Insufficient Folding of Type IV Collagen and Formation of Abnormal Basement Membrane-like Structure in Embryoid Bodies Derived from Hsp47-Null Embryonic Stem Cells

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Hsp47 is a molecular chaperone that specifically recognizes procollagen in the endoplasmic reticulum. Hsp47-null mouse embryos produce immature type I collagen and form discontinuous basement membranes. We established Hsp47−/− embryonic stem cell lines and examined formation of basement membrane and production of type IV collagen in embryoid bodies, a model for postimplantation egg-cylinder stage embryos. The visceral endodermal cell layers surrounding Hsp47−/− embryoid bodies were often disorganized, a result that suggested abnormal function of the basement membrane under the visceral endoderm. Rate of type IV collagen secretion by Hsp47−/− cells was fourfold lower than that of Hsp47+/+ cells. Furthermore, type IV collagen secreted from Hsp47−/− cells was much more sensitive to protease digestion than was type IV collagen secreted from Hsp47+/+ cells, which suggested insufficient or incorrect triple helix formation in type IV collagen in the absence of Hsp47. These results indicate for the first time that Hsp47 is required for the molecular maturation of type IV collagen and suggest that misfolded type IV collagen causes abnormal morphology of embryoid bodies.

INTRODUCTION

Collagen is one of the most abundant proteins of the extracellular matrix (Kuhn et al., 1987); 26 subclasses of collagen molecules, types I through XXVI, have been identified (Kuhn et al., 1987; Prockop and Kivirikko, 1995; Sato et al., 2002). Although the fibril-forming types I and III collagen are abundant in most extracellular matrices, type IV collagen, a nonfibrillar network-forming collagen (Timpl et al., 1981; Kuhn et al., 1987), is the major component of basement membrane (BM). Collagen molecules contain helical domains, known as collagenous domains, that are composed of X-Y-Gly triplet repeats; often, the Y residue is hydroxyproline (Kuhn et al., 1987; van der Rest M, 1991). The type IV procollagen molecule consists of three domains; 7S, a short, N-terminal triple-helical domain; NC1, a noncollagenous, C-terminal C-propeptide domain; and a long triple-helical collagenous domain centrally (Kuhn et al., 1987).

During collagen biosynthesis, procollagen chains interact with several endoplasmic reticulum (ER)-resident molecular chaperones and protein folding catalysts (Lamandé and Bateman, 1999), including the 47-kDa heat shock protein (Hsp47) (Nagata, 1996), the 78-kDa glucose-regulated protein (GRP78, also called BiP) (Chessler and Byers, 1993), protein disulfide isomerase (PDI) (Wilson et al., 1998), and prolyl 4-hydroxylase (P4H) (Chessler and Byers, 1992; Walmsley et al., 1999). Formation of triple helices within collagenous domains proceeds from the C terminus to the N terminus (Bachinger et al., 1980; Engel and Prockop, 1991; Bulleid et al., 1997), and hydroxylation of proline residues at the Y positions stabilizes the triple helices of procollagen (Uitto and Prockop, 1974). After triple helix formation, procollagen is exported through the general secretion pathway and then mature collagen forms higher order complexes in the extracellular matrix (Timpl et al., 1981; Kuhn et al., 1987).

Hsp47 is a collagen-binding protein (Nagata and Yamada, 1986) that assists in the molecular maturation of procollagen (Nagai et al., 2000; Tasab et al., 2000). Hsp47 can bind to procollagen in vivo (Nakai et al., 1990) and in vitro (Natsume et al., 1994; Koide et al., 2002) and preferentially binds the triple-helical region of procollagen (Koide et al., 2000; Tasab et al., 2000). In vitro analyses have indicated that Hsp47 recognizes Pro-Pro-Gly triplet repeats (Koide et al., 2000) and preferentially binds to Pro-Arg-Gly triplet repeats (Koide et al., 2002; Tasab et al., 2002). Hsp47 dissociates from procollagen in the ER-Golgi intermediate compartment or in the cis-Golgi during transport to the Golgi apparatus (Nakai et
Immunostaining

EBs were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde at 4°C. Paraffin sections (4 μm) were treated with 0.3% hydrogen peroxide/methanol at room temperature for 30 min. After blocking non-specific protein binding using 5% horse serum for 30 min, sections were incubated with rabbit antibodies against Hsp47, type IV collagen, or laminin (1:200), followed by incubation with biotinylated anti-rabbit IgG (Elite ABC kit; Vector Laboratories, Burlingame, CA). Specific antibody binding was visualized using Elite ABC reagent (Vector Laboratories) and the EnVision kit/HRP (DakoCytomation, California, Carpiniteria, CA). The percentage of EBs that contained a normal VE cell layer was estimated using the following four criteria for normal: 1) EB diameter >100 μm; 2) extracellular thin layer (between the outer VE cell layer and the epiblast/inner cell mass) stained strongly with anti-type IV collagen antibody; 3) VE cell layer showed normal simple epithelial morphology; and 4) >20% of the outer surface of the EB was surrounded by VE cells.

Metabolic Labeling

Ascorbic acid phosphate (136 μg/ml) was added to monolayer endodermal EBs 16 h before metabolic labeling. Cells were incubated with 3.9 MBq/ml 2-3H-Pro (Amersham Biosciences UK) for 10 h. Aliquots of medium containing equal amounts of TCA-insoluble radioactivity were treated with a prostatease mixture consisting of 100 μg/ml trypsin and 250 μg/ml chymotrypsin in 0.05 M Tris-HCl (pH 7.4), 0.2 M NaCl, and 0.25 M glucose at 37°C or 4°C. Digests of type IV collagen were analyzed using 5% SDS-PAGE; proteins were fixed using 30% methanol/10% acetic acid, and gels were soaked in EN/HANCE (PerkinElmer Life and Analytical Sciences) and exposed to x-ray film.

Protease Digestion of Secreted Type IV Collagen

Monolayer endodermal EBs were cultured in medium that contained ascorbic acid phosphate (136 μg/ml) and ditalyzed 10% fetal calf serum in the presence of 3.7 MBq/ml 2-3H-Pro (Amersham Biosciences UK) for 10 h. Aliquots of medium containing equal amounts of TCA-insoluble radioactivity were treated with a prostatease mixture consisting of 100 μg/ml trypsin and 250 μg/ml chymotrypsin in 0.05 M Tris-HCl (pH 7.4), 0.2 M NaCl, and 0.25 M glucose at 37°C or 4°C. Digests of type IV collagen were analyzed using 5% SDS-PAGE; proteins were fixed using 30% methanol/10% acetic acid, and gels were soaked in EN/HANCE (PerkinElmer Life and Analytical Sciences) and exposed to x-ray film.

Binding of Type IV Collagen to Fibronectin

Secreted type IV collagen was labeled with 1-23-3H-Pro (described previously) by using a shortened labeling time of 6 h. Bovine plasma fibronectin (ICN, Costa Mesa, CA) was coupled with cyanogen bromide in excess to Sepharose 4B (Pharmacia Biotechnology). Fibronectin-coupled beads (20 μl) were mixed with cell culture medium (200 μl), incubated for 2 h at 4°C with gentle mixing, and washed with 0.05 M HEPES (pH 7.5) containing 0.15 M NaCl, 0.15 mM EDTA. Bound proteins were extracted by boiling for 5 min in Laemmli's sample buffer supplemented with 0.1 M dithiothreitol. Extracted proteins were separated using 5% SDS-PAGE; proteins were fixed using 30% methanol/10% acetic acid, and gels were soaked in EN/HANCE (PerkinElmer Life and Analytical Sciences) and exposed to x-ray film.

RESULTS

Abnormal Morphology of VE Cell Layer in EBs of Hsp47−/− ES Cells

BM has been reported to be discontinuously disrupted in Hsp47-null mouse embryos (Nagai et al., 2000). We first
examined the morphology of *Hsp47*/*-" EBs by immuno-
staining of sections (Figure 1) and found that the mor-
phology of VE cell layers composing outer surface of EBs
are abnormal in *Hsp47*/*-" EBs. At day 8, anti-*Hsp47*
antibody strongly stained VE cell layers in *Hsp47*/*-" EBs
(Figure 1A), whereas no staining was observed in
*Hsp47*/*-" EBs, confirming the absence of *Hsp47* protein
(Figure 1, B and C). *Hsp47*/*" and *Hsp47*/*-" EBs
both contained BM-like structures that separate the outer
VE cell layer from the inner epiblast cells (Figure 1, D–K).
In both genotypes, most EBs formed cavities (Figure 1,
D–K), which suggested that differentiation of EBs were
not affected by absence of *Hsp47* at this stage of develop-
ment.

Figure 1. VE cell layer shows disorganized morphology in EBs of *Hsp47*/*-" ES
cells. The distribution of *Hsp47* (A–C), type IV collagen (D–F, J, and K), and
laminin (G–I) in EBs differentiated from *Hsp47*/*" and *Hsp47*/*-" ES cells at day
8 were analyzed by immunostaining. (A, D, G, and J) *Hsp47*/*-" clone 7. (B, E, H, and K)
*Hsp47*/*-" clone 10. (L) Percentage of EBs that contained normal morphology of VE cell layers adjacent to BM-like structures
(see Materials and Methods for details) was determined. Bars, 100 μm (A–I); and 10
μm (J and K). EE, epiblast epithelium; asterisk, cavity.
However, we found significant difference in the morphology of VE cell layer situated along the BM-like structures. In most Hsp47−/− EBs, the VE cell layers exhibited disorganized morphology, and weak dispersed staining with anti-type IV collagen and anti-laminin antibodies was observed at the extracellular regions (Figure 1, E, F, H, I, and K). In contrast, the VE cell layers exhibited smooth simple epithelium-like morphology in Hsp47+/+ EBs (Figure 1, D, G, and J). Most Hsp47+/+ EBs contained thin BM-like structures strongly and continuously stained with anti-type IV collagen and anti-laminin antibodies. In two Hsp47−/− ES clones, normal VE cell layer epithelial morphology was seen only in 13% (12 of 95) and 15% (21 of 144) of EBs, whereas normal morphology was seen in 57% (158 of 276) of Hsp47+/+ EBs (Figure 1L). Because the BM is important for maintaining epithelial cell layers, the abnormal morphology of the VE cell layer adjacent to the BM in Hsp47−/− EBs may be due to a defect in BM function.

**Expression of Type IV Collagen Is Not Affected by the Absence of Hsp47**

Because the morphology of VE cell layers along the BM-like structures was disordered in Hsp47−/− EBs (Figure 1) and Hsp47 is known to recognize type IV collagen in vitro (Natsume et al., 1994), we next examined expression levels of type IV collagen, a component of BM, in Hsp47−/− cells during differentiation (Figure 2). Using Western blotting, we found Hsp47 in Hsp47−/− monolayer endodermal cells at day 3 and in Hsp47−/− EBs at day 8. The level of Hsp47 and type IV collagen both increased over the course of differentiation (Figure 2A), a result that is consistent with previous observations that indicated Hsp47 and type IV collagen are
Figure 3. Rate of type IV collagen secretion is significantly slower in Hsp47−/− cells than in Hsp47+/+ cells, as determined by pulse-chase experiment. (A–C) Radioactivity of intracellular and secreted type IV collagen of Hsp47+/+ cells (A), Hsp47−/− clone 7 cells (B), and Hsp47−/− clone 10 cells (C). (D) Relative radioactivity of intracellular type IV collagen was plotted in a logarithmic scale (n = 3). (E) Radioactivity of fibronectin secreted into the medium.
both expressed in early-stage mouse embryos and in differentiating F9 cells (Leivo et al., 1980; Takechi et al., 1992; Nagai et al., 2000). In contrast, type I collagen was not detected at any stage. These results suggest that the up-regulation of Hsp47 during ES cell differentiation may play a role in the production of type IV collagen.

The level of type IV collagen and of type IV collagen mRNA was similar between Hsp47+/+ and Hsp47−/− cells (Figure 2, B and C). In both cell types, most type IV collagen was present in the NP-40–soluble fraction (unpublished data), which suggests that type IV collagen does not aggregate in these cells. These results indicate that disruption of the Hsp47 gene does not affect the level of expression of type IV collagen.

**Type IV Collagen Secretion Rate Is Significantly Slower in Hsp47−/− Cells**

To examine whether the secretion of type IV collagen is affected by the absence of Hsp47, we determined the secretion rate of type IV collagen by pulse-label and chase experiments by using 35S-Met and 35S-Cys. Intracellular and secreted type IV collagen were detected by immunoprecipitation by using the specific antibody (Figure 3). The decrease in the intracellular amounts of type IV collagen directly corresponded to the increase in the amounts of type IV collagen secreted into the medium both in Hsp47+/+ and Hsp47−/− cells (Figure 3, A–C). The level of labeled intracellular type IV collagen decreased during chase periods, and the rate of decrease was fourfold slower in Hsp47−/− cells than in Hsp47+/+ cells (Figure 3D), which was consistent with the rate of increase in the type IV collagen secreted into the medium, that is, the rate of secretion was significantly higher in Hsp47+/+ cells compared with that in Hsp47−/− cells (Figure 3, A–C). In contrast, the secretion of fibronectin analyzed as a control was not affected by the absence of Hsp47 (Figure 3E), which indicated that general secretion pathways were not impaired by the disruption of Hsp47 gene. These results indicate that the absence of Hsp47 specifically caused a marked decrease in the rate of secretion of type IV collagen, a finding that is consistent with the previous observation that the secretion of type IV collagen was enhanced in Hsp47 overexpress cells (Tomita et al., 1999; Rocnik et al., 2002).

**Type IV Collagen Secreted from Hsp47−/− Cells Binds with High Affinity to Fibronectin**

Fibronectin is an extracellular matrix protein that interacts with various types of collagen (Engvall and Ruoslahti, 1977; Jilek and Hormann, 1978). Interestingly, fibronectin preferentially binds via ionic interaction (Vuontu et al., 1982) to denatured, unfolded collagen (gelatin) rather than to native collagen (Engvall and Ruoslahti, 1977; Engvall et al., 1982). We therefore used fibronectin as a high-affinity indicator of denatured or misfolded collagen. Binding of secreted type IV collagen to fibronectin was examined using fibronectin-coupled Sepharose beads (Figure 5). Fibronectin-Sepharose bound a much higher (10-fold) quantity of type IV collagen, which had been secreted by Hsp47−/− cells compared with that secreted by Hsp47+/+ cells (Figure 5, A and C). Similarly, heat-denatured type IV collagen exhibited marked increase (14-fold) in binding to fibronectin-Sepharose (Figure 5, B and D), a result that confirmed that fibronectin binds to denatured type IV collagen with high affinity. These results support our earlier observation; type IV collagen secreted by Hsp47−/− cells is not correctly folded.

**DISCUSSION**

In this study, we found that type IV collagen secreted by Hsp47−/− cells is much more susceptible to protease digestion than those with correct triple-helical structures (Dolz et al., 1988; Nagai et al., 2000). We compared the protease sensitivity of type IV collagen derived from differentiating ES cells by digesting the collagen by using a mixture of trypsin and chymotrypsin to address the molecular features of secreted collagen (Figure 4). Type IV collagen molecules of 210 and 200 kDa secreted by Hsp47−/− cells yielded a 190-kDa fragment probably consisting of triple-helical domain after protease treatment at 37°C for 5 min. In contrast, type IV collagen molecules secreted by Hsp47−/− cells that were treated under the same conditions were undetectable by SDS-PAGE (Figure 4). Complete digestion of type IV collagen secreted by Hsp47−/− cells also was observed after treatment at 4°C for 5 min (unpublished data). In contrast, protease sensitivity for intracellular type IV collagen of Hsp47−/− cells was similar to that of Hsp47+/+ cells (Supplemental Data 1), suggesting that properly folded type IV collagen is rapidly secreted from the ER and only minor portion was remained in the Hsp47+/+ cells. These results indicate that type IV collagen secreted by Hsp47−/− cells is not in the form with a correctly folded triple-helix, even at temperatures well below the melting point of normal collagen. These results clearly show that Hsp47 is essential for triple-helix formation of type IV collagen.

![Figure 4](image)

**Figure 4.** Type IV collagen secreted from Hsp47−/− cells is susceptible to protease digestion. Differentiating ES cells were cultured in the presence of l-[2,3-3H]Pro, and aliquots of medium containing equivalent amounts of TCA-insoluble radioactivity were treated with a mixture of trypsin and chymotrypsin at 37°C for indicated periods. The medium before and after protease digestion was analyzed by SDS-PAGE.
tion than that secreted by Hsp47+/+ cells (Figure 4), a finding that is consistent with our previous results for type I collagen (Nagai et al., 2000). Type IV collagen molecules secreted by Hsp47+/− cells were completely digested by trypsin/chymotrypsin at 37°C and at 4°C, whereas type IV collagen secreted by Hsp47+/+ cells were not digested completely, suggesting that collagen secreted by Hsp47+/− cells is unfolded, even at temperatures that are well below the melting point of normal collagen. These results clearly show that Hsp47 plays an essential role in the productive folding of type IV collagen.

Type IV collagen secreted by Hsp47−/− cells readily binds fibronectin at an affinity similar to that of fibronectin binding to heat-denatured type IV collagen (Figure 5). Because fibronectin preferentially binds denatured forms of collagen such as gelatin (Engvall and Ruoslahti, 1977; Engvall et al., 1982), these observations support the idea that type IV collagen secreted by Hsp47−/− cells is unfolded configuration. It is noteworthy that the α2 chain secreted from Hsp47−/− cells preferentially bound to fibronectin, whereas the α1 and α2 chains of heat-denatured collagen exhibited a similar binding activity to fibronectin (Figure 5, A and B). Folding and/or denaturing states may be different in the type IV collagen secreted from Hsp47−/− cells from those of heat-denatured one.

We also revealed here that secretion of type IV collagen was significantly delayed in the absence of Hsp47 (Figure 3), although the absence of Hsp47 does not affect the expression of type IV collagen (Figure 1). Delayed secretion is consistent with previous observations that indicated that secretion of type I collagen is enhanced with overexpression of Hsp47 (Tomita et al., 1999; Rocnik et al., 2002). Quality control in the ER is accomplished by cooperation among several ER-resident proteins (Hammond and Helenius, 1995; Ellgaard et al., 1999) that interact with unfolded proteins and retain them in the ER until they adopt correct conformation. During colla-
gen synthesis, PDI associates with the C-propeptides of monomeric procollagen chains before initial chain assembly (Wilson et al., 1998), which may maintain them in the unfolded state until the trimer formation is initiated in the C-propeptide region (Bottomley et al., 2001). Thus, these ER chaperones, including PDI, may cause delayed secretion of type IV collagen in the absence of Hsp47.

Triple helix formation occurs from the C terminus to the N terminus (Bachinger et al., 1980; Engel and Prockop, 1991; Bulleid et al., 1997), and hydroxylation of proline residues at the Y position of X-Y-Gly repeats by P4H helps to stabilize the triple-helical structure (Berg, 1973). Hsp47 preferentially binds the triple-helical form of procollagen rather than the monomeric α-chain (Koida et al., 2000; Tasab et al., 2000, 2002). Hsp47 dissociates from procollagen during transport from the ER to the Golgi apparatus, presumably at the ER-Golgi intermediate compartment or at the cis-Golgi (Nagai et al., 1992; Satoh et al., 1996). Although Hsp47 is reported to prevent formation of collagen fibrils and aggregation of collagen molecules in vitro (Thomson and Ananthanarayan, 2000; Tasab et al., 2002), we suggest that Hsp47 has another role in facilitating productive folding of collagen in the ER.

We previously reported that Hsp47 knockout mouse embryos cannot survive beyond 11.5 d postcoitum (Nagai et al., 2000). These embryos are severely deficient in collagen fibril formation, and Hsp47−/− fibroblasts established from Hsp47 knockout mouse embryos produce immature type I collagen that does not adopt an appropriate triple-helical conformation. In addition to these abnormalities, BMs of Hsp47−/− embryonic tissues are discontinuous. Hsp47 is therefore an important molecular chaperone during murine development. However, the molecular mechanisms that produce disrupted BMs were not clarified. From the data presented in this study, we suggest that the collagen fibril deficiency is caused by misfolding of type IV collagen. The BM that contains misfolded type IV collagen may be more fragile than one that contains properly folded type IV collagen.

Expression of Hsp47 correlates with expression of various types of collagen (Nagata and Yamada, 1986; Nakai et al., 1990; Takechi et al., 1992; Masuda et al., 1994); here, we showed that during differentiation of ES cells, expression of Hsp47 correlated with that of type IV collagen but not with that of type I collagen during the differentiation of ES cells (Figure 2), consistent with previous observations that both Hsp47 and type IV collagen are expressed in early-stage mouse embryos and in differentiating F9 cells (Leivo et al., 1980; Takechi et al., 1992; Nagai et al., 2000). The up-regulation of Hsp47 during ES cell differentiation may facilitate the folding and assembly of type IV collagen.

Although histochemical analysis indicated that Hsp47−/− EBs contained type IV collagen in the BM-like structure beneath the VE cell layer, the VE cell layer adjacent to this structure exhibited disorganized morphology (Figure 1). The percentage of Hsp47−/− EBs with normal VE cell morphology was only one-fourth that of Hsp47+/+ EBs. These results suggest that the BM-like structures in Hsp47−/− EBs are functionally abnormal, which is consistent with the observation that BMs are discontinuous in Hsp47−/− mouse embryos (Nagai et al., 2000), because EBs are an in vitro model for postimplantation egg-cylinder stage embryos. The type IV collagen that is secreted by Hsp47−/− cells into the extracellular matrix may not be competent to form a meshwork in BMs.

Type IV collagen is important for BM function. For example, Alport syndrome is a genetic disease caused by any of >50 different mutations in the gene encoding the type IV collagen α5 chain, mutations that include single-base mutations and large deletions (Hudson et al., 1993). These mutations cause abnormal structure and function of the type IV collagen, resulting in derangement of BMs and defects in kidney function. Moreover, mouse embryos that lack the laminin γ1 subunit lack BMs and die by day 5.5 postcoitum (Smyth et al., 1999).

The results of this report combined with those of our previous report (Nagai et al., 2000) clearly show that Hsp47 in vivo is indispensable for productive folding of collagen types I and IV. Although Hsp47 can bind to collagen types I through V in vitro (Natsume et al., 1994), the in vivo chaperone function of Hsp47 for other types of collagen has not yet been established, because early-stage embryos die.

To address this issue, we are now analyzing the role of Hsp47 in vivo by making mice with conditionally disrupted Hsp47 gene by adopting Cre-LoxP system.

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REFERENCES


Type IV Collagen Misfolding in Hsp47-Null Cells


