Type VII collagen in Alport syndrome

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Abstract

Background. Absence or segmental distribution of the α5(IV) collagen chain along the epidermal basement membrane (EBM) is diagnostic of X-linked Alport syndrome (X-AS), but the typical morphologic alterations usually observed along the glomerular basement membrane (GBM) are lacking. However, several differences in protein composition exist between GBM and EBM, and such differences could account for a different phenotype with the same genetic defect. Type VII collagen is one of the major collagenous components of the EBM; the purpose of this study was to investigate the modifications of protein synthesis and expression of type VII collagen in the skin of patients with X-AS.

Methods. The distribution of type VII collagen has been studied in 15 skin biopsies (10 from X-AS patients and 5 controls) by means of electron microscopy, immunofluorescence and confocal microscopy; type VII collagen mRNA expression was also measured by RT-PCR on the same skin fragments.

Results. Protein and mRNA amounts for type VII collagen were significantly higher in skin samples from X-AS patients than in controls (P<0.001); highest values were in cases in which α5(IV) was completely absent.

Conclusions. Our results indicate that lack of α5(IV) molecule significantly alters the assembly of extracellular matrix molecules other than αx(IV) chains also at the EBM level. We suggest that the increased synthesis and deposition of type VII collagen is likely to balance the absence of stabilizing activity normally exerted by α5(IV).

Keywords: Alport syndrome; confocal microscopy; dermoepidermal junction; RNA analysis; type VII collagen

Introduction

Alport syndrome is an inherited disorder of type IV collagen, the major collagenous constituent of the basement membrane (BM) [1]. The hallmark of the disease is persistent microscopic haematuria, often associated with proteinuria, progressive renal failure, ocular abnormalities and high-tone sensorineural hearing loss [2]. The disease is genetically heterogeneous, but the majority (~85%) of Alport syndrome kindreds show X-linked dominant inheritance and are caused by mutations in the COL4A5 gene located in the Xq22 region [3].

The type IV collagen family of proteins comprises six isomeric chains, designated α1 through α6. These chains form heterotrimers through association between their carboxyterminal NC1 domains, associated with folding of the collagenous domains into triple elices. Such triple elices are also capable of forming networks through several types of intermolecular interactions [4]. These various linkages between type IV collagen molecules produce a non-fibrillar polygonal assembly that serves as a scaffolding for the deposition of other matrix glycoproteins and for the attachment of cells [5].

Mutations of the COL4A5 gene result in complete or segmental loss of the α5(IV) chain in those BM which normally contain significant amounts of it, such as kidney glomerulus and skin [6]. Interestingly, the glomerular basement membrane (GBM) of patients with X-linked Alport syndrome (X-AS) displays characteristic ultrastructural features (diffuse thickening with splitting of the lamina densa into multiple interweaving strands) while no significant morphologic alterations at the light or electron microscopic level have been demonstrated so far along the epidermal basement membrane (EBM) [7]. The reason for such a different reaction to the same genetic defect is not known but it could be due to differences in protein(s) composition that exist between the two BMs. For instance, while α1(IV) and α2(IV) chains are normally present in all BMs, the GBM contains also...
Table 1. Clinical data of affected patients

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Sex</th>
<th>Age</th>
<th>FH</th>
<th>GBM alterations</th>
<th>α5(IV) in EBM</th>
<th>Group</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
<td>G1</td>
<td>Ex 29 2544G/T(Gly/Stop)</td>
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<tr>
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<td>M</td>
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<td>+</td>
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<td>Ex 31869G/A (Gly/Arg)</td>
</tr>
<tr>
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<td>M</td>
<td>4</td>
<td>−</td>
<td>+</td>
<td>Absent</td>
<td>G1</td>
<td>Ex 374bp ins. (frame-shift)*</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>1</td>
<td>+</td>
<td>n.p.</td>
<td>Absent</td>
<td>G1</td>
<td>n.i.</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>37</td>
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<td>G1</td>
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<td>6</td>
<td>F</td>
<td>3</td>
<td>+</td>
<td>n.p.</td>
<td>Segmental</td>
<td>G2</td>
<td>Ex 37 Gly1107G/A (Gly/Arg)</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>64</td>
<td>+</td>
<td>n.p.</td>
<td>Segmental</td>
<td>G2</td>
<td>n.i.</td>
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<tr>
<td>8</td>
<td>F</td>
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<td>+</td>
<td>n.p.</td>
<td>Segmental</td>
<td>G2</td>
<td>Ex 30828G/A (Gly/Arg)</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>10</td>
<td>+</td>
<td>n.p.</td>
<td>Segmental</td>
<td>G2</td>
<td>Ex 30828G/A (Gly/Arg)</td>
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<tr>
<td>10</td>
<td>F</td>
<td>35</td>
<td>+</td>
<td>+</td>
<td>Segmental</td>
<td>G2</td>
<td>n.i.</td>
</tr>
</tbody>
</table>

*de novo mutation.
FH, family history; M, male; F, female; n.p. = renal biopsy not performed; n.i. = mutation not identified.

α3(IV) and α4(IV) chains, but not the EBM [8]. However, significant differences between GBM and EBM other than in α2(IV) chains also exist. Type VII collagen, for instance, is not found in normal glomeruli, being detectable only in sclerotic lesions [9], but is present in large amounts in the normal EBM, where it is the predominant component of anchoring fibrils, attachment structures which ensure the integrity of the cutaneous basement membrane zone, and maintain dermal–epidermal integrity [10]. Also, type VII collagen plays a major role in those conditions characterized by a damaged BM, such as surgical or chronic wounds, in which its overexpression is required for the restoration of a functional BM zone [11]. A defective BM, as observed in X-AS, could induce in the epidermis significant effects on extracellular matrix assembly other than ultrastructure of the EBM, e.g. on the interaction between EBM and the underlying dermis.

Our purpose was then to investigate the possible modifications of type VII collagen synthesis and expression by molecular and morphological methods, using skin biopsies in which α5(IV) was absent or with segmental distribution.

Materials and methods

Patient selection

Ten symptomatic patients (five males, five females) with X-AS were studied. Clinical features of the patients are shown in Table 1. The diagnosis was based on the presence of at least two of the following features: (i) positive family history of persistent haematuria; (ii) demonstration of typical ultrastructural appearance of GBM in renal biopsy performed on the studied patient or on a symptomatic male relative; (iii) partial or complete lack of α5(IV) chain along the EBM; (iv) identification of a mutation on the COL4A5 gene.

Punch (3 mm) skin biopsies were performed at the axillary fold; mean age at biopsy was 26.6 years (range 2–64 years). Informed consent was obtained from all patients and/or their parents. Normal human skin tissue obtained from five patients undergoing surgery for malignant tumours (squamous cell carcinoma or melanoma) was also used as control.

Skin samples from all cases were immediately divided into three fragments: one was embedded in OCT, snap frozen in liquid nitrogen-cooled isopentane and stored at −80°C until used; a second fragment was snap-frozen in liquid nitrogen without OCT for the RNA isolation procedure, and the third fragment was fixed in phosphate-buffered 4% formaldehyde, post-fixed in osmium tetroxide and routinely embedded in Epon for ultrastructural observation.

Immunohistochemical procedure and confocal microscopy

Immunohistochemistry was performed in each case onto adjacent sections, using the following primary antibodies:

(i) monoclonal antibody against α5(IV) chain (Weislab AB, Lund, Sweden) to confirm absence or segmental distribution of the chain;
(ii) monoclonal antibody against NC1 peptide of collagen VII (Chemicon International, Inc., Temecula, CA, USA);
(iii) polyclonal antibody against laminin 5 (Sigma Corp., St Louis, MI, USA), whose distribution is not modified significantly in the EBM of X-AS patients (unpublished data).

A double immunostaining procedure was also performed, using the antibodies against NC1 peptide of type VII collagen and laminin 5, for colocalization purposes. Immunostaining for type VII collagen was also performed on the available renal biopsy specimens (cases #1, 2, 3 and 10), to verify the presence of such collagen at the glomerular level.

FITC-conjugated, affinity purified F(ab')2 goat anti-mouse antibody and Texas Red-conjugated, affinity purified F(ab')2 goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies.

Four to six-micron cryostat sections of frozen tissues were fixed in acetone and stained by the indirect method; primary antibodies were diluted 1:50 (laminin 5, type VII collagen) or 1:5 [α5(IV)] in phosphate-buffered saline with 0.5% bovine serum albumin (PBS/BSA). Incubation of primary antibodies was carried out for 1 h at room temperature, followed by 30 min incubation with 1:20 solution of secondary antibody. Sections for α5(IV) immunostaining were denatured in 6 M
urea 0.1 glycine HCL buffer, pH 3.5, 30 min at room temperature, before the immune reaction.

Sections of all patients and controls were independently examined by two observers with a Nikon microscope equipped with epifluorescence illumination optics. The same sections were subsequently examined with a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM). In particular, images were collected either with the usual scanning method (i.e. laser scans of subsequent x-y planes at various z-positions in the specimen), or with cross sections through data sets along the x-z plane (i.e. vertical scans), which provide a direct insight into the axial extension of scanned structures.

The following parameters were considered in each sample:

(i) spatial arrangement and relative distribution of the two fluorochromes
(ii) total area occupied by each fluorochrome. Briefly, a vertical scan was performed to determine the plane of highest intensity of the fluorescent signal within the specimen; a single horizontal scan was subsequently performed at that plane and the fluorescence intensity, linearly correlated with the number of pixels was quantitatively analysed. Areas were calculated by pixel count and expressed in square microns (μ²). All scans were performed using the same objective (60X); laser intensity, confocal aperture, gain and black level settings were also kept constant.

Results were compared by Student's t-test and analysis of variance, and linear regression (χ²). Results were expressed as mean ± SD; P < 0.05 is considered statistically significant. Data were processed using Microsoft Excel.

**RNA isolation and reverse transcription**

Total RNA was extracted from frozen tissue using Trizol (GIBCO, Gaithersburg, MD, USA) and following the manufacturer’s instructions. Its concentration was estimated from absorption values at 260 nm and its quality was assessed by conventional gel electrophoresis. One microgram of total RNA from each sample was reverse transcripted in a 20 μl reaction mix for cDNA synthesis containing the following reagents at final concentrations: 0.05 mg/ml random primers (Invitrogen, Paisley, UK), 0.5 mM each of dNTPs (Invitrogen, Paisley, UK), 10 mMDTT (Invitrogen, Paisley, UK), 40 units RNase inhibitor (Invitrogen, Paisley, UK), 200 units Monkey Moloney Leukemia Virus reverse transcriptase enzyme (Invitrogen, Paisley, UK) and 5× First-Strand buffer (Invitrogen, Paisley, UK). The cDNA synthesis consisted of the following steps: 65°C for 5 min, 42°C for 50 min and 72°C for 15 min. The cDNAs were then diluted 1:5 in nuclease-free H2O (GIBCO, Gaithersburg, MD, USA).

Samples were kept at −20°C, or immediately processed by TaqMan PCR.

**TaqMan real-time PCR**

Assay-on-Demand reaction mix 20× (Applied Biosystems, Inc., Boston, MA, USA) was used for collagen type VII and beta actin quantification. Based on the 5’ nuclelease chemistry, each assay consists of two unlabelled PCR primers and a FAM™ or VIC™ dye-labelled TaqMan® MGB probe. Primers and probes were designed intron spanning to avoid co-amplification of genomic DNA; 2.5 μl of cDNA was incubated with 12.5 μl of 2× TaqMan Universal PCR Master Mix, 1.25 μl of TaqMan probe and sense and reverse primers (20× assay on demand reaction mix). The reaction mixture was brought up to a final volume of 25 μl with RNase-free water, and was incubated in a 96-well plate on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Inc., Boston, MA, USA) using the following temperature cycling: 10 min at 95°C for activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and amplification. In each plate, serial dilutions of cDNA from human RNA universal (BD Franklin Lakes, NJ, USA) were used as template under the same conditions (same sets of primers and probe) to generate standard curves relating the threshold cycle (Ct) to the log input amount of cDNA. A blank assay without an RNA template was utilized as negative control.

The amount of collagen mRNA (cDNA) in each sample was determined using the standard curve method, as described in detail in ABI PRISM Sequence Detection System User Bulletin 2 (Applied Biosystems, Inc., Boston, MA, USA). MicroAmp Optical 96-Well Reaction Plates and Optical Caps (Applied Biosystems, Inc., Boston, MA, USA) were used.

To minimize variability caused by differences in RT efficiency, collagen quantity was normalized to the amount of beta actin cDNA. Collagen type VII gene-specific probes were fluorescently labelled at the 5’-end with 6-carboxy-fluorescein phosphoramidite (FAM, reporter dye), the beta actin probe was 5’-labelled with VIC™ (Applied Biosystems). Relative changes in gene expression as measured by real-time PCR were calculated by comparing pathological samples with normal controls. Data are presented as the fold change in gene expression normalized to beta actin and relative to the control.

**Results**

**Ultrastructural morphology**

Electron microscopy of the skin of X-AS patients revealed a normal-appearing epidermis with a continuous BM and anchoring fibrils, which correspond to normal type VII collagen fibres. In particular, morphology and number of fibrils did not differ from what was appreciable in the control samples. Significant thinning and/or splitting of the lamina densa of the EBM was never observed (Figure 1).

**Standard immunofluorescence and confocal microscopy**

Immunostaining with antibodies against z5(IV) chain identified two separate groups among the patients: Group 1 (G1) in which z5(IV) was completely absent and Group 2 (G2) in which a segmental distribution of the fluorochrome was detected. The extent of the staining in G2 varied from very limited (one or two short segments of stained EBM) to longer tracts; however, it never exceeded half the length of the skin fragment (Figure 2).
Antibodies against laminin and type VII collagen showed excellent immunostaining along the EBM; the fluorochromes displayed a linear, uninterrupted distribution in all cases of X-AS (either with absent or segmental \( \alpha_5(IV) \) chain) and in controls. Laminin 5 stained also the BM of epidermal appendages and of capillaries of the superficial dermis. Standard observation with conventional immunofluorescence microscopy failed to reveal appreciable differences in staining intensity and/or distribution of the same antibody in the different slides (cases and controls) (Figure 3). Confocal microscopy of the double-stained specimens demonstrated that the two antibodies always co-localized along the EBM zone. However, the area occupied by type VII collagen within the BM zone was constantly higher in the skin samples from each X-AS patient than in controls (Figure 4; Table 2); interestingly, area values were irregularly distributed along the single BMs of the G2 cases (segments with increased values alternating with segments with reduced values), while they were uniform in G1 cases. Differences in the area occupied by type VII collagen were statistically significant between G1 and controls and G2 and controls, but not between G1 and G2 (Table 3); on the contrary, the area occupied by laminin 5 was not different among the three groups (patients and controls) (Figure 5; Table 3).

No immunostaining for type VII collagen was evident in the renal biopsy samples.

**RNA analysis**

By RT-PCR, type VII collagen mRNA was detected in significantly higher amounts in all the skin fragments in which the \( \alpha_5(IV) \) chain was immunohistochemically absent. Segmental distribution of \( \alpha_5(IV) \) was associated with mRNA expression that ranged from slight increase to high values, when compared with

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**Fig. 1.** Ultrastructural appearance of dermal-epidermal junction in X-AS patients. Anchoring fibrils are normally distributed (arrows) and epidermal basement membrane displays no significant variation in thickness (Uranyl Acetate-Lead citrate). Scale bar = 2 \( \mu \)m.

**Fig. 2.** Standard immunofluorescence microscopy of skin biopsies from X-AS patients. Primary monoclonal antibody anti-\( \alpha_5(IV) \) chain; FITC-conjugated secondary antibody. (a) Group 1 patients, with absence of signal along the dermal-epidermal junction. (b) Group 2 patients, with segmental distribution (arrow) of signal along the dermal-epidermal junction. Scale bars = a: 25 \( \mu \)m; b: 50 \( \mu \)m.

**Fig. 3.** Skin biopsies from X-AS patients. Standard immunofluorescence microscopy, using anti-type VII Collagen (a,c,e) and anti-Laminin-5 (b,d,f) primary antibodies, followed by FITC- and Texas Red-conjugated secondary antibodies, respectively. No significant differences in immunostaining intensity and/or distribution are visible when comparing normal skin (a,b) with X-AS of Group 1 (b,c) or Group 2 (c,f) patients. Scale bars = 15 \( \mu \)m.
control samples. Mean values of mRNA copies of the three groups (G1, G2, controls) are depicted in Figure 6.

Discussion

We have shown that significant increase of type VII collagen occurs in the skin of patients affected by X-AS, in which $\alpha5(IV)$ chain is lacking or is irregularly incorporated in the EBM. Increased synthesis and deposition of collagen VII was particularly evident in those cases in which the $\alpha5(IV)$ was completely absent; irregular presence of $\alpha5(IV)$ was associated with lower, but still higher than normal, amounts of collagen VII, though a significant statistical (reverse) relationship could not be demonstrated, probably because of the limited number of the cases studied. The difference in signal increase observed between RT-PCR and immunohistochemistry, although impressive, is not surprising: RT-PCR is a much more sensitive method and such apparent discrepancies are common when comparing the two methods [12,13]. In addition, post-translational events are critical for protein function, assembly into multi-subunit complexes and trafficking through the exocytic pathway; in this line, the lack of an important constituent of the EBM may trigger an uncoordinated up-regulation, with a resulting inefficient assembly of well formed microfibrils.

That a given component of the extracellular matrix should be laid down in larger amounts, or even synthesized de novo, in the presence of modifications of other components is not surprising. In Alport syndrome, either X-linked or autosomal, $\alpha1(IV)$ and $\alpha2(IV)$ chains, as well as type V and type VI collagens, accumulate abnormally in the GBM and their distribution changes from exclusively subendothelial to the entire width of the GBM [14]. Also, in systemic sclerosis, in which the skin displays abnormally high amounts of collagens I and III, a net increase of type VII collagen production is evident [15]. Type VII collagen represents the major structural component of the anchoring fibrils, specifically devoted to maintaining basement membrane stability and its adhesion to the underlying dermis [10]. It consists of three identical $\alpha1(IV)$ chains, each composed of a long collagenous triple helix flanked by globular amino (NC1)- and carboxy (NC2)- terminal non-collagenous domains, both located within the lamina densa of the EBM [16]. The NC1 domain is able to bind to type IV collagen, several laminins, and fibronectin [17]; it has a characteristic three-armed structure, with each individual arm capable of interactions with different

Table 2. Area values for collagen VII and laminin 5 in patients and controls

<table>
<thead>
<tr>
<th>Case No.</th>
<th>group</th>
<th>C. VII ($\mu^2$)</th>
<th>Laminin ($\mu^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G1</td>
<td>44.21</td>
<td>11.53</td>
</tr>
<tr>
<td>2</td>
<td>G1</td>
<td>40.43</td>
<td>8.33</td>
</tr>
<tr>
<td>3</td>
<td>G1</td>
<td>41.01</td>
<td>10.55</td>
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<td>4</td>
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</tr>
<tr>
<td>5</td>
<td>G1</td>
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<td>12.21</td>
</tr>
<tr>
<td>6</td>
<td>G2</td>
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<td>G2</td>
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</tr>
<tr>
<td>15</td>
<td>C</td>
<td>31.92</td>
<td>8.73</td>
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</table>

Table 3. Mean area values for collagen VII and laminin 5 in Group1 ($\alpha5(IV)$ absent), Group2 ($\alpha5(IV)$ segmental) and in controls

<table>
<thead>
<tr>
<th>$\alpha5(IV)$</th>
<th>C. VII ($\mu^2$)</th>
<th>Laminin ($\mu^2$)</th>
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</thead>
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<tr>
<td>Absent (G1)</td>
<td>41.19 ± 2.77</td>
<td>10.08 ± 1.94</td>
</tr>
<tr>
<td>Segmental (G2)</td>
<td>35.9 ± 8.52</td>
<td>8.75 ± 1.15</td>
</tr>
<tr>
<td>Controls</td>
<td>21.44 ± 8.26</td>
<td>7.62 ± 0.72</td>
</tr>
<tr>
<td>$P$</td>
<td>&lt;0.001</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Fig. 4. Immunofluorescence staining with primary antibody against type VII collagen; confocal microscopy analysis. The average pixel intensity of the signal has been reproduced as colour variation (see colour reference columns). Intensity in X-AS cases with complete $\alpha5(IV)$ absence (a) or segmental $\alpha5(IV)$ distribution (b) is much higher than in normal skin (c). Segmental cases display also focal reduction of signal intensity (arrow) compared to adjacent BM tracts (arrowheads). Scale bar: 20 µm.
basement membrane components, allowing a single type VII collagen molecule to establish trivalent interactions with other macromolecules [10].

The relationship between collagen IV and collagen VII is of particular interest. Type IV collagen alpha chains and type VII collagen chains are synthesized at the skin level by the same cell types, namely basal keratinocytes of the epidermis and dermal fibroblasts [18]. Organotypic skin models have shown that α5(IV) chain and α1(VII) chains appear late in the course of the embryiological development of the dermal–epidermal junction [19], and that collagen IV and collagen VII synthesis is stimulated preferentially by TGFβ [20,21]. It is conceivable that the change in the EBM of type IV collagen phenotype may modify collagen VII expression in Alport EBM, perhaps by stimulating the expression of the genes encoding for such collagen. Interestingly, evidence exists of increased expression of renal TGFβ mRNA in a murine model of Alport syndrome [22].

The absence of type VII collagen fibrils in the normal-appearing renal tissue of our patients is not surprising; such collagen is normally not synthesized in significant amounts in normal glomeruli, being expressed only in glomeruli with obvious scars, therefore representing a late marker of sclerosis [9].

Increased synthesis and deposition of type VII collagen is particularly evident in the process of wound healing and scar formation: its incorporation into anchoring fibrils is a prerequisite to restore and maintain the stability and integrity of the basement membrane zone, which is instrumental in order for healing to proceed [11]. Indeed, collagens with ancestral structural characteristics are preferentially expressed in tissue repair states, and type VII collagen has been shown to be an existing equivalent of the progenitor of the major fibrillar collagens [11]. It is therefore tempting to speculate that in X-AS a ‘microscar’ takes place, in which higher amounts of type VII collagen represent a compensatory response to the loss of the stabilizing effect normally exerted in the EBM by α5(IV) chain [19]. In this line, the focal reductions of area values for collagen VII observed in G2 cases (i.e. areas with normal expression of collagen VII) could represent BM segments in which α5(IV) chain is normally expressed, therefore accounting for a segmental up-regulation of type VII collagen in a pattern inversely correlated with α5(IV) expression.

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Conflict of interest statement. None declared.
References


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