

Thrombospondin-1 Acts as a Fence to Inhibit Angiogenesis That Occurs During Cervical Carcinogenesis

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PURPOSE

The acquisition of an angiogenic phenotype (angiogenic switch) is essential for cervical carcinogenesis. This study was aimed to examine the spatial and temporal relationship of thrombospondin-1 (TSP-1) expression in patients with precursor lesions and squamous cell carcinoma of uterine cervix and to correlate its expression with tumor angiogenesis.

PATIENTS AND METHODS

TSP-1 expression and microvessel density were assessed by immunohistochemistry in samples obtained from patients with pathological diagnoses of cervical intraepithelial neoplasm I, carcinoma in situ, invasive squamous cell carcinoma (SCC), and benign disease ($N = 12$ from each group). Two representative blocks that contained serial changes of cervical lesions from these 48 subjects were examined, and the pathological findings were categorized into the four groups of (1) normal cervical epithelia, (2) low-grade squamous intraepithelial lesions (LSILs), (3) high-grade SILs (HSILs), and (4) SCC.

RESULTS

A total of 120 foci with various cervical lesions from 98 slides were examined and classified into normal (48), LSIL (36), HSIL (24), and SCC epithelium (12). Immunohistochemical studies showed that TSP-1 was mainly localized at the basal epithelial cells, and we named it as the "TSP-1 fence." The mean microvessel density counts and TSP-1 scores for normal, LSIL, HSIL, and

SCC epithelium were 7.3 ± 2.9 , 9.9 ± 3.4 , 17.7 ± 5.1 , and 22.8 ± 8.6 , and 3.8 ± 0.4 , 3.8 ± 0.4 , 1.8 ± 0.4 , and 1.5 ± 0.5 , respectively. The TSP-1 intensities were significantly higher and the MVD counts lower in the groups of normal and LSIL epithelium than in those with HSIL and SCC epithelium. In addition, microvessel density count was negatively associated with the intensity of TSP-1.

DISCUSSION

Our results indicate that the disruption of TSP-1 fence and the switch to angiogenic phenotype occurred during the transition from LSIL into HSIL. This concordance suggests that TSP-1 plays a role in the regulation of angiogenic switch. We conclude that the onset of angiogenesis is an early event in cervical carcinogenesis due, in part, to the down-regulation of TSP-1 by the dysplastic epithelium. (*Cancer J* 2004;10:27–32)

KEY WORDS

Thrombospondin-1, angiogenesis, microvessel density, cervical neoplasms

Cervical cancer is the second most frequent causes of death from malignant neoplasms among women worldwide.¹ It usually develops by a sequence of gradual, stepwise events starting at low-grade squamous intraepithelial lesion (LSIL) and progressing through high-grade SIL (HSIL), until invasive cancer is present.² Tumor development and metastasis are complex processes that includes transformation, proliferation, neovascularization, and metastatic spread. The angiogenic switch—acquisition of an angiogenic phenotype that is induced by a change in the balance of angiogenesis activators and inhibitors—is essential for tumor growth and metastasis.^{3,4} It has been reported that tumor microvasculature, accompanied by the overexpression of vascular endothelial growth factor (VEGF), was progressively up-regulated during the process of cervical carcinogenesis.⁵ However, the timing of angiogenic switch during cervical carcinogenesis remains to be debated.⁶

The thrombospondins (TSPs) are a family of extracel-

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lular proteins that participate in cell-to-cell and cell-to-matrix communication. Five family members have been identified.⁷ Recent work has identified the roles of TSP-1 as an endogenous angiogenic inhibitor that plays an important role in the angiogenic switch in skin, prostate, and bladder cancers.^{4,8,9} Evidence revealing the role of TSP-1 in cervical carcinogenesis is currently lacking. The purpose of this study was aimed to examine the spatial and temporal expression of TSP-1 in patients with preinvasive and invasive squamous cell carcinoma of the uterine cervix and correlated TSP-1 expression with microvessel density (MVD).

PATIENTS AND METHODS

Sample Selections

Surgical specimen collections, obtained from cervical punch biopsy, conization, or hysterectomy, were retrieved from the Department of Pathology, Chi Mei Foundation Hospital. Thirty-six patients with a mean age of 49.2 years (range, 31–65 years) who had pathological diagnoses of cervical intraepithelial neoplasm (CIN) 1, carcinoma in situ, and International Federation of Gynecology and Obstetrics stage Ib squamous cell carcinoma (SCC) ($N = 12$ from each group) were recruited in this study. They underwent cervical punch biopsy, conization, or radical abdominal hysterectomy, respectively. Two representative blocks that contained serial changes of cervical lesions from each specimen were analyzed. In each block, the cervical lesions of various severity as well as adjacent normal cervical epithelia were categorized into normal, LSIL, HSIL, and SCC epithelium groups. In addition, two tissue sections that contained normal cervical epithelium from 12 patients with benign uterine disease who underwent hysterectomy for uterine leiomyoma or adenomyosis were used as normal controls. MVD counts and TSP-1 expression were assessed in these samples by immunohistochemistry.

Immunohistochemical Staining

Formalin-fixed, paraffin-embedded tissue blocks were cut with serial sections of 4- μ m thickness. One section from each sample was stained with hematoxylin-eosin for the confirmation of histologic diagnoses by one of the authors (C.-C.T.). Adjacent sections were stained for von Willebrand factor antigen and TSP-1 by the use of standard immunoperoxidase staining methods. The paraffin was removed in xylene, and the tissue sections were rehydrated in descending dilutions of ethanol. After sections were heated and boiled for 13 minutes in a microwave oven in 10 mM of citrate (pH 6.0) buffer, they were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Specimens were then

incubated with mouse anti-human von-Willebrand factor monoclonal antibody (clone F8/86; Dako Corp., Glostrup, Denmark) diluted to a 1:50 ratio or with mouse anti-human TSP-1 monoclonal antibody (Calbiochem Co. La Jolla, CA) with a 1:250 dilution. Slides were then incubated with supersensitive immuno-detection system of BioGenex (San Ramon, CA). The 3,3'-diaminobenzidine tetra-hydrochloride was used as chromogen. Human placenta, which was known to exhibit TSP-1 expression, was used as the positive control. A negative control, for which the primary antibody was substituted with the same concentration of the appropriate immunoglobulin G, was used in each staining run. Sections were counter-stained lightly with hematoxylin. All stained slides were examined by the author (C.-C.T.) and associates who were blinded to the patients' status.

Microvessel Density

Sections were scanned at low magnification (100 \times) to identify areas of capillaries. The vessels that were defined as any positively stained single cell or cluster of cells were counted to determine the MVD count per high-power microscopic field (200 \times ; 20 \times objective lens and 10 \times ocular lens, 0.785 mm² per field). MVD were scored from three fields for each lesion by use of the microvessel counting protocol and criteria developed by Weidner et al,¹⁰ with minor modifications. The quantization was simultaneously performed (with use of a multiheaded microscope) by three investigators who had to concur on the areas to be analyzed and on vessel identification and count.

TSP-1 Expression

Preliminary results showed that TSP-1 expression was largely localized in the basal layer of uterine cervical epithelium. Thus, we measured TSP-1 intensity and classified TSP-1 scores into four arbitrary units according to the percentage of TSP-1-expressing basal epithelial cells. Scores of 4, 3, 2, or 1 were given under the conditions that TSP-1 was expressed in 75%–100%, 50%–75%, 25%–50%, or < 25% of the basal epithelial cells, respectively.

Statistical Analysis

All values were reported as mean \pm standard deviation. Because of small sample size and ordinal property of TSP-1 expression measurement, TSP-1 and MVD were subjected to nonparametrical data analysis method. The Kruskal-Wallis test was performed to detect any difference of MVD and TSP-1 expression intensities among normal, LSIL, HSIL, and SCC epithelium and to detect any difference of MVD among the four levels of TSP-1 expression intensity. A value of $P < 0.05$ was considered

as statistically significant. The Dunn test was adapted as the post hoc comparing method to test the differences between groups in a pairwise fashion. Based on the Bonferroni correction method, the alpha level of each post hoc comparison was corrected into 0.008. In addition, statistical comparisons between different lesions on the same slide were made with Wilcoxon signed-rank test.

RESULTS

A total of 96 tissue sections were recruited for this study. Among them, different extent of cervical lesions, that is, LSIL, HSIL, and invasive cancer, that appeared in the same slide were scored individually and regarded as different lesions. Accordingly, normal cervical epithelia in 48 slides, LSILs in 36 slides, HSILs in 24 slides, and SCCs in 12 slides were eligible for analysis. The MVD count and TSP-1 expression in the four groups are summarized in Table 1.

MVD

A representative immunohistochemical staining of von Willebrand factor is shown in Figure 1. Microvessels were only scarcely visible in the groups of normal cervical epithelium and LSIL, whereas in HSIL and SCC groups, microvessels were abundant (Fig. 1). It is noteworthy that the neovascularization in HSIL is confined to a narrow zone immediately underneath the dysplastic epithelium and along the basement membrane. The mean MVD counts for normal, LSIL, HSIL, and SCC epithelium were 7.3 ± 2.9 , 9.9 ± 3.4 , 17.7 ± 5.1 , and 22.8 ± 8.6 , respectively. Statistical analyses with the Kruskal-Wallis test revealed that the MVD counts were significantly different among these four groups ($P < 0.001$). Furthermore, the MVD counts were significantly lower in the groups of low vascularity, that is,

normal and LSIL epithelium, compared with those with high vascularity, that is, HSIL and SCC ($P < 0.001$, Dunn post hoc comparison). Together, these results clearly indicate that MVD counts increase during the transition from LSIL into HSIL.

Because more than one category of cervical epithelium may apply to a block and two blocks were taken from each patient, we also analyzed the data as dependent samples. Pairwise comparisons between different lesions on the same slide with Wilcoxon signed-rank test showed that the mean MVD counts for normal/LSIL epithelium, LSIL/HSIL epithelium, and HSIL/SCC epithelium were $7.1 \pm 3.0/9.9 \pm 3.4$, $9.8 \pm 3.5/17.7 \pm 5.1$, and $18.4 \pm 4.7/22.8 \pm 8.6$, respectively, a significant difference (Table 2).

TSP-1 Expression

A representative immunostaining of TSP-1 in various cervical epithelial lesions is shown in Figure 2. In the groups of normal and LSIL epithelium, intense TSP-1 expression was found and localized mainly in the basal epithelial cell layer. It arrayed like a barrier; therefore, we named it "TSP-1 fence" (Fig. 2). In contrast, this TSP-1 fence disappeared partially in HSIL and became nearly invisible in SCC epithelium. The mean TSP-1 scores for normal, LSIL, HSIL, and SCC epithelium were 3.8 ± 0.4 , 3.8 ± 0.4 , 1.8 ± 0.4 , and 1.5 ± 0.5 , respectively. The TSP-1 scores were significantly different among these four groups ($P < 0.001$, Kruskal-Wallis test), and Dunn post hoc comparison further confirmed that the TSP-1 intensities were significantly higher in the groups of low vascularity than in those with high vascularity ($P < 0.001$). These results indicate that the disruption of TSP-1 fence occurred during the transition from LSIL into HSIL.

When two blocks from the same patient were considered as dependent samples, TSP-1 score was signifi-

Distribution of Mean Microvessel Density Count and Thrombospondin-1 Score in the Four Groups of Normal Cervical Epithelia (Normal), Low-grade Squamous Intraepithelial Lesions, High-grade SIL, or Invasive Squamous Cell Carcinoma (SCC)

Cervical Lesions	No.	MVD		TSP-1 Score	
		Mean \pm SD	P Value	Mean \pm SD	P Value
Normal	48	7.3 ± 2.9	< 0.001	3.8 ± 0.4	< 0.001
LSIL	36	9.9 ± 3.4		3.8 ± 0.4	
HSIL	24	17.7 ± 5.1		1.8 ± 0.4	
SCC	12	22.8 ± 8.6		1.5 ± 0.5	

Abbreviations: MVD, mean microvessel density; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; TSP-1, thrombospondin-1.

TSP-1 decreased significantly during the transition from LSIL to HSIL, which was concomitant with the increase of MVD counts. Statistical analyses with Kruskal-Wallis test revealed that MVD counts and TSP-1 scores significantly differed among the four groups. In addition, MVD counts were significantly lower ($P < 0.001$) and TSP-1 scores higher ($P < 0.001$) in subjects with normal and LSIL epithelium than in those with HSIL and SCC epithelium (Dunn post hoc comparison test).

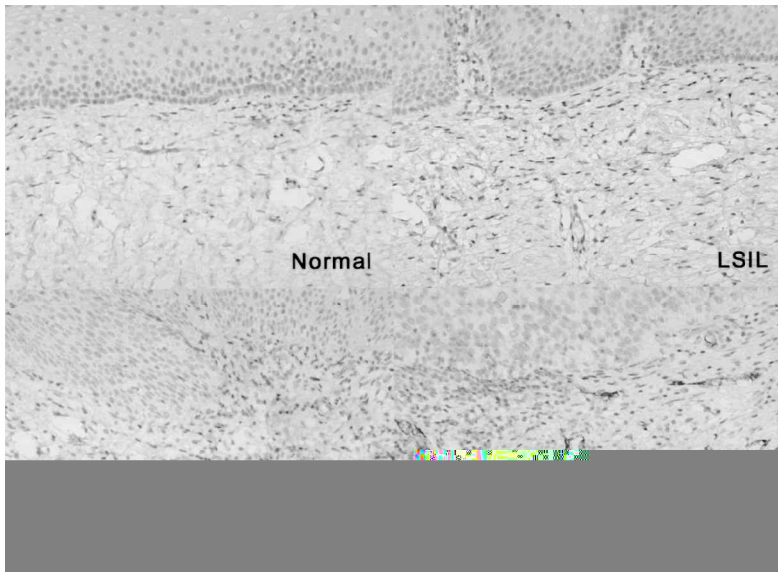


FIGURE 1 Immunohistochemistry ($\times 200$) of representative subjects with normal cervical epithelia (normal), low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), or invasive squamous cell carcinoma (SCC) revealed that microvessels were only scarcely visible in normal and LSIL epithelium. In contrast, microvessels were abundant in HSIL and SCC epithelium.

TABLE 2 Paired Comparisons of MVD and TSP-1 Score Between Normal/LSIL, LSIL/HSIL, and HSIL/SCC Epithelium Groups

Cervical Lesions	No.	MVD		TSP-1 score	
		Mean \pm SD	P Value	Mean \pm SD	P Value
Normal/LSIL	36	7.1 \pm 3.0 / 9.9 \pm 3.4	< 0.001	3.8 \pm 0.4 / 3.8 \pm 0.4	NS
LSIL/HSIL	24	9.8 \pm 3.5 / 17.7 \pm 5.1	< 0.001	3.8 \pm 0.4 / 1.8 \pm 0.4	< 0.05
HSIL/SCC	12	18.4 \pm 4.7 / 22.8 \pm 8.6	< 0.05	1.8 \pm 0.5 / 1.5 \pm 0.5	NS

Abbreviations: HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; TSP-1, thrombospondin-1; NS, not significant.

Wilcoxon signed-rank test was applied for paired comparisons between different lesions on the same slide. MVD was significantly different between normal/LSIL epithelium, LSIL/HSIL epithelium, and HSIL/SCC epithelium. In contrast, the TSP-1 score was significantly different only between LSIL/HSIL epithelium.

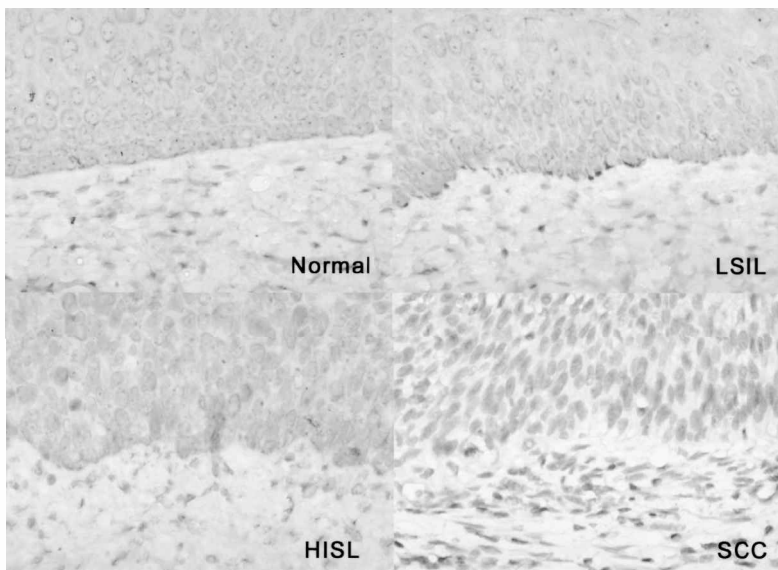


FIGURE 2 Immunohistochemistry ($\times 400$) of representative subjects with normal cervical epithelia (normal), low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), or invasive squamous cell carcinoma (SCC) revealed that thrombospondin-1 (TSP-1) expression was localized mainly in the basal epithelial cell layer (upper panel). The staining intensity gradually decreased in cells of superficial layers; therefore, we named it "TSP-1 fence." This TSP-1 fence existed only in normal and LSIL epithelium, became much less obvious in HSIL epithelium, and was hardly visible in SCC epithelium.

cantly different only between LSIL/HSIL epithelium (Table 2). The mean TSP-1 scores for normal/LSIL, LSIL/HSIL, and HSIL/SCC epithelium were $3.8 \pm 0.4/3.8 \pm 0.4$, $3.8 \pm 0.4/1.8 \pm 0.4$, and $1.8 \pm 0.4/1.5 \pm 0.5$, respectively.

Association of MVD and TSP-1

Among the 120 cervical lesions examined, 66 cases were grade 4 positive for TSP-1 staining, 18 cases were grade 3 positive, 24 cases were grade 2 positive, and 12 were grade 1 positive. The mean MVD counts for TSP-1 grades 1, 2, 3, and 4 cervical lesions were 21.0 ± 9.2 , 18.6 ± 5.3 , 8.0 ± 3.1 , and 8.6 ± 3.4 , respectively. These results indicate that the down-regulation of TSP-1 grade 3 into TSP-1 grade 2 was accompanied by a significant elevation of MVD counts. A Kruskal-Wallis analysis revealed that MVD count was negatively associated with the intensity of TSP-1 ($P < 0.001$). A P value less than 0.001 from Dunn post hoc comparison test further pointed out the difference of MVD counts between low TSP-1 expression group (score 1 or 2) and high TSP-1 expression group (grade 3 or 4).

DISCUSSION

The timing of angiogenic switch during cervical carcinogenesis remains controversial. A debate exists regarding the ability of CIN to induce angiogenesis.^{6,11,12} Smith-McCune and Weidner⁶ found a significant increase of MVD in the CIN III lesions compared with those underlying low-grade lesions, such as condyloma and CIN I. On the contrary, reports from Abulafia et al¹² showing that microinvasive squamous cell carcinoma is angiogenic, but not carcinoma in situ. In the present study, we intended to examine the slides that contain different severities of cervical lesions in the same slide, so that every lesion can be used as an internal control for the other. Thus, the heterogeneity in the inborn characters of angiogenesis can be eliminated. Our data showed that the angiogenic switch in cervical carcinogenesis occurred during the transition from LSIL to HSIL, and the neovascularization was largely confined to a narrow zone immediately underneath the dysplastic epithelium. This is in concordance with the results from Smith-McCune and Weidner⁶ and further suggests that cervical carcinogenesis is angiogenesis dependent.

Our results also showed that TSP-1 was mainly localized on basal cervical epithelial cells and was arrayed like a barrier. We therefore named it the "TSP-1 fence." TSP-1 decreases significantly during the transition from LSIL to HSIL, which is concomitant with the increase of MVD counts. The temporal and spatial concordance of TSP-1 down-regulation and the emergence of angiogenic imply that the TSP-1 fence may act as an angio-

genic barrier to inhibit angiogenesis, which occurred in the early phase of cervical carcinogenesis. The disappearance of the angiogenic barrier may induce a vigorous angiogenic response for tumor growth and perhaps tumor metastasis.¹³ Evidence from Kodama et al¹⁴ showed that TSP-1 messenger RNA expression was significantly lower in advanced-stage cervical cancer, and its expression is of value as a prognostic factor in cervical cancer. The origin of TSP-1 is currently unknown, and two possible origins, including tumor cells themselves and host cells (endothelial cells), may be responsible for the production.¹⁵ Our data suggest that the basal epithelial cells contribute to the production of TSP-1 under physiologic conditions and lose the ability to secrete TSP-1 during the transformation from LSIL to HSIL.

The argument has been raised that our statistical comparisons were made between different lesions, irrespective of the patients. Because more than one category of cervical epithelium may apply to a block and two blocks were taken from each patient, we also analyzed the data as dependent samples. Pairwise comparisons with Wilcoxon signed-rank test further confirmed the significant decrease of TSP-1 and elevation of MVD during the transition from LSIL to HSIL.

Accumulating evidence indicates that for most tumors, the switch to the angiogenic phenotype depends on the outcome of a balance between angiogenic stimulators and angiogenic inhibitors.¹⁵ Up-regulation of angiogenesis activators alone may not be enough for the emergence of angiogenic switch; it accompanies down-regulation of some angiogenesis inhibitors in the same time.¹⁶ TSP-1 and VEGF appear to be the constituents of a "switch" that regulates in concert many components of the angiogenic and differentiated phenotypes of endothelial cells.¹³ In a skin cancer model, down-regulation of TSP-1 and up-regulation of VEGF happened coincidentally and had spatial correlation throughout the consecutive stages of tumorigenesis.⁴ In contrast, down-regulation of TSP-1 secretion is a key event in the switch from an antiangiogenic to an angiogenic phenotype, whereas VEGF seems to play little role in bladder cancer.⁹ VEGF has been found to increase significantly in high-grade cervical intraepithelial lesions as compared with low-grade intraepithelial lesions and benign epithelium.⁵ We suggest that TSP-1, as one of the endogenous angiogenic inhibitors, may play the comparable role to other angiogenic activators, such as VEGF, in the angiogenesis balance during cervical carcinogenesis.

The angiogenic response, which is induced by the disappearance of "TSP-1 fence," modulates the pericellular environment and can potentially change the cell-matrix interactions associated with cell movement and further progression. TSP-1 does not appear to contribute directly to the structural integrity of connective tissue

elements. Instead, TSP-1 acts by modulating the activity and bioavailability of protease and growth factors and by interaction with cell-surface receptors.^{17,18} Matrix metalloproteinases (MMPs) play an active role in the neovascularization of tumors through their ability to degrade the extracellular matrix.^{19,20} Bergers et al²¹ showed that the switch from vascular quiescence to angiogenesis involves MMP-9, which is up-regulated in angiogenic islets and tumors, rendering VEGF more available to its receptors. Notably, MMP-9 is negatively modulated by TSP-1. Thus, TSP-1 acts as a multifunctional modulator of angiogenesis by modulating the activity and bioavailability of MMP-9. How TSP-1 modulates MMPs deserves further studies.

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