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Type VII and XVII Collagen mRNA Expressions in Regenerated Epidermal Laminae in Chronic Equine Laminitis

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To confirm ability forming the basement membrane of the regenerated laminar epidermis (rLE) in chronic laminitis, expression of type VII and type XVII collagen mRNAs in the rLE was studied applying sequences of two type of murine collagens. On northern blot analysis, complement DNA (cDNA) probes adjusted from the murine type VII and type XVII collagen could hybridize with the equine mRNAs, and each signal was detected as single-bands at approximately 9.5 kb and 5.6 kb, respectively. Contrasting with the expression level of equine glyceraldehyde-3-phosphate dehydrogenease mRNA, the band of type VII collagen mRNA in laminitis was stronger than normal, but the type XVII collagen mRNA in laminitis was less than normal. By in situ hybridization, positive signals in response to the murine type VII and type XVII collagen mRNA probes could be detected in the equine laminitic rLE region. From these results, it is concluded that the keratinocytes constructing the rLE in chronic stage of laminitis can express type VII and type XVII collagen mRNAs and these expression patterns were different from the normal.

Key words: *equine, laminitis, type VII collagen, type XVII collagen*

In equine laminitis, the prodromal or initial stage is associated with laminar circulation dysfunction [15] or zinc-dependent proteinase overload [17] of the laminar region. The pathologic evidence at first stage is disconnection between the laminar epidermis (LE) and the laminar dermis (LD) resulted from basement membrane zone (BMZ) degradation [16–18]. While the detached epidermis structure loses vascular support and rapidly necrotizes, some keratinocytes attaching to the BMZ subsequently regenerate the LE [10, 16] and produce finally the ectopic white line tissue [9]. In this healing process, the regenerated laminar epidermis (rLE) could re-organize the BMZ in which the type VII and XVII collagens are included [10]. In this pilot study, we tried to confirm whether the rLE could express above these two types of extracellular matrix in two laminitis cases (case 1: two year old filly, two weeks after onset; case 2: three year old female, four weeks after onset) contrasting with one control horse (four year old female). Production ability of these matrices can be a good mark to know how the regenerated keratinocytes recovered because these collagens are produced mainly by keratinocytes [1, 5, 8]. The type VII collagen is one of main components constructing the microscopic structure of anchoring fibril [23] and the type XVII collagen is a part of the hemi-desmosome in the BMZ [13]. It is known that these microscopic structures complete the joining of the epidermis and dermis [2].

For sampling, the laminar interface in proximo-distal 50% hoof wall height of the each right fore-hoof was trimmed by the previously described method [10, 19]. One section was rapidly frozen in liquid nitrogen for

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Manufacture addresses:
[1]Promega Co. Madison, WI
[2]Nippon Roche, Tokyo, Japan
[3]Roche Diagnostics Gmbh., Im Nonnenwald, Penzberg, Germany
[4]Hyperfilm ECL, Amersham, Arlington Heights, IL, USA
[5]Fuji Photo Film, Tokyo, Japan
[6]CS-930, Shimadzu, Kyoto, Japan

Table 1. Sequences of PCR primer sets used in this study

primer sequence	annealing temperature	product size
equine GAPDH (GenBank accession No. AF083897)	57.6°C	266 bp
5'-CCCTGCTTCTACTGGTGCTG-3' (sense, 76–95 nt)		
5'-CGAAGGTGGAAGAGTGGGTG-3' (antisense, 322–341 nt)		
murine type VII collagen (GenBank accession No. MMU32107)	64.5°C	860 bp
5'-AGGGTGACAAAGGTGATCGTGGGG-3' (sense, 776–799 nt)		
5'-GCTGCCATTCCGTCCGTCTTCTCC-3' (antisense, 1612-1635 nt)		
murine type XVII collagen (GenBank accession No. NM_007732)	62.4°C	541 bp
5'-GAAGAAAAACGTGCCCCAGCCTCC-3' (sense, 1217-1240 nt)		
5'-CAGCAGCAGCAGCCAGGTGAGCAG-3' (antisense, 1734-1757 nt)		

northern blot analysis. Another was fixed by methacarn fixative for conventional histology and *in situ* hybridization (ISH) analysis. Meanwhile, the equine type VII and XVII collagens mRNA sequences were still not verified at the beginning of this study. So that we prepared the murine type VII collagen alpha 1 (776 nt to 1,635 nt) and XVII collagen alpha 1 (1,217 nt to 1,757 nt) complementary DNA (cDNA) fragments amplified from the BALB/c mouse body skin RNA by the listed primer sets (Table 1) to analyze with molecular biology. These cDNA probes labeled with digoxigenin (DIG) were generated by PCR using the PCR DIG probe synthesis kit[3] with the RT-PCR products cloned into pGEM-T Easy plasmid vector[1] as the templates. An ABIPRISM 377 DNA auto-sequencer was used for each cDNA probe, and the base sequences were then confirmed to be the murine's collagen sequences by Sanger's dideoxy termination method [20]. On the other hand, previously reported cDNA fragment corresponding the equine glyceraldehyde-3-phosphate dehydrogenease (GAPDH) cDNA (1.4 kb) was utilized as housekeeping gene probe [4]. Nonradioactive northern blot hybridization analysis was performed according to a previous method [6]. Poly(A+) RNA samples of the laminar region were purified using Oligotex dT30 Super[2]. One μg of poly(A+) RNA of each sample was separated on 2.2M formaldehyde-containing 1.0% denaturing agarose gel, and transferred onto a nylon membrane[3]. After UV crosslinking of this membrane, conventional procedure to detect the DIG-labeled cDNA probe was according to a chemiluminescence method [4]. The chemiluminescent signals were detected on X-ray films[4]. The film developed with RENDOL[5] according to the manufacturer's protocol and the intensities of signals were scanned with a dual-wavelength scanner[6], followed by quantifying in a DR-2 data recorder[6] with a CCS-1 program[6] to measure the densitometric intensity of each positive signal on the film. The value of each mRNA is expressed relative to the GAPDH mRNA value and differences between the relative values of each collagen mRNAs were compared. For ISH analysis, horizontal paraffin sections (6 μm) of the each laminar epidermis were prepared by the conventional methods. After preparing the tissue sections, digoxigenin-labeled sense/antisense RNA probes were synthesized by Sp6 or T7 RNA polymerase with DIG-dUTP using a DIG RNA labeling kit[3]. Then, type VII and type XVII collagen mRNA expressions in laminar region were determined using these RNA probes and consequently signalized by alkaline-phosphatase-labeled anti-digoxigenin antibody[3] and Nitro Blue Tetrazolium (NBT), 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and 5 mM $MgCl_2$ in 100 mM Tris-HCl buffer (pH 9.6). The nucleus was comparatively stained with safranin. Each sense strand probe was used as a negative control. In results on northern blotting hybridization analysis, murine type VII and type XVII collagen cDNAs hybridized with the equine mRNAs to show signals of approximately 9.5 kb and 5.6 kb single bands, respectively (Fig. 1). This size of equine type VII collagen mRNA was approximately same as the type VII collagen mRNA of human [22] and murine [11]. On the other hand, the size of equine type XVII collagen mRNA was also approximately same as the type XVII collagen mRNA of human [7] and murine [12]. This indicated that these murine collagen cDNA probes could detect the equine collagen mRNAs and that keratinocytes constructing the rLE could express the type VII and XVII collagen mRNAs. The band of type VII collagen mRNA in laminitis showed distinctly stronger than normal contrasting with the level of equine GAPDH mRNA. On the contrary, the band of type XVII collagen mRNA in laminitis was weaker than

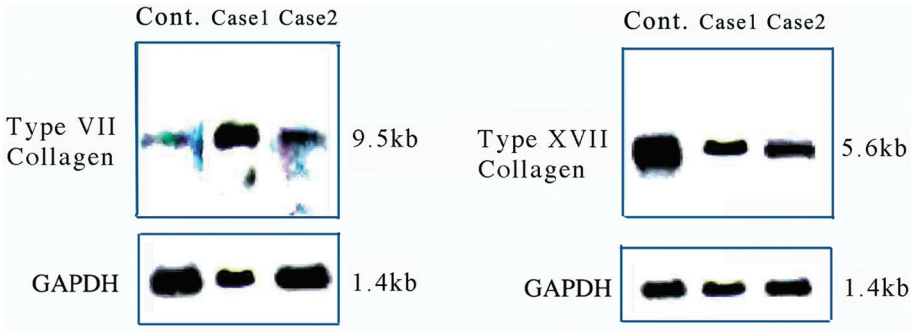

Fig. 1. Expression of type VII and type XVII collagen mRNAs in the normal (Cont.) and laminitic regenerated laminar epidermis (Case 1 and Case 2) by nonradioactive northern blot analysis. In contrasting with the housekeeping gene of equine glyceraldehyde-3-phosphate dehydrogenease (GAPDH), signal strength of type VII collagen mRNAs in two cases are stronger than normal, while type XVII collagen mRNA signals in laminitis are less than normal.

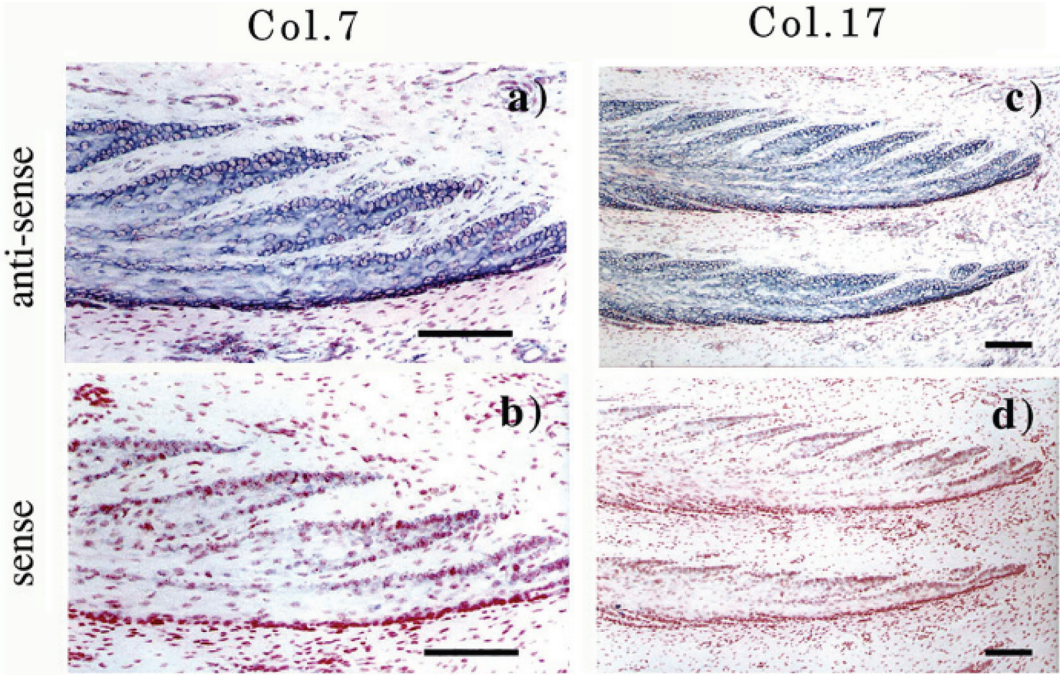

Fig. 2. In situ hybridization results in the case 2 (four weeks after onset). Positive blue signal was recognized mainly in the keratinocytes of regenerated laminar epidermis with both of antisense probes of type VII (a) and type XVII (c) collagen mRNAs. The sense probes do not show positive reaction to the keratinocytes (b and d). Col.7: type VII collagen mRNA. Col.17: type XVII collagen mRNA. Bar: 100 μm.

normal. The level of type VII collagen mRNA in two cases was approximately 7-fold greater than that in normal case. In contrast, the level of type XVII collagen tended to decrease in the laminitis (an approximate fifth part of the normal) based on the level of mRNA for GAPDH. From these results, it was recognized that expression patterns of these two types of collagen in the rLE differed from the normal one in healing manner of the chronic stage. By ISH, each antisense probe showed a positive reaction to type VII

and type XVII collagen mRNAs not only in the LE of the normal horse (data not shown) but also in the rLE of laminitic horses (Fig. 2). These positive reactions in all horses were basically observed in the non-keratinized epidermal tissue. Furthermore, each sense probe showed negative or obviously weak reactions on serial sections.

In conclusion, the keratinocytes in the laminitic rLE can express type VII and type XVII collagen mRNAs. Strong signal intensity of type VII collagen mRNA in laminitic hooves suggested that the rLE enhanced population of the type VII collagens in healing process. In the reported study to assess the generation of cultured human keratinocyte autografts by the electron microscopy, the anchoring fibril density restored and was equal to those of normal biopsy skin within two weeks after grafting [5]. As same as the human keratinocyte autografts, equine keratinocytes of the rLE can aggressively form the anchoring fibrils within two weeks after onset of laminitis. Meanwhile, the signal intensity of type XVII collagen mRNA in laminitis showed lower than normal in blotting analysis. It suggested that capability to form the hemidesmosomes in rLE might be lower than normal. This hypothesis is consistent with the previous report [10] in which existence of few hemidesmosomes were recognized in the BMZ. At current, we cannot propose any regulatory mechanism that can promote the type VII collagen and suppress the type XVII collagen, simultaneously in horse's foot. Dermatologist has already elucidated that tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β) [21] and transforming growth factor-beta (TGF-β) [3] can up-regulate the type VII collagen gene (Col7 A1) expression. Meanwhile, the type XVII collagen gene expression was not influenced by above cytokines [14]. It seems that these two collagens are controlled under different regulation systems. Further study regarding the production manner of these BMZ-ECMs in laminitis is needed to more clarify the expression pattern in total healing process using probes of the equine collagen mRNAs.

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