LN; 100× original magnification). (B) Fibroblasts and endothelial cells of large vessels in the submucosa were strongly positive, as well as platelets (P) (200×). (C and D) TSP-1 expression in colonic mucosa. A selected area of the APCMin/H11001 intestine, demonstrating a progressive decrease in TSP-1 immunoreactivity (arrows) (C; 100×) along with a well-developed adenoma (D, 100×) showing very low expression of TSP-1. vWF staining of adenomatous (E; 200×) and normal (F; 200×) intestine show numerous capillaries, mainly at the top (arrows) of the adenomas (E). Staining is stronger in epithelial cells of the crypts (arrows) and becomes weaker through the tips of the villi. (H) VEGF immunostain of normal mouse intestine (200×) shows that VEGF is expressed mainly in epithelial cells of the crypts (arrows) and becomes weaker through the tips of the villi. (H) VEGF immunostain of an adenoma (200×). Staining is stronger in the adenoma, mainly at the adenomatous areas at the top and luminal surface epithelium (arrows). Staining is localized to the luminal borders of the epithelial cells and adenomatous (H) mouse intestine shows that VEGF levels are significant in both sections. This down regulation of TSP-1 in the mature adenoma (D) coupled with the presence of VEGF favors the neovessel formation observed in (F). In normal tissue, the higher levels of TSP-1 would inhibit the angiogenic tendency of the VEGF observed in (G), resulting in a lack of neovessel formation in (E).

...and tumor vascularity and survival. There is evidence as well of increased vascularity and angiogenic factors, such as VEGF, in human adenomas (36,37).

VEGF immunostaining of normal (Figure 1G) and adenomatous (Figure 1H) mouse intestine shows that VEGF is clearly present in both samples, but demonstrates a somewhat different expression pattern with variability between regions of the intestine. VEGF is expressed mainly in the proliferative zones of the crypts in normal intestine (Figure 1G). Immunoreactivity of the glandular epithelium decreases gradually from the bases of the crypts to the luminal surfaces. In adenomas (Figure 1H), the strongest cytoplasmic staining was present in the epithelial cells of the mucosal surface. The increase in VEGF intensity at the tops of the adenomas correlate with higher areas of vascularity.

The data obtained suggest that the down regulation of TSP-1 in the mature adenoma (Figure 1D), coupled with the presence of VEGF (Figure 1H), shifts the balance toward neovascularization that would be stimulated by VEGF. These observations offer an explanation of the vascularization seen in the adenoma in Figure 1E. However, in normal tissue, the presence of TSP-1 (Figure 1A and B) would inhibit the angiogenic tendency of the VEGF observed in Figure 1G, thus producing the lack of neovessels in Figure 1F. Thus, a regulatory switch in TSP-1 levels may play a role in angiogenic changes of normal and adenomatous tissue leading to carcinoma of intestinal polyps.

General features of APCMin/H11001/TSP−/− mice

Because of the relationships suggested above between TSP-1 levels and angiogenesis, TSP-1-deficient mice were crossed with mice to generate APCMin/−/TSP−/− mice in order to more directly assess the role of TSP-1 in this genetic model of colonic carcinogenesis. The APCMin/−/TSP−/− mice showed the characteristic lordotic spine curvature observed in TSP−/− mice (33). They rapidly developed anemia (extremely pale skin and internal organs), bloody feces and weight loss. They expired earlier than their controls (one mouse died from severe anemia before 30 days and one died before 90 days). For this reason, most of the APCMin/−/TSP−/− mice were killed before 90 days after birth. No mammary, skin or stomach tumors were observed.
Adenoma counts and diameters in APC<sup>Min+</sup> and APC<sup>Min+TSP-1</sup> mice

Mean adenoma counting revealed a significant increase (P = 0.037) in adenoma number in APC<sup>Min+/TSP-1</sup> mice (39 adenomas, n = 7), compared with APC<sup>Min+</sup> control littermates (25 adenomas, n = 13) (Figure 3A–C). Whereas the differences in the adenoma diameters were not statistically significant (P = 0.54), adenomas in APC<sup>Min+/TSP-1</sup> mice tended to be larger (3.6 mm) compared with their controls (2.1 mm).

Histopathological evaluation of colonic adenomas from APC<sup>Min+/TSP-1</sup> mice

Each tissue section stained with H&E was evaluated. Features, such as dysplastic changes, carcinoma <i>in situ</i> and stromal invasion were evaluated in each adenoma (Figure 4). Intestinal tumors in APC<sup>Min+/TSP-1</sup> mice shared many similar properties. Both showed adenomas with different grades of inflammation (especially eosinophilic), dysplasia and carcinoma <i>in situ</i>. However, after evaluation of each lesion, histologic features, such as carcinoma <i>in situ</i> and invasion were a more typical pattern in the APC<sup>Min+/TSP-1</sup> mice. While most of the lesions were exophytic in the APC<sup>Min+</sup> mice (Figure 4A and D), most of the tumors in the APC<sup>Min+/TSP-1</sup> mice appeared as complete intramucosal carcinomas, showing central necrosis (Figure 4B and E), intense leukocytic infiltrate, and complete loss of glandular and nuclear polarity (Figure 4B, C, E and F). Carcinoma <i>in situ</i> was present in almost all the lesions, even those that were smaller (Figure 4C). Also, thickening of the walls, glandular invasion of the muscularis and venous embolism were more frequently seen in APC<sup>Min+/TSP-1</sup> mice (Figure 4F). TSP<sup>−/−</sup> littermate intestines were also evaluated. Intestinal mucosa of these mice showed some evidence of greater epithelial proliferation, increased mucosal depth growth and villi diameters (data not shown).

Vascularity in adenomas of APC<sup>Min+/TSP-1</sup> mice

As TSP-1 is an anti-angiogenic agent, a higher number of vessels in mice lacking TSP-1 was expected. No significant differences were observed between APC<sup>Min+</sup> (vascularity in
Adenomas, 22.3; vascularity in carcinomas, 29.4) and APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} (vascularty in adenomas, 21.1; vascularity in carcinomas, 27.5) mice. As it has been reported, an increase in angiogenesis was observed in the transition of normal-adenoma to carcinoma in both genotypes, but the vessel counts were similar between both groups (data not shown).

**Apoptotic indices**

TSP-1 induces apoptosis in endothelial cells and is upregulated by p53 (29). As p53 is a major regulator of apoptosis, we examined *in situ* apoptosis in APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice using the TUNEL assay. Apoptotic indices were significantly lower in adenomas and carcinoma of APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice (adenomas, 4.5; carcinomas, 1.0) compared with the lesions of APC\textsuperscript{Min+/+} mice (adenoma, 8.8; carcinomas, 3) (Figure 5). Adenomas of the APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice showed apoptotic indices very close to those found in the carcinomas of APC\textsuperscript{Min+/+} (Figure 5), demonstrating that most of the tumors, even in early lesions in APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice are indeed carcinomas.

**PCNA immunohistochemistry**

All the tumors from both genotypes showed increase of PCNA labeled cells compared with normal adjacent intestinal mucosa. Most of the adenomas of APC\textsuperscript{Min+/+} mice displayed fewer PCNA-positive cells than carcinomas and lesions of the APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice. Positive cells were located mainly in glandular foci and showed fewer positive cells in the upper epithelium (Figure 6A). In contrast, carcinomas of APC\textsuperscript{Min+/+} and all lesions of APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice, showed higher numbers of positive PCNA cells (Figure 6B–D). PCNA-positive cells were found to be more diffuse in the upper epithelium and the entire glandular component. Adenomas in APC\textsuperscript{Min+/+} mice displayed significantly lower PCNA indices as

<table>
<thead>
<tr>
<th>Histopathology Classification</th>
<th>APC\textsuperscript{Min+/+} (N=13)</th>
<th>APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoma</td>
<td>100 (153/153)</td>
<td>100 (86/86)</td>
</tr>
<tr>
<td>&gt;50% carcinoma <em>in situ</em></td>
<td>30 (46/153)</td>
<td>70 (60/86)</td>
</tr>
<tr>
<td>+ Stromal expansion/invasion</td>
<td>14 (21/153)</td>
<td>66 (57/86)</td>
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Fig. 4. Histological evaluation of adenomas in APC\textsuperscript{Min+/+} and APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice. (Top) Each adenoma from the corresponding H&E stain section was evaluated. Most of the adenomas were exophytic lesions, mainly in the adenomas (A, 200×) and carcinomas (D, 100×) that developed in APC\textsuperscript{Min+/+} mice. APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice showed more dysplasic areas and carcinoma *in situ* than their controls (Table 1) even in small lesions (B and C, 200× and 100×, respectively). Central necrosis (arrows in B and E, 100×), stromal invasion, and venous embolisms (arrow in F, 200× and magnified insert, 400×) were more frequently seen in lesions of APC\textsuperscript{Min+/+}/TSP-1\textsuperscript{−/−} mice. (Bottom) Summary of features evaluated in H&E stained sections. n = number of animals; A, total number of adenomas evaluated by histology; a, number of adenomas with the indicated feature.

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providing a basis for the lack of differences found in adenoma and perhaps no additional vascularization beyond that found in adenomas, already dysplastic, ultimately become carcinomas, APCMin/TSP-1 indices were higher in adenomas of APC Min/TSP-1–/– mice compared with carcinomas and lesions developed in APCMin/TSP-1–/– mice. These results indicate that higher proliferation and accelerated carcinogenesis are typical features in the lesions which arose in APCMin/TSP-1–/– mice, and even smaller adenomas showed elevated PCNA indices. The latter may be the result of the behavior of malignant cell clones, which eventually will display a more aggressive and invasive phenotype.

VEGF immunohistochemistry

VEGF immunostaining was observed in the endothelial cells lining vessels of normal intestine and adenomas. The stain was more intense in the normal colonic mucosa. In contrast to the normal mucosa, adenomas showed a variable intensity, with focal areas of epithelial cells usually strongly positive. The stroma of the lamina propria was positive for VEGF in both groups, and this staining was usually confined to the top area of the adenoma, where vessels and stroma were usually positive. In general, no clear differences in intensity and localization were observed between APCMin/TSP-1–/– and APCMin mice.

Discussion

The aim of this study was to evaluate the importance of the angiogenic inhibitor TSP-1 in naturally occurring tumors in vivo. As an indicator of the value of this approach, it has been demonstrated that mice with a deficiency of the tumor suppressor gene, p53, showed decreased survival with an additional deficiency of the TSP-1 gene (38). In our case, using an animal model for intestinal adenomatosis, we report the immunolocalization of TSP-1 in normal and pre-malignant intestine. Evidence is presented of a decrease of TSP-1 expression in adenomas and its inverse relationship with more proliferative and vascularized intestine.

On the other hand, no significant differences in vascularization between adenomas and carcinomas in APCMin mice and all lesions of APCMin/TSP-1–/– mice were found. These adenomas, already dysplastic, ultimately become carcinomas, and perhaps no additional vascularization beyond that found in the adenomas is required for the transformation, thus providing a basis for the lack of differences found in adenoma and carcinoma vascularization. These results suggest that TSP-1 may be required in the initial stages of angiogenesis when the vascular supply is low. However, when angiogenesis in the tumor is well established, endogenous TSP-1 has less influence. It is possible that during carcinogenesis, specific clones of cells, less dependent on vessel supply and more resistant to hypoxia, overgrow (39). Therefore, at this stage, vascular supply and angiogenic regulators may play a more secondary role. Also, angiogenesis may be genetically driven (40), and tumor cells at later time points may self-determine their vascular microenvironment. These findings may explain recent reports showing that the efficiency of anti-angiogenic therapy is related to the type, size and location of the tumor (41). Alternatively, TSP-1 is not the only inhibitor of angiogenesis, since other anti-angiogenic factors, e.g. angio-statin and TSP-2, could be produced after the onset of tumor development.

Our results further show TSP-1 to have properties beyond its anti-angiogenic effects. APCMin mice, additionally lacking the TSP-1 gene, were more tumorigenic, indicating that this combination of altered genes leads to a more malignant phenotype. TSP-1 is a complex protein, with the ability to interact with a number of pro-apoptotic and growth factors. Its interactions with these proteins in the tumor microenvironment may regulate apoptosis and proliferation, not only in neovessels, but also in malignant epithelial cells. TSP-1 regulates activation of endogenous proteins, including TGF-β (40,41), which is involved in multiple pathological events, such as wound healing, proliferation and tumor angiogenesis (42). Also, the loss of tumor suppressor genes may decrease TSP-1 expression and lead to an angiogenic phenotype in tumors (43).

Other studies have indicated that TGF-β RI expression is diminished in intestinal adenomas of APCMin mice with an associated increase in cyclin D1 and cyclin-dependent kinase 4 (Cdk4) expression, which would facilitate cell proliferation and progression of the disease (44). Additionally, a mutation in the APC gene induces nuclear expression of β-catenin, which is involved in E-cadherin-mediated cell adhesion and is also a key effector of the pronoecigenic Wnt signaling pathway. This latter property has been suggested as a potential cause of development of carcinoma from adenoma in colorectal carcinoma (45). Catenin accumulation induces activation of proliferation-associated genes, cyclin D and c-myc, and the invasion-associated genes, MMP-1 and MMP-7 (46). TSP-1 may be additionally involved in this pathway as it modulates tumor cell adhesion (47) and also induces increased levels of members of the catenin family, e.g. γ-catenin and p120 (Cas), in endothelial cells (48). Of potential importance is the observation that TSP-1 increases the secretion of protease inhibitor PAI-1 (49), which has been reported as a regulator of angiogenesis and tumor growth potentially via its effects on VEGF expression (50). Additionally, TSP-1 is up regulated by the WT product of the P53 gene (51). This induction by p53 may cause tumor cell apoptosis thus directly or indirectly inhibiting tumor angiogenesis.

Numerous reports have shown that high TSP-1 expression in human cancers appears to be a good prognostic factor (52–54). The clinical data, as well as some animal data, emphasize the role of TSP-1 as a protective factor against cancer and a strong modulator of tumorigenesis (38). This has been further reinforced in studies of a spontaneous mammary tumor model (transgenic neu/erbB2 oncogene under control of the mouse mammary tumor virus) with an additional deficiency or over...
expression of TSP-1 where it was demonstrated that TSP-1 regulates angiogenesis and tumor burden (55). The current study reports the first in vivo analysis of the impact of TSP-1 in an intestinal carcinogenesis model at early stages of tumor initiation and development. These findings agree with the concept of TSP-1 as a tumor suppressor gene, as its absence...
enhances tumorigenesis by regulating proliferation and apoptosis, and accelerating the transformation of adenomas to carcinomas. Tumor apoptosis and vascularity are the main outcome predictors in many tumors. These findings are relevant as they highlight the dual properties of TSP-1 as an early antiangiogenic and pro-apoptotic agent, making it a potential powerful tool against colorectal cancer. Lastly, the presence of early gene mutations, and/or environmental factors, may affect TSP-1 production and secretion. Down regulation of TSP-1 may play an important role in early events of colonic carcinogenesis, and this should be considered in an overall strategy for delaying polyposis in patients with a genetic predisposition to colorectal carcinoma. Thus, this protein may be a valuable marker of malignant transformation.

Acknowledgements

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Received August 7, 2002; revised October 4, 2002; accepted October 16, 2002