Journal of Dermatological Science 74 (2014) 106–115

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Thrombospondin-2 overexpression in the skin of transgenic mice reduces the susceptibility to chemically induced multistep skin carcinogenesis

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ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form 8 December 2013
Accepted 6 January 2014

Keywords:
Angiogenesis
Skin cancer
Thrombospondin
Carcinogenesis

ABSTRACT

Background: We have previously reported stromal upregulation of the endogenous angiogenesis inhibitor thrombospondin-2 (TSP-2) during multistep carcinogenesis, and we found accelerated and enhanced skin angiogenesis and carcinogenesis in TSP-2 deficient mice.
Goals: To investigate whether enhanced levels of TSP-2 might protect from skin cancer development.
Methods: We established transgenic mice with targeted overexpression of TSP-2 in the skin and subjected hemizygous TSP-2 transgenic mice and their wild-type littermates to a chemical skin carcinogenesis regimen.
Results: TSP-2 transgenic mice showed a significantly delayed onset of tumor formation compared to wild-type mice, whereas the ratio of malignant conversion to squamous cell carcinomas was comparable in both genotypes. Computer-assisted morphometric analysis of blood vessels revealed pronounced tumor angiogenesis already in the early stages of carcinogenesis in wild type mice. TSP-2 overexpression significantly reduced tumor blood vessel density in transgenic mice but had no overt effect on LYVE-1 positive lymphatic vessels. The percentage of desmin surrounded, mature tumor-associated blood vessels and the degree of epithelial differentiation remained unaffected. The antiangiogenic effect of transgenic TSP-2 was accompanied by a significantly increased number of apoptotic tumor cells in transgenic mice.
Conclusion: Our results demonstrate that enhanced levels of TSP-2 in the skin result in reduced susceptibility to chemically-induced skin carcinogenesis and identify TSP-2 as a new target for the prevention of skin cancer.

1. Introduction

In contrast to the plethora of reports on tumor angiogenesis factors, much less is known about the biological role of endogenous inhibitors of angiogenesis during tumor development, in particular during the early stages of tumor promotion. Several endogenous inhibitors of tumor angiogenesis have been identified including thrombospondin- (TSP)-1 [1], TSP-2 [2], angiostatin [3], endostatin [4], vasostatin [5] and tumstatin [6]. Although TSP-1 and TSP-2 are members of the same family of glycoproteins with considerable structural similarities [7,8], the expression of TSP-2 differs spatially and temporally from TSP-1 during embryonic development [9,10]. Moreover, the regulation of TSP-2 gene expression by growth factors is distinct from TSP-1 [7]. Previously, TSP-2 was shown to diminish the angiogenic activity of basic fibroblast growth factor [11] and the formation of focal adhesions in aortic endothelial cells [12],

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0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2014.01.002

indicating its role in controlling angiogenesis. TSP-2 deficient mice are characterized by increased vascular density in several tissues including the skin [13] and display accelerated healing of excisional wounds by virtue of their highly vascularized granulation tissue [14].

Previously, we identified stromal up-regulation of TSP-2 expression as a potential host anti-tumor mechanism during multistep skin carcinogenesis [15], and we found that stable overexpression of TSP-2 in human squamous cell carcinoma xenotransplants inhibited tumor growth and vascularization even more potently than TSP-1 [2]. Furthermore, systemic treatment with an N-terminal 80 kDa recombinant fragment of TSP-2 inhibited angiogenesis and tumor growth in squamous cell carcinoma (SCC) bearing mice [16].

However, human cancers arise through a multistep progression pathway, and the role of TSP-2 in the early stages of cancer development has remained unknown. Based on our previous observation that TSP-2 deficient mice display enhanced skin carcinogenesis and angiogenesis, we hypothesized that TSP-2 might play a role in the control of early tumorigenesis. To directly evaluate the biological effects of TSP-2 in multistep epithelial tumor development, we established transgenic mice with targeted overexpression of TSP-2 in epidermal keratinocytes of the skin. Hemizygous TSP-2 transgenic mice and their wild-type littermates were subjected to a standard two-step skin carcinogenesis regimen, using topical application of 7,12-dimethylbenz (α) anthracene (DMBA) for tumor initiation and phorbol 12-myristate 13-acetate (PMA) for tumor promotion. This established model of skin carcinogenesis [17] allows detailed insights into the premalignant as well as the malignant stages of skin cancer development.

We found that targeted overexpression of TSP-2 in the skin of transgenic mice reduced the incidence of early, premalignant stages of tumor development as well as the formation of squamous cell carcinomas. Tumor angiogenesis was significantly inhibited in all stages of skin carcinogenesis in TSP-2 transgenic mice, but lymphangiogenesis remained unaffected. Moreover, the number of apoptotic tumor cells in TSP-2 transgenic mice was significantly increased over wild-type controls, identifying TSP-2 as a potential factor for the prevention of skin cancer.

2. Materials and methods

2.1. Generation of TSP-2 transgenic mice

We cloned a 4.1-kb mouse TSP-2 cDNA sequence, comprising the full TSP-2 coding sequence, into a pGEM-3Z vector containing the human keratin 14 (K14) promoter (kindly provided by Dr. Elaine Fuchs, Chicago) that targets transgene expression to epidermal keratinocytes of the skin [18]. We have previously used the identical expression vector to establish transgenic mice overexpressing the mouse VEGF164 gene [19] and the human TSP-1 gene [20]. The correct sequence and orientation of the TSP-2 insert were verified by restriction mapping and direct sequencing using the Sanger dideoxy method.

After digestion of the complete construct with the restriction enzymes *KpnI* and *HindIII*, the 8.3-kb expression vector (Fig. 1A) was used to generate transgenic mice as described [19]. Transgenic founders were detected by Southern blot analysis of *BamHI* digested genomic tail DNA obtained 2 weeks after birth. For rapid identification, genomic tail DNA was subjected to PCR using an 18-mer primer and a 21-mer primer that bind, respectively, to positions 321–338 and 650–630 of the human growth hormone gene in the transgene construct, leading to selective amplification of a 330-bp fragment when the transgene construct was incorporated into the genome. Transgenic lines were established in the FVB/J

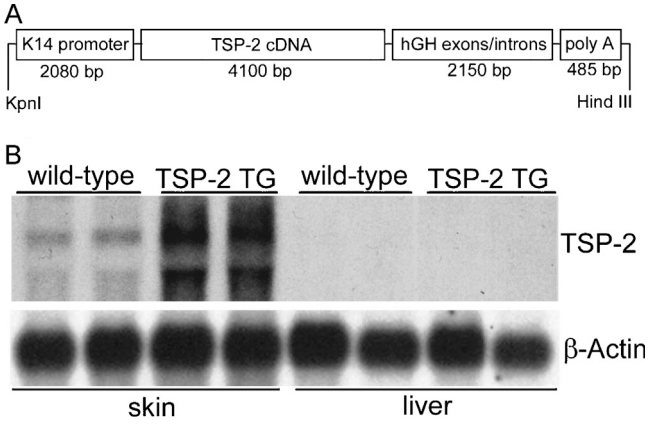

Fig. 1. Schematic representation of the K14-TSP2 transgene construct. A 4100 bp mouse TSP-2 *BamHI* cDNA fragment was ligated into the *BamHI* restriction site of the keratin 14 promoter expression cassette (A). Overexpression of TSP-2 mRNA in the skin of 3-week-old K14-TSP-2 transgenic mice was confirmed by Northern blotting. TSP-2 mRNA was not increased above wild-type (wt) levels in the liver of TSP-2 transgenic mice. Hybridization with a murine b-actin probe served as control for equal loading (B).

genetic background. Transgene expression was confirmed by Northern hybridization of total RNA extracted from the skin of mice (Fig. 1B). Northern blot analysis confirmed transgenic TSP-2 mRNA expression in the skin of TSP-2 transgenic mice whereas TSP-2 mRNA was not significantly increased above baseline in the liver of transgenic mice demonstrating selective transgenic expression in the skin. This was further confirmed by in situ hybridization demonstrating strong TSP-2 overexpression in basal epidermal keratinocytes and in outer root sheath keratinocytes of hair follicles (Fig. 3B and D).

2.2. Western blot analysis

For Western blot analyses, murine skin and squamous cell carcinomas (SCCs) were obtained from four different wild-type and hemizygous TSP-2 transgenic mice and were homogenized as described [20]. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Samples were boiled in denaturating Laemmli sample buffer (Bio-Rad) with β-mercaptoethanol. Thirty microliters of each sample were electrophoresed on 12% SDS polyacrylamide gels and blotted onto nitrocellulose membranes (Bio-Rad). Membranes were incubated overnight in phosphate-buffered saline (PBS) containing 5% skim milk to block nonspecific binding and were then incubated with a goat anti-mouse VEGF-A antibody (R&D Systems, Minneapolis, MN) or a polyclonal rabbit anti mouse TSP-2 antibody (kindly provided by Paul Bornstein, University of Washington, Seattle, WA). After incubation with horseradish peroxidase-conjugated anti-goat IgG or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), immunoreactive proteins were visualized using a chemiluminescence detection system (ECL; Amersham). To confirm equal loading, the same membranes were stained with Naphthol Blue Black solution (Sigma, St. Louis, MO).

2.3. Chemical skin carcinogenesis regimen

For tumor initiation, 50 μg of DMBA (Sigma), dissolved in 200 μl acetone, were topically applied to the shaved back skin of 8-weeks-old female hemizygous TSP-2 transgenic mice ($n = 25$) and their wild-type littermates ($n = 25$), followed by weekly topical application of 5 μg of the tumor promoter PMA (Sigma) over 20 weeks, as described [21,22]. In addition, 5 wild-type and 5 TSP-2