Tsukushi is involved in the wound healing by regulating the expression of cytokines and growth factors

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Abstract During the wound-healing process, macrophages, fibroblasts, and myofibroblasts play a leading role in shifting from the inflammation phase to the proliferation phase, although little is known about the cell differentiation and molecular control mechanisms underlying these processes. Previously, we reported that Tsukushi (TSK), a member of the small leucine-rich repeat proteoglycan family, functions as a key extracellular coordinator of multiple signalling networks. In this study, we investigated the contribution of TSK to wound healing. Analysis of wound tissue in heterozygous TSK-lacZ knock-in mice revealed a pattern of sequential TSK expression from macrophages to myofibroblasts. Quantitative PCR and in vitro cell induction experiments showed that TSK controls macrophage function and myofibroblast differentiation by inhibiting TGF- β 1 secreted from macrophages. Our results suggest TSK facilitates wound healing by maintaining inflammatory cell quiescence.

Keywords TGF- β 1 · Tsukushi · Wound healing · Quiescence

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Introduction

Wound healing is a highly dynamic process and involves complex interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating inflammatory cells (Eming et al. 2007). The classic model of wound healing is divided into three sequential phases: inflammatory, proliferative and remodeling (Gurtner et al. 2008). But, little is known about the cell differentiation and molecular control mechanisms underlying these processes.

Previously, we identified a secreted molecule, Tsukushi (TSK), which belongs to the small leucine-rich proteoglycan family (SLRP) (Schaefer and Iozzo 2008) and inhibits signalling molecules such as BMP and Wnt (Ohta et al. 2004, 2011). We also reported that TSK controls cell differentiation during the hair cycle by regulating TGF- β 1 (Niimori et al. 2012). Because follicular stem cells not only supply hair follicles, sebaceous glands, and epidermis, but also regenerate tissue during wound healing (Cotsarelis 2006), we anticipated that TSK could be involved in wound healing. In this study, we investigated the contribution of TSK to wound healing.

Materials and methods

Mice

TSK null mutant mice were generated by inserting a LacZ/Neo cassette into the TSK coding exon (Ito et al. 2010). The mice were backcrossed to the C57BL/6 J strain for at least six generations and can be considered to be of a nearly uniform genetic background. All experiments were conducted in accordance with the guidelines of the Kumamoto University Center for Animal Resources and Development.

Tissue processing

Deeply anaesthetised mice were fixed by transcardial perfusion with 4 % paraformaldehyde (PFA) in PBS. Full-thickness back skin (0.25-cm² squares) was excised from 3-week-old TSK heterozygous mice. The wounds were harvested after 2, 7, or 11 days for LacZ or HE staining and immunohistochemistry. These samples were preserved in 20 % sucrose overnight at 4 °C and then embedded in a mould containing cold Tissue Tek® OCT compound and stored at −80 °C. Frozen samples were sliced 10-µm sections with a cryostat (Leica).

LacZ staining

Slides were dried for 30 min at 37 °C and stained for 6–24 h at 37 °C in a solution containing 400 µg/mL X-gal. After washing in H₂O for 1 min at room temperature (RT), the slides were counterstained with eosin or 1 % Neutral Red (Wako) solution for 3 min, washed, dehydrated in graded ethanol and xylene, and mounted with xylene-based medium. We observed no endogenous β-gal activity with WT mice in our experiments.

Immunohistochemistry

Sections were blocked with PBS containing 5 % normal goat serum for 30 min at RT; the following antibodies diluted in PBS were applied: rat anti-F4/80 (1:200 Abcam), Cy3-conjugated rabbit anti-α-smooth muscle actin (1:500 SIGMA), and FITC-conjugated rabbit anti-β-galactosidase (1:100 Abcam). The sections were incubated with primary antibody at RT for 16 h in a moist chamber. After washing, the secondary antibody was applied to the sections for 1–2 h at RT. During washing with PBS, the slides were counterstained with Hoechst 33342 and mounted.

Quantitative PCR

Total RNA was prepared from the normal and wounded skin of WT and TSK^{−/−} mice. For wound healing analysis, 1-cm² squares of full-thickness back skin were excised from 3.5-week-old mice; wounded skin was harvested after 2, 5, and 8 days. Total RNA was extracted from each sample using Qiagen RNeasy spin columns (QIAGEN) and reverse-transcribed to cDNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems). The cDNA was stored at −20 °C until needed for quantitative PCR. Transcript levels of TGF-β1, IL-6, Stat3 VEGF, FGF, PDGF, and IGF2 were examined by quantitative PCR using sequence-specific primers and probes, pre-developed TaqMan assay reagents (40 cycles of denaturing at 92 °C for 15 s and annealing at 60 °C for 60 s), and an ABI StepOne™ (Applied Biosystems). Glyceraldehyde-3-phosphate (GAPDH) was used for normalisation. Relative expression was determined

by quantitative PCR using the $\Delta\Delta C_t$ method to compare target gene and housekeeping gene expression levels.

Cell culture and differentiation

NIH3T3 cells were cultured in DMEM containing 10 % FCS and maintained in a humidified incubator at 37 °C with 5 % CO₂. The cells were seeded on sterile square coverslips in 6-well plates. Upon reaching 70 % confluence, the medium was exchanged for serum-free DMEM. After 48 and 72 h, a subset of wells was filled with the following solutions: TGF-β1 (Cell Signaling Tech.) 2 ng/µl in all wells; TSK protein supernatant diluted to 0, 4, or 20 % with DMEM; the wells were incubated at 37 °C. At 48 and 72 h, all wells were washed twice with PBS. A subset of wells was fixed with 4 % PFA for 5 min at RT and washed with PBS; the coverslips were removed, placed on slides, and blocked with PBST containing 5 % skim milk for 30 min. The cells were immunostained with Cy3-conjugated anti-rabbit α-SMA antibody (1:300 Sigma) at RT for 1 h. After being washed with PBS, the cells were counterstained with Hoechst 33342 and mounted.

Statistical analysis

Student's *t*-test was used to determine the levels of significance between samples. A *P* value less than 0.05 was considered statistically significant.

Results

TSK is expressed during wound healing

To investigate TSK expression patterns in wound healing, we analysed the wound repair process in TSK heterozygous mice. Wounds were excised and sections prepared 2–11 days after wounding; LacZ-stained transverse sections were observed. TSK was expressed 2 days post-wounding (dpw) in the inflamed wounds (Fig. 1a). Immunofluorescence staining with an anti-macrophage antibody showed that macrophages expressed TSK (Figs. 1b and c). At 7 dpw, TSK was expressed from epidermis and extracellular matrix in the wound edge to neoepidermis and granulation tissue (Fig. 1d and e). Images of TSK-expressing granulation tissue (β-gal) were merged with those of α-smooth muscle actin (α-SMA), which was used as a myofibroblast marker in the wound (Fig. 1f). Further, TSK was expressed in

Fig. 1 TSK-expressing cells facilitate wound healing. (a, b, d, g, h) β -Galactosidase staining of wound cross-sections from TSK heterozygous mice. Counterstaining was performed using neutral red (a, b, d, g) and eosin (h). a TSK is expressed in inflammatory cells and neoepidermis 2 days post-wounding (dpw). b High magnification of the wounded area in (a) shows that TSK is expressed in polygonal cells, which are shaped differently than polymorphonuclear leukocytes (neutrophils). c Immunostaining with antibodies against macrophages (red) and β -gal (green) was performed on serial section with (b). The white box indicates high

magnification of the merged image (dotted line). d TSK is expressed from epidermis and extracellular matrix in the wound edge to granulation tissue and basal layer of neoepidermis (arrowheads) at 7 dpw. e HE staining of serial section with (d). f Merged image of α -SMA (red) and β -gal (green) staining. Hoechst shows nuclear stain (blue) (c, f). The arrows in (d) and (e) indicate the wound edge. Scale bars indicate 100 μ m (a, d, e, f, g) and 50 μ m (b, c, h). g TSK is expressed in musculus panniculus carnosus (dotted line) at 7 dpw. h TSK disappears in dermal area after fibrosis at 11 dpw

musculus panniculus carnosus under the granulation tissue (Fig. 1g). After fibrosis, TSK disappeared in dermal area at 11dpw (Fig. 1h). As negative controls, we performed the LacZ staining of wounded tissues with wild type mice and found no endogenous signal (data not shown).

Loss of TSK causes TGF- β 1 upregulation and excess inflammation in wound healing

To investigate the functional role of TSK during wound healing, mRNA expression levels of several cytokines and growth factors were examined by quantitative PCR in samples of wounded skin tissue (Fig. 2a). TSK $^{-/-}$ mice expressed substantially higher levels of TGF- β 1, IL-6, and Stat3 in comparison to WT mice during wound healing (Fig. 2a). Growth factor expression

(VEGF, FGF, PDGF, and IGF2) did not significantly differ between WT and TSK $^{-/-}$ mice (data not shown).

TSK inhibits myofibroblast differentiation by competing with TGF- β 1

To address how TSK functions in TGF- β signalling, we performed in vitro cell induction assay. Myofibroblast differentiation from NIH3T3 cells is induced by TGF- β 1 (Chen et al. 2009). The impact of TGF- β 1 and/or TSK on NIH3T3 cells was monitored by measuring expression of the myofibroblast marker α -SMA. Immunostaining analyses showed that TGF- β 1 alone induced myofibroblasts for 48 and 72 h (Fig. 2b), but TSK did not induce myofibroblast differentiation (data not shown). TSK combined with TGF- β 1 inhibited myofibroblast differentiation in a manner dependent on extracellular TSK protein concentration (Fig. 2b).

Fig. 2 **a** Expression of wound-related genes in the wounded skin tissue of WT and TSK $^{-/-}$ mice. Relative expression of TGF- β 1, IL-6, and Stat3 was determined by quantitative PCR and normalized to endogenous GAPDH. ** p < 0.005. Error bars indicate standard deviation. **b** TSK inhibits myofibroblast differentiation by competing with TGF- β 1.

Immunofluorescence pictures of NIH3T3 cells treated with TGF- β 1 and TSK protein supernatant (0, 4, 20 %) for 48 and 72 h. Myofibroblast differentiation (red) was inhibited gradually in a manner dependent on the TSK protein amount. Hoechst shows nuclear stain (blue). Scale bars indicate 50 μ m

Discussion

Our analysis of wound tissue in TSK heterozygous mice revealed that TSK is expressed sequentially from

macrophages to myofibroblast and from follicular cells to neoepidermis and granulation tissue. Wound macrophages derived from circulating monocytes appear after wounding (Brancato and Albina 2011). Myofibroblasts differentiate

Fig. 3 A schematic model of TSK function during wound healing. TSK controls macrophage function and myofibroblast differentiation by inhibiting TGF- β 1 secreted from macrophages. Further, TSK may maintain myofibroblast quiescence

from fibroblasts, capillary endothelial cells, and fibrocytes (Hinz 2007). Regenerated epidermis is reconstructed by the migration and proliferation of hair follicle stem cells or by dedifferentiated epidermis at the wound edge (Blanpain and Fuchs 2006; Oshima et al. 2001). Interestingly, TSK is not expressed in these progenitor cells such as circulating monocytes, epidermis and fibroblasts at the homeostatic state. Our findings suggest that TSK expression is characterised by spatially and temporally changing patterns of wound healing.

TGF- β 1 is produced by macrophages after wounding and contributes to myofibroblast differentiation (Brancato and Albina 2011). During wound healing, TSK $^{-/-}$ mice showed increased mRNA expression levels of TGF- β 1, the inflammatory cytokine IL-6, and the downstream signalling molecule Stat3. These results suggest TSK controls macrophage function; thus, TSK deletion causes excess inflammation. Other SLRP family molecules such as decorin also enhance macrophage activation by inhibiting endogenous TGF- β (Comalada et al. 2003). Furthermore, the cell-induction experiment results demonstrated TSK control of myofibroblast differentiation via inhibition of TGF- β 1 secreted from macrophage.

Thus, we suggest that TSK facilitates wound healing by maintaining inflammatory cell quiescence (Fig. 3). These findings help characterise the niche environment during wound healing.

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Conflict of interest The authors have no conflict of interest to declare.

References

- Blanpain C, Fuchs E (2006) Epidermal stem cells of the skin. *Annu Rev Cell Dev Biol* 22:339–373
- Brancato SK, Albina JE (2011) Wound macrophages as key regulators of repair: origin, phenotype, and function. *Am J Pathol* 178:19–25
- Chen H, Yang WW, Wen QT, Xu L, Chen M (2009) TGF- β induces fibroblast activation protein expression; fibroblast activation protein expression increases the proliferation, adhesion, and migration of HO-8910 PM. *Exp Mol Pathol* 87:189–194
- Comalada M, Cardó M, Xaus J, Valledor AF, Lloberas J, Ventura F, Celada A (2003) Decorin reverses the repressive effect of autocrine-produced TGF- β on mouse macrophage activation. *J Immunol* 170:4450–4456
- Cotsarelis G (2006) Epithelial stem cells: a folliculocentric view. *J Invest Dermatol* 126:1459–1468
- Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 127:514–25
- Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. *Nature* 453:314–321
- Hinz B (2007) Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127:526–537
- Ito A, Shinmyo Y, Abe T, Oshima N, Tanaka H, Ohta K (2010) Tsukushi is required for anterior commissure formation in mouse brain. *Biochem Biophys Res Commun* 402:813–818
- Niimori D, Kawano R, Felemban A, Niimori-Kita K, Tanaka H, Ihn H, Ohta K (2012) Tsukushi controls the hair cycle by regulating TGF- β 1 signaling. *Dev Biol* 372:81–87
- Ohta K, Lupo G, Kuriyama S, Keynes R, Holt CE, Harris WA, Tanaka H, Ohnuma S (2004) Tsukushi functions as an organizer inducer by inhibition of BMP activity in cooperation with chordin. *Dev Cell* 7:347–358
- Ohta K, Ito A, Kuriyama S, Lupo G, Kosaka M, Ohnuma S, Nakagawa S, Tanaka H (2011) Tsukushi functions as a Wnt signaling inhibitor by competing with Wnt2b for binding to transmembrane protein Frizzled4. *Proc Natl Acad Sci U S A* 108:14962–14967
- Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y (2001) Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell* 104:233–245
- Schaefer L, Iozzo RV (2008) Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J Biol Chem* 283:21305–21309