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Expression of Hsp47 in Fibroblasts Derived from Fetal and Neonatal Rat Tongues

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Abstract : Scar formation, caused by the abnormal expression and accumulation of collagen molecules accompanying the expression of heat shock protein 47 (Hsp47), a collagen-specific molecular chaperone, is a serious problem after surgery in the postnate, whereas in the fetus, the wound heals without scarring. In this study, we compared the expression patterns of Hsp47 and type I and type III collagens induced by TGF- β 1 between fetal and neonatal fibroblasts in the primary cultures of rat tongues. In the neonate, high level expressions of both Hsp47 and collagen mRNAs and proteins were observed to be induced by TGF- β 1, but not in the fetus. We performed a reporter assay using pLUC 5.5(III), which carried the enhancer/promoter region of the mouse Hsp47 gene, to compare promoter/reporter activity. In the neonate, high promoter/reporter activity in fibroblasts treated with TGF- β 1 was observed, but was unchanged in the fetus. Thus, the expression of Hsp47 is enhanced by TGF- β 1 in the neonate but not in the fetus. This different Hsp47 expression pattern between the fetus and neonate appears to be attributed to the different transcriptional regulation of the gene. Elucidation of the regulatory mechanism of Hsp47 production in developmental processes may provide a therapeutic modality for the scarless healing of postnatal wounds.

抄録：コラーゲンの異常な発現と蓄積に起因する瘢痕形成は、生後の外科処置後の深刻な問題である。一方、胎児の創傷は瘢痕を形成することなく治癒する。Heat shock protein 47 (Hsp47) はコラーゲンに特異的な分子シャペロンであり、瘢痕部での発現の増加が認められる。本研究では、胎児および新生仔のラット舌由来の線維芽細胞を用いて、TGF- β 1 に誘導される Hsp47、I および III 型コラーゲンの発現パターンを比較検討した。新生仔由来線維芽細胞では、TGF- β 1 処理により Hsp47 およびコラーゲンの発現は mRNA、タンパク質ともに増加した。これに対して、胎児由来線維芽細胞ではそれらの増加は認められなかった。さらに、プロモーター/レポーター活性を比較するために、マウス Hsp47 遺伝子のエンハンサー/プロモーター領域を含んだ pLUC 5.5 (III) を用いてレポーターアッセイを行った。その結果、新生仔由来線維芽細胞では TGF- β 1 処理により高い活性が認められたが、胎児由来線維芽細胞では認められなかった。以上のことより、Hsp47 の発現は新生仔ラットでは TGF- β 1 により増加することが明らかになった。さらに、胎児と新生仔における Hsp47 の発現様相の差は、遺伝子の転写レベルですでに存在することが明らかになった。個体発生および発育過程における Hsp47 生成のメカニズムの解明は、生後の創傷を無瘢痕性に治癒させる治療法に寄与すると思われる。

Introduction

Heat shock protein 47 (Hsp47), a 47-kDa stress protein, is a molecular chaperone, which is localized in the endoplasmic reticulum (ER) as inferred from the presence of a carboxyl-terminal RDEL sequence similar to the ER retrieval signal, KDEL¹⁻³. Hsp47 is an essential protein for the synthesis and processing of various types of collagens^{4,5}. Collagen-producing cells always accompany the expression of Hsp47, but conversely, non-producing cells do not^{1,6-10}.

There is a remarkable morphological and biochemical difference in the wound healing process between fetal and neonatal animals. Neonatal skin wounds heal with scar formation, whereas fetal skin wounds show minimal inflammatory response and heal without scar formation, fibrosis and contracture¹¹⁻¹³. Scar formation is characterized by an abnormal deposition and placement of collagen fibers, especially type I and type III collagens, accompanied with an increased expression of Hsp47^{14,15}.

It has been shown that fetal scarless wound repair is not attributed to systemic factors involved in the immune system, serum, or amniotic fluid. The capacity for scarless healing is considered to be inherent in fetal tissue itself, and fibroblasts are thought to be the main effectors of scarless healing in fetal tissues¹⁶⁻²³. However, the details of this mechanism are largely unknown.

Scar formation during skin wound healing of neonates has been reported to be closely related to the activity of TGF- β family cytokines²⁴. In particular, TGF- β 1 is a key cytokine involved in the initiation and termination of tissue repair^{25,26} and has important roles in wound healing, which accompanies the deposition of collagen and other extracellular matrix components²⁷. However, the over-expression of TGF- β 1 during neonatal wound healing causes the enhanced expression of Hsp47 and type I and III collagens.

The aim of this study was to elucidate the mechanism of different expression patterns of Hsp47 as well as type I and III collagens between fetal and neonatal fibroblasts.

Materials and Methods

1. Primary cell culture and treatment with recombinant human TGF- β 1

Fibroblasts outgrown from the healthy tongues of fetuses (17-day gestation) and neonates of Sprague-Dawley (SD) rats (gestation term=21 days) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Nissui, Japan) containing 10% fetal bovine serum (FBS) (Invitrogen life technologies, California, U. S. A.) and 1% penicillin-streptomycin (Invitrogen life technologies) in 100-mm plates under humidified 5% CO₂ at 37°C. The medium was changed twice a week and the cells were passaged with PBS (-) containing 0.02% trypsin (Invitrogen life technologies) and 0.25% EDTA. After 3-5 passages, the penicillin-streptomycin solution was removed from the medium. All the following experiments were performed in 60-mm plates. After cultivation in serum-free DMEM for 24 h to eliminate the effect of cytokines present in the serum, the cells were cultured in medium containing 10 ng/ml recombinant human TGF- β 1 (R & D Systems, Inc., Minneapolis, U. S. A.) up to 48 h.

2. Western blotting analysis

Cells were dissolved in lysis buffer [150 mM NaCl, 50 mM Tris-HCl : pH 8.0, 0.1% Nonidet P-40 containing 0.25% Protease Inhibitor Cocktail Tablets : Complete, Mini (Roche, Germany)] . Protein concentration was measured using a protein assay kit (Bio-Rad) according to the manufacturer's instructions. One μ g of protein was applied per lane on a 10% SDS-polyacrylamide gel followed by electrophoresis²⁸. Separated proteins were transferred to a nitrocellulose membrane (Bio Trace NT Blotting Membrane : Gelman Sciences Japan)²⁹. After blocking with 3% skim milk in Tris-buffered saline containing 1% Tween 20, pH 7.4 (TBS-T) for 30 min at room temperature, the membranes were incubated with the mouse anti-Hsp47 (Colligin) monoclonal antibody (StressGen Biotechnologies Corp, Canada), goat anti-procollagen α 2(I) (Y-18) polyclonal antibody (Santa Cruz Biotechnology, Inc., U. S. A.), or goat anti-

Table 1 Sequences of primers

Gene		Primer sequence	Base pairs	Annealing temperature (°C)	Cycle number	Reference
Hsp47	Forward	5'-AAGATGGTAGACAACCGTGG-3'	604	62	30	30)
	Reverse	5'-GTCTCGCATCTTGTCTCCCTT-3'				
$\alpha 1(I)$	Forward	5'-CCCACCCCAGCCGCAAAGAGT-3'	352	51	35	31)
	Reverse	5'-TTGGGTCCCTCGACTCCTACA-3'				
$\alpha 1(III)$	Forward	5'-TGCCCACAGCCTTCTACACCT-3'	244	58	25	32)
	Reverse	5'-CAGCCATTCCCTCCCACTCCAG-3'				
GAPDH	Forward	5'-TCCCTCAAGATTGTCAGCAA-3'	308	55	30	33)
	Reverse	5'-AGATCCACAACGGATACATT-3'				

$\alpha 1(I)$ and $\alpha 1(III)$: procollagen $\alpha 1(I)$ and $\alpha 1(III)$, respectively, GAPDH : glyceraldehyde-3-phosphate dehydrogenase.

procollagen $\alpha 1(III)$ (N-18) (Santa Cruz Biotechnology, Inc.) at 1 : 4000 dilution overnight at 4°C. Proteins were detected with ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc. : U.K.) and visualized using an enhanced chemiluminescence system (ECL : Amersham Pharmacia Biotech Inc.). The relative density of the protein bands was quantified using a Scion Image. The density of a band untreated with TGF- $\beta 1$ (0 h) was set as 1.

3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured fibroblasts using ISOGEN (Wako, Japan). Reverse transcription-PCR was carried out with total RNA (0.5 μ g for Hsp47, 3 ng for procollagen $\alpha 1(I)$ and 10 ng for procollagen $\alpha 1(III)$) by SUPERScript One-Step RT-PCR with PLASTINUM *Taq* (Invitrogen life technologies). Samples were incubated in a PCR Thermal Cycler (PERSONAL, TaKaRa) at 51–62°C for 25–35 cycles, depending on the primer sets (see Table 1). The PCR products were separated by electrophoresis on a 1.6% agarose gel and the bands were visualized by ethidium bromide staining³⁴⁾. The density of each band was quantified using a Scion Image. The density of the band untreated with TGF- $\beta 1$ (0 h) was set as 1.

4. Transient transfection of a Hsp47 gene reporter plasmid

Cells were plated at a density of 1×10^5 /60-mm/culture dish. After being cultured for 20 h, cells were transfected by the calcium phosphate method (DAC-30 : EUROGENTEC BEL., Belgium) with 2.5 μ g of pLUC 5.5(III)³⁵⁾ as a reporter plasmid for the Hsp47 gene. One μ g of pRL-TK (Wako) was co-transfected as the internal control to normalize the variation in transfectional efficiency. After 4 h, the medium with the transfection precipitate was replaced with fresh DMEM containing 10% FBS. After 14 h, the medium was changed to serum-free conditions to eliminate the effect of cytokines present in the serum. Cells were further incubated for 24 h. TGF- $\beta 1$ (10 ng/ml) was then added and the cells were harvested after 24 h. Quantification of the luciferase activity was carried out with a Lumat LB 9501 (Berthold) as described previously³⁵⁾. The activity of pRL-TK (internal control) was set as 100.

5. Statistics

All of the values were expressed as the mean $\pm$ S. D. Statistical significance was evaluated by Student's *t*-test.

Results

1. Western blotting analysis

Immunoblotting analysis revealed a 47-kDa band, specific for Hsp 47. The intensity was unchanged in

Fig. 1 Western blotting analysis of Hsp47 and type I and type III collagens.

(A) Hsp47, (B) procollagen $\alpha 2(I)$ and (C) procollagen $\alpha 1(III)$ proteins were immunoblotted after the treatment of (left) fetal and (right) neonatal fibroblasts with 10 ng/ml TGF- β 1 for 0–48 h. Values are expressed as means $\pm$ S. D. ($n=6$).

the fibroblasts derived from the fetal tongue following treatment with TGF- β 1 (0, 1, 5 and 10 ng/ml). In contrast, in the neonatal fibroblasts, the expression increased in a concentration-dependent manner for TGF- β 1 at 36 h (data not shown). When the cells were exposed to TGF- β 1 at 10 ng/ml for 0, 24, 36 and 48 h, the Hsp47 expression was unchanged in the fetal fibroblasts, while in the neonatal fibroblasts, the intensity of the Hsp47 band treated with TGF- β 1 for 36 h was 3.3 times greater than that of the non-treated specimen (Fig. 1 A). The relative expression level of Hsp47 in the fetal and neonatal fibroblasts at 0 h was almost identical (1 : 0.95). Concomitantly, TGF- β 1 increased the expression of procollagens $\alpha 2(I)$ and $\alpha 1(III)$, 2.6- and 2.3-fold, respectively, in the neonatal fibroblasts after treatment with TGF- β 1 for 36 h, but not in the fetal cells (Fig. 1 B, C).

2. RT-PCR analysis

The Hsp47 mRNA level slightly decreased during

Fig. 2 RT-PCR analysis of Hsp47 and procollagen $\alpha 1(I)$ and $\alpha 1(III)$ mRNAs.

RT-PCR was performed for (A) Hsp47, (B) procollagen $\alpha 1(I)$, (C) $\alpha 1(III)$ mRNAs after the treatment of (left) fetal and (right) neonatal fibroblasts with TGF- β 1 for 0–48 h. Values are expressed as means $\pm$ S. D. ($n=6$). M, 100-bp ladder (TAKARA SHUZO CO.).

the TGF- β 1 treatment period in fetal fibroblasts (Fig. 2 A). There was also little difference in the expression levels of procollagen $\alpha 1(I)$ and $\alpha 1(III)$ mRNAs in fetal fibroblasts with or without TGF- β 1 treatment (Fig. 2 B, C). However, in neonatal fibroblasts, the amount of Hsp47 mRNA after treatment with TGF- β 1 for 24 and 36 h was 2.6- and 3.8-fold higher than that of non-treated cells, respectively (Fig. 2 A). In the case of procollagen $\alpha 1(I)$, the expression was 5.2- and 2.8-fold increased after 36 and 48 h treatments, respectively (Fig. 2 B), and the procollagen $\alpha 1(III)$ expression was 3.2-fold higher at 24 h (Fig. 2 C). Thus, the expressions of Hsp47 protein and mRNA were responsive to TGF- β 1 in the fibroblasts derived from the neonatal but not the fetal tongue. Apparently, the increase in the protein contents was either

preceded by the increase in the Hsp47 and procollagen $\alpha 1(\text{III})$ mRNAs or coincided with the increase in procollagen $\alpha 1(\text{I})$ mRNA.

3. Reporter assay

In the neonatal fibroblasts, the luciferase activity was 2.5-fold up-regulated by TGF- β 1, while in the fetal fibroblasts, the activity was not altered (Fig. 3). This finding was consistent with the results of Western blotting (Fig. 1) and RT-PCR analyses (Fig. 2), and further confirmed that the expression of Hsp47 mRNA was directly enhanced by TGF- β 1.

Discussion

Why does a fetal wound heal without a scar? Many studies have attempted to answer this question, but it remains to be elucidated. We recently reported that the up-regulation of type I collagen and Hsp47 expression is directly responsible for scar formation in neonatal rat wound³⁶⁾. Subsequently, in this study, we compared the expression of Hsp47 between fibroblasts derived from fetal and neonatal tissues.

Type I and III collagens are the main components of the extracellular matrix in the scar, and heat shock protein 47 (Hsp47) is also co-expressed in this region^{1,8)}. Hsp47 is a collagen-specific molecular chaperone and an essential proteins for collagens syntheses. Therefore, it was reasonably assumed that the different patterns of Hsp47 expression between the fetus and neonate were responsible for their resultant wound healing.

We used 17-day gestation rats to propagate the fibroblasts, because the transitional point from scarless healing to scar formation is at 18–19 days of gestation in rats^{37,38)}. It has been reported that the expression of TGF- β 1 was scarcely altered during fetal wound healing²⁹⁾, and this has been considered to be one of the reasons why fetal wounds heal without scar. We used TGF- β 1 as the inducer of collagen production, because it is an important factor in wound healing, and moreover, its over-expression induces scar formation during wound healing^{39–41)}.

In fetal fibroblasts, TGF- β 1 had little effect on the expression of Hsp47 and type I and III collagens. In

Fig. 3 Reporter assay with pLUC 5.5(III).

The luciferase activity of fetal and neonatal fibroblasts, transfected with pLUC 5.5(III) was determined as described in Materials and Methods. Transfected cells treated (open) without or (hatched) with TGF- β 1 for 24 h. Values are expressed as means $\pm$ S. D. (n=6).

contrast, in the neonatal fibroblasts, they were increased by TGF- β 1 at both the protein and mRNA levels. Thus, the coordinated expression of Hsp47 and collagens^{1,8)} was maintained, when the fibroblasts were treated with TGF- β 1 *in vitro*. The difference in their biological behavior appears to be maintained in fibroblasts derived from fetal and neonatal rat tongues for at least several passages.

In order to determine whether TGF- β 1 directly modulates the expression of Hsp47, we performed a transfection assay of the fibroblasts with pLUC 5.5(III). The mouse Hsp47 gene consists of 6 exons and 5 introns, of which pLUC 5.5(III) encodes the first and second introns and the first exon followed by the luciferase gene³⁵⁾. The luciferase activity of pLUC 5.5(III) has been reported to be up-regulated in Hsp47-producing cells but not in nonproducing cells³⁵⁾. This study also demonstrated the gene activation of Hsp47 by TGF- β 1 in the neonatal fibroblasts.

The expression of TGF- β receptor I (T β RI) was unchanged by TGF- β 1 in both neonatal and fetal fibroblasts (Ohba, S. and Inokuchi, T., unpublished observation). Durham, L. A. 3rd, *et al.* reported that, as fibroblasts differentiate during development from embryo through fetus on to adult, there is a progressive reduction in the number of T β Rs with adult fibroblasts expressing the minimum⁴²⁾. Since TGF- β 1

is an essential growth factor in ontogenesis, it is not considered that T β R in the fetus is inactive. Altogether, it is not presumed that the number of T β Rs are pertinent to the different expression of Hsp47 induced by TGF- β 1 between fetal and neonatal fibroblasts.

This study demonstrated that scar formation in the neonatal wound is partly attributed to TGF- β 1. However, this study also revealed that the scarless healing characteristic of a fetal wound may be independent of the concentration of TGF- β 1, because exogenous TGF- β 1 did not induce the expression of Hsp47 and collagens. Accordingly, unknown factor(s) may contribute to this phenomenon. There are several possibilities for this hypothetical factor(s) : The first possibility is that this might involve the transcription factor(s) or suppressor(s) of genes for Hsp47 and collagenous proteins. Hirata, *et al.*³⁵⁾ reported the presence of the Hsp47 gene-binding factor specific for Hsp47-producing cells. Accordingly, it is of great interest to investigate the developmental kinetics of this factor during gestation and the neonatal period. Secondly, it is conceivable that a factor(s) may play a role downstream of TGF signaling, eg, Smad family proteins. For instance, there is an inhibitory Smad in TGF- β signaling, Smad7, and the addition of IFN- γ induces Smad7 expression, causing the suppression of the collagen expression⁴³⁾.

Conclusion

The expression of Hsp47 as well as type I and III collagens is induced by TGF- β 1 in neonatal but not fetal fibroblasts. This process appears to be readily controlled at the transcriptional level.

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