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Localization of thrombospondin-1 and its cysteine-serine-valine-threonine-cysteine-glycine receptor in colonic anastomotic healing tissue

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Summary. Thrombospondin-1 (TSP-1) is a matrix protein implicated in mechanisms of wound healing. TSP-1 contains the sequence cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) that has been shown to function primarily as a cell adhesion domain. Our laboratory has isolated a novel receptor specific for the CSVTCG adhesive domain of TSP-1. Immunohistochemical staining techniques and computerized image analysis were used to identify and quantitate TSP-1 and its CSVTCG receptor in surgically created colon anastomotic wounds. Histopathologic and quantitative examination demonstrated increased expression of TSP-1 and its CSVTCG receptor in areas of wound healing. These findings suggest a role for TSP-1 and its CSVTCG receptor in wound healing. The control of expression and activity of these molecules may eventually be the basis for the development of wound healing agents that could significantly reduce the morbidity from surgical intervention.

Key words: Thrombospondin-1, CSVTCG receptor, Bowel surgery, Wound healing, Immunohistochemistry

Introduction

Thrombospondin-1 (TSP-1), a 450 kilodalton glycoprotein released by platelets in response to physiological activators (such as thrombin and collagen), was first discorvered in 1971 (Lahav, 1993; Lawler and Hynes, 1986). A variety of cells synthesize and secrete TSP-1 including fibroblasts (Jaffe et al., 1983), smooth muscle cells (Raugi et al., 1982), and endothelial cells (McPherson et al., 1981). The structure of TSP-1 is conserved among various animal species as shown by amino acid sequence homology and antibody crossreactivity (Switalska et al., 1985; Lahav, 1993). Like fibronectin, TSP-1 is composed of linear polypeptide domains that specifically interact with a number of macromolecules, including heparin (Yabkowitz et al., 1989), fibrinogen (Tuszynski et al., 1985), collagen (Mumby et al., 1984), and plasminogen (DePoli et al., 1989).

TSP-1 plays a major role in cell adhesion and cell-cell interaction. TSP-1 has been shown to promote the cell-substratum adhesion of a variety of cells, including platelets, melanoma cells, smooth muscle cells, endothelial cells, fibroblasts, and epithelial cells (Tuszynski et al., 1987). Cells with specialized functions, such as keratinocytes (Varani et al., 1988) and osteoblasts (Robey et al., 1989), attach to TSP-1. TSP-1 also promotes the irreversible aggregation of platelets (Tuszynski et al., 1988). TSP-1 appears to be a key element in the regulation of wound healing, as seen in a murine model, in which inhibition of TSP-1 by TSP-1 antisense oligonucleotides delayed healing and epithelization in dermally wounded animals (DiPietro et al., 1996).

TSP-1 has been shown to play a significant role in cellular proliferation, a process central to wound healing and neoplastic growth. TSP-1 potentiates the mitogenic activity of epidermal growth factor for smooth muscle cells (Majack et al., 1986). Platelet-derived growth factor induces TSP-1 synthesis in smooth muscle cells (Majack et al., 1985). TSP-1 stimulates activation of smooth muscle cell S6 kinase, a protein kinase involved in the transition of cells from the quiescent to the proliferative state (Scott-Burden et al., 1988). TSP-1 also promotes the proliferation of human fibroblasts in culture (Phan et al., 1989). Anti-TSP-1 antibodies inhibit the growth in culture of smooth muscle cells (Majack et al., 1988). TSP-1 is believed to destabilize cell matrix contacts, and facilitate mitosis, and migration of cells during wound healing and angiogenesis (Murphy-Ulrich and Hook, 1989).

Our laboratory has isolated a unique receptor for

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TSP-1, which recognizes th cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) adhesion domain of TSP-1. Since this receptor has been shown to promote TSP-1 dependent adhesion of lung and breast carcinoma (Tuszynski et al., 1993; Wang et al., 1996) and tumor cell invasion (Wang et al., 1995, 1996), similar biological processes that occur during wound healing, we decided to investigate whether TSP-1 and its receptor are expressed in colonic wound healing.

Materials and methods

Eleven male Sprague Dawley rats underwent a laparotomy with transection and anastomosis of their descending colon. Rats were allowed ad libitum rat chow and water post operatively. On post operative day 7, one centimeter segments of perianastomotic tissue ranging from 0.5 centimeters proximal and 0.5 cm distal to the anastomosis were harvested.

Normal colon was harvested from a segment of colon corresponding to the perianastomotic tissue in rats which had not undergone any intraabdominal manipulation. All sections of tissue were fixed in formalin and embedded in paraffin blocks, Samples of 5 μm thickness were taken from the blocks and placed on glass slides.

Immunohistochemical staining of the wound tissue for TSP-1 was accomplished in accordance with the protocol supplied by Vectastain Corporation (Vector Laboratories, Burlingame, California): deparaffinization was accomplished, followed by ethanol treatment, and quench (3% H_2O_2 in methanol). Blocking solution (1% bovine serum albumin (BSA)/phosphate buffered saline (PBS), 1% blocking serum) was then applied. Primary antibody (1% BSA/PBS, 1.5% blocking solution as diluent) was subsequently applied to the samples.

For TSP-1 evaluation, the primary antibody used was goat anti-TSP-1 IgG antibody. Goat IgG was used as the control. The samples were incubated with primary antibody for 60 minutes. Secondary antibody consisting of biotinylated rabbit anti-goat IgG was applied at a 1/300 dilution in 1.5% blocking serum, with 0.1% BSA/PBS, and this was incubated for 30 minutes. The Avidin Biotin Complex (ABC), made 20 minutes before application, was applied for 30 minutes to the samples, followed by 5 mg/ml 3,3'-diaminobenzidine (DAB), with 4.5 ml PBS, and 5 μl of 30% H_2O_2 (added last) was applied to the slides. Hematoxylin counterstain was then applied. The slides were mounted wet.

For CSVTCG receptor evaluation, the primary antibody was a polyclonal rabbit serum against human receptor. Rabbit serum was used as the control. The blocking serum in the Vectastain kit consisted of 1% BSA/PBS, 1.5% blocking serum, and 4% horse serum. The samples were incubated with primary antibody for 60 minutes. The secondary antibody was biotinylated goat anti-rabbit IgG. Vectastain streptavidin-peroxidase complex was used, followed by DAB (5 mg/ml), 4.5 ml PBS, 5 μl of 30% H_2O_2 (added last) was applied to the slides. Hematoxylin counterstain was applied. The slides were mounted wet.

Quantification of the staining was accomplished with the use of computer assisted image analysis by using the CAS 200 R Image Analyzer (Becton-Dickenson, Inc., San Jose, California). This system is a computerized video microscope that emits light as a single wavelength (620 nm) and measures the optical density of stained sections. The intensity of the immunohistochemical expression is directly proportional to the light absorbed by the section and translated optical density of the analyzed sample. Software for Quantitative Estrogen/Progesterone Analysis (Cell Analysis Systems, Inc.), provided by Becton-Dickenson, Inc., was used for the quantification of the samples.

Each glass slide had two colonic anastomosis samples applied to it. One tissue sample served as the background absorbance control, the other sample was the tissue expressing the molecule (TSP-1 or the CSVTCG receptor) to be investigated. Goat IgG was used for negative control staining of TSP-1. Rabbit serum was used for negative control in the staining of the CSVTCG receptor. Ten image fields form the control side of the sample were obtained to determine the background absorbance. The objective antibody threshold for specific staining was defined for each specimen by using the negative control section as reference. Ten image fields from the specimen side were then obtained. Control values were subtracted from the sample values to obtain the amount of absorbance, thus eliminating background staining from the study measurements.

Results

Quantitative evaluation

A significant difference in expression of TSP-1 and its CSVTCG receptor was seen between the non-wounded control tissue of the anastomosis and perianastomosis. Significant differences were not seen between anastomotic and peri-anastomotic tissue. Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis noted a significance of $p<0.05$ vs. control by ANOVA evaluation. Positive controls were sections of platelet-rich-plasma which express TSP-1 and its receptor. The values of the positive controls are: TSP-1, 63.43, and CSVTCG, 58.72, respectively.

Table 1. Quantitative examination of TSP-1 and CSVTCG receptor expression.

	Control (N)	Peri-anastomosis (N)	Anastomosis (N)
TSP-1	5.02±10.17 (6)	22.40±3.36* (5)	17.70±3.68* (5)
Receptor	11.14±2.28 (5)	22.42±4.56* (5)	29.53±6.40* (5)

Results are expressed as mean±SEM. *: $p<0.05$ vs control by ANOVA. Postive control: TSP-1, 63.43; CSVTCG, 58.72.

Histological evaluation

The control sample is counterstained blue. The positive stain is brown in color. TSP-1 localized in the stroma and glandular tissue. Expression of CSVTCG receptor was localized primarily in the individual cells, glandular tissue, and polymorphonuclear leukotyctes (PMN's).

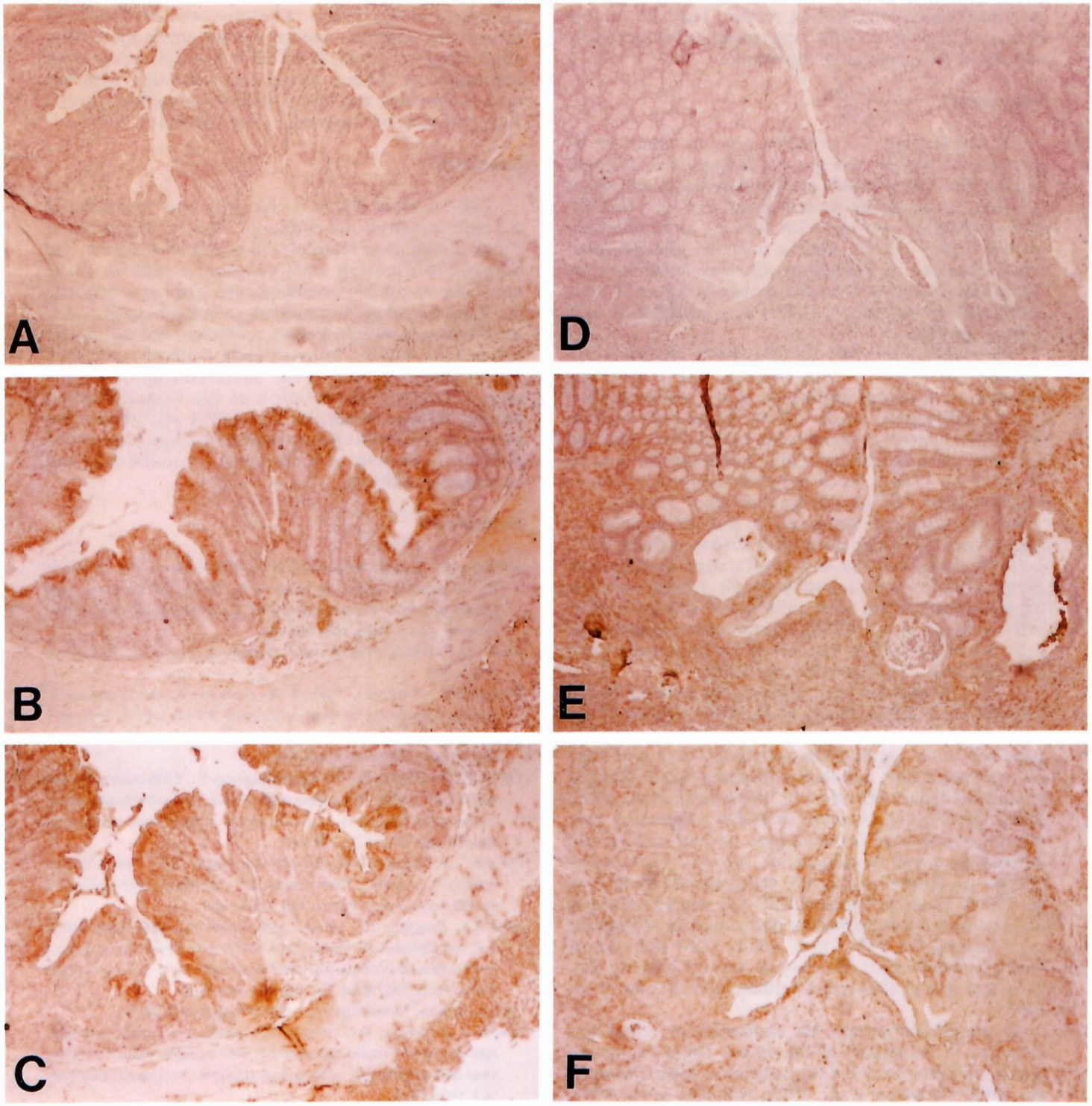

Fig. 1. Histological examination of TSP-1 and the CSVTCG receptor. **A.** Transverse sample. Control. **B.** Transverse sample, TSP-1. **C.** Transverse sample, CSVTCG receptor. **D.** Longitudinal sample, Control. **E.** Longitudinal sample, TSP-1. **F.** Longitudinal sample, CSVTCG receeptor. x 10

The histological findings of the CSVTCG receptor include similar expression in the epidermis and muscle. However, the CSVTCG receptor was also expressed more intensely in the pericytes of blood vessels. Pericytes are cells found immediately adjacent to the neovasculature of the healing wound. They are present during angiogenic activity. Angiogenesis was primarily seen in the area adjacent to the wound.

Discussion

Wound healing is a complex process, mediated by multiple cytokines and growth factors that promote healing. Examples include platelet-derived growth factor (PDGF), transforming growth factor beta-1, (TGF-ß), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF). Matrix proteins also promote wound healing by providing adhesive substrates for migrating cells that remodel damaged tissue (Clark, 1989). Thrombospondin-1 (SP-1) is an example of such a matrix protein.

TSP-1 is a molecule that has been postulated to promote the wound healing process. TSP-1 promotes adhesion of cells that participate in the wound healing process (Tuszynski et al., 1987). These cells include fibroblasts, smooth muscle cells, endothelial cells, and epithelial cells. In addition TSP-1 has been shown to be an adhesive matrix for neutrophils and macrophages (Suchard et al., 1991), and keratinocytes (Varani et al., 1988). In addition to being able to provide an adhesive matrix for cells during the wound healing process, TSP-1 can promote motility and proliferation of cells. For example, TSP-1 has been shown to be a potent mitogen for fibroblasts (Phan et al., 1989) and smooth muscle cells (Majack et al., 1988). Furthermore, endothelial cells are stimulated to migrate to surfaces containing adsorbed TSP-1 (Taraboletti et al., 1990).

TSP-1 is rapidly up-regulated in injured tissue (Munjal et al., 1990; Raugi et al., 1990; Miano et al., 1993). In full-thickness skin wounds TSP-1 is rapidly produced in the extracellular matrix of the wound edge and neovasculature, and then is downregulated when the wound has healed (Raugi et al., 1987; Reed et al., 1993, 1995). This initial increase in TSP-1 expression may stimulate cell proliferation (see above) and matrix degradation and angiogenesis through proteolytic enzyme production as recently demonstrated by Qian et al., 1997). The importance of TSP-1 in wound healing is underscored by experiments performed in transgenic mice lacking the TSP-1 gene which show that dermal wounds demonstrated delayed wound healing and persistent granulation tissue formation (Polverini et al., 1995).

In the present studies, the expression of TSP-1 was observed in the stroma of the tissue. These results are similar to those observed for neoplastic tissue (Wong et al., 1992; Clezardin et al., 1993; Arnoletti et al., 1994; Tuszynski and Nicosia, 1994). The expression of the CSVTCG receptor was increased in the proliferating cells and pericytes. We have previously observed expression of the CSVTCG receptor in highly proliferating lung carcinoma (Tuszynski et al., 1993), breast carcinoma (Tuszynski and Nicosia, 1994; Roth et al., 1997) and squamous carcinoma of the head and neck (Arnoleti et al., 1994). The presence of CSVTCG receptor in pericytes and areas adjacent to the wound edge suggest a role for TSP-1 and its receptor in angiogenesis. These results are consistent with our studies showing that TSP-1 modulates angiogenesis in vitro (Nicosia and Tsuzynski, 1994; Qian et al., 1997).

Conclusion

TSP-1 and its CSVTCG receptor were expressed in colonic anastomotic wounds. The localization of expression of these molecules in matrix, vasculature, and highly proliferating cells suggests a role for these molecules in colonic wound healing. They may facilitate the biochemical steps important in wound healing such as cell mitosis, differentiation, migration, and cell matrix interactions. Further study of these molecules and their interactions may help in the understanding of colonic wound healing. Modulation of the wound healing process through molecules like TSP-1 and its receptor may someday enable us to speed the healing process and decrease the risk of anastomotic breakdown and subsequent morbidity.

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