主論文の要旨

Negative Regulation of GADD34 on Myofibroblasts during Cutaneous Wound Healing

（創傷治癒における繊維芽細胞の働きをGADD34は負に制御する）

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Introduction

Cutaneous wound healing process depends on three major phases---inflammation, tissue formation and tissue remodeling. Among them tissue formation begins with re-epithelialization of wounds by epidermal cells migrating from skin appendages such as hair follicles. Next, myofibroblasts, a subgroup of cells with expression of α-smooth muscle actin (α-SMA) within cytoplasmic stress fibers, proliferate and are recruited to wound site. One of the most important function of myofibroblasts is to generate contractile forces to shrink wound size by deposition of collagen-rich extracellular matrix, including collagen type I, type III, fibronectin and proteoglycan. As the wound becomes epithelialized and the scar forms, myofibroblasts undergo apoptosis and disappear from granulation tissue, although this mechanism has not been entirely understood until now.

GADD34, as a member of growth arrest and DNA damage-inducible protein family, is up-regulated in response to a variety of cellular stresses, including endoplasmic reticulum stresses, nutrient deprivation, oxidative stress, heat shock and energy depletion. Expression of GADD34 is correlated with apoptosis and its overexpression facilitates apoptosis associated with ionizing radiation. Recently, GADD34 has been shown to be involved in TGFß signal pathway by cooperating with Smad7 to dephosphorylate TGFß type I receptor, which plays a crucial role in myofibroblast differentiation. The observed association of GADD34 with cellular apoptosis and TGFß signal pathway promoted us to ask whether GADD34 might participate in myofibroblast differentiation and apoptosis during wound healing.

Materials and Methods

Animal experiments.

For wound healing studies, 10 weeks WT (C57BL/6) and GADD34-deficient ($GADD34^{-/-}$, KO) male mice were anesthetized by intraperitoneal injection of avertin (250ul/20g mice) and a full-thickness 3 mm skin wound was made by punch biopsy onto the middle back skin. For each time point examined, wound tissue biopsies were collected with 6 mm diameter punch at day 0, 2, 4, 6, 8, 12, 16 days after wounding and either snap-frozen in liquid nitrogen or embedded in optimal cutting temperature (OCT) medium for further experiments.

Western blotting, immunohistochemistry and Immunofluorescence.

Tissue lysates were prepared with SDS lysis buffer. The primary antibodies used were Smad2/3, phospho-Smad2/3, GAPDH, caspase 3 (Cell Signaling). The blots were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Sigma). Western blots were developed by ECL detection system (GE Healthcare). To measure histological characteristics of wounds, each wound was

embedded and sectioned through its entirety. For histology analysis, cryosections were fixed in 4% formaldehyde and stained with Hematoxylin and Eosin. Masson`s trichrome staining was performed using a standard staining protocol. For immunofluorescence analysis, the cryosections were incubated with anti-α-SMA-Cy3 (Sigma C6198) antibody. After counterstaining with DAPI, the slides were mounted. To examine apoptotic myofibroblasts, TUNEL staining was performed using an In situ Apoptosis Detection Kit (Takara, MK500).

Real-time PCR.

RNA isolation (QIAGEN) and Taqman reverse transcriptase reactions (Applied Biosystems) were performed according to the manufacturer's instructions. Real time PCR reactions (SYBR Green system) were conducted. Each value was normalized to the ß-actin level as a reference.

Statistical Analysis

The unpaired two-tailed Student`s t test was used to identify statistical significance，multiple comparisons of which were corrected by using the Holm-Sidak method, and a p value of <0.05 was considered significant. The formula of α-SMA$^+$ area $\%=\frac{\alpha-SMA+\ area\ within\ granulation\ tissue}{granulation\ tissue\ area}\times 100\%$ was used for calculating the percentage of α-SMA$^+$ area.

Results

Refer to the next page.

Discussion

In this study, we showed that GADD34 could inhibit phosphorylation of Smad3 during myofibroblast differentiation in proliferative phase. Thus GADD34 suppresses α -SMA expression via inhibition of Smad3 phophorylation. When we examined α -SMA expression and Smads phosphorylation, we found that more fair follicle formed in GADD34-/- mice and simultaneously Smad2 phosphorylation also increased. Previous investigation has shown that GADD34 can be recruited by Smad7 to dephophorylate TGF ß type I receptor. Thus, through being recruited by Smad7 to inhibit Smad2/3 phosphorylation, GADD34 possibly has a role of universe suppressing activity in other biological processes that are regulated by Smad-dependent TGF ß signal pathway.

The contribution of GADD34 to apoptotic process remains unclear. The existing reports indicate a double role of GADD34 in cellular apoptosis. The expression of GADD34, caused by both VSV infection and energy depletion, can protect cells from apoptotic cell death by activating caspase3. However, overexpression of GADD34

facilitates cellular apoptosis associated with ionizing radiation. Why there exist conflicting molecular mechanisms has not been elucidated until now. Our data presented here support that GADD34 elicits cellular apoptosis through activating caspase3, at least during dermal wound repair.

Conclusion

1. GADD34 suppresses myofibroblast differentiation through inhibiting phosphorylation of Smad3.
2. GADD34 facilitates myofibroblast apoptosis by activating caspase-3 pathway.