Expression of Type VI Collagen mRNA During Wound Healing

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During the highly regulated process of wound healing the expression of the interstitial collagens I and III is increased in a time-dependent fashion. Although ultrastructural and in vitro studies suggest a physiologic role of collagen VI in the organization of extracellular matrix deposition, nothing is known about its role in wound healing. Therefore, we studied collagen VI gene expression during wound healing in humans compared to that of collagens I and III. The presence of specific α1(VI) and α3(VI) mRNA species in scar tissue was demonstrated by Northern blot analysis. Quantification of mRNA expression by dot blot analysis and in situ hybridization indicated that like for the interstitial collagens I and III collagen VI gene expression was increased during wound healing, reaching its maximum 2 weeks after initial insult. In the late phase of wound healing like α1(I) the α1(VI) gene expression was not down regulated significantly. In contrast, a reduction of α3(VI) collagen gene expression was observed, as was for the α1(III) collagen gene, indicating a non-coordinate regulation of these chains. Collagen VI gene expression could be localized to fibroblast-like cells and to endothelial cells of newly formed vessels. Collagen VI gene expression was undetectable in smooth muscle cells and myoepithelial cells of eccrine glands. These results indicate that collagen VI gene expression is regulated in a time-dependent fashion and that fibroblasts and endothelial cells appear to play an important role in collagen VI synthesis during wound healing.

Wound healing is a highly regulated event. The different phases include the infiltration by inflammatory cells, the formation of granulation tissue and the deposition of connective tissue, which finally leads to the restoration of the normal architecture [1]. Previous studies have investigated the expression of the interstitial collagens type I and III during wound healing both at the protein and at the gene level [2-5]. Collagen gene expression appears to be regulated in a time-dependent fashion. In the early phase of wound healing the expression of α1(I) and α1(III) collagen chains is coordinately increased throughout the entire dermis. In later stages the gene expression is rapidly down regulated, first in the lower dermis, and later also in the papillary dermis as well. In contrast, little is known about the role of type VI collagen in the process of wound healing.

Type VI collagen is a ubiquitous component of extracellular matrices present in most connective tissues [6-8]. It is a heterotrimer composed of three polypeptide chains, α1(VI), α2(VI) and α3(VI), with molecular masses of approximately 140, 140, and 260 kDa, respectively [9,10]. Whereas the α1(VI) and α2(VI) collagen genes are both located on chromosome 21, the α3(VI) collagen gene is located on chromosome 2 [11]. Major mRNA species encoding these chains have a size of 4.2 kb (α1), 3.5 kb (α2), and 8.5 kb (α3) [11]. In vitro experiments have indicated that mRNA expression of the constituent chains is differentially regulated and that the synthesis of the α3(VI) chain appears to be the rate-limiting step for the production of intact collagen VI [12]. The monomer of type VI collagen is characterized by a rather short triple helix (length 105 nm) flanked on each side by large globular domains resulting in a dumbbell-shaped appearance. These monomers have the potential to form disulfide-linked dimers and tetramers that are thought to represent the building blocks of tissue microfibrils [6]. The biologic functions of type VI collagen are not yet fully understood. Type VI collagen networks have been identified in the vicinity of interstitial collagen and basement membranes of nerves and blood vessels by immunoelectronmicroscopy suggesting that these type VI collagen microfibrils may function as a mechanical link between these structures and the surrounding connective tissues [13].

In vitro experiments have shown that peptic-solubilized and intact type VI collagen substrates mediate attachment and spreading of several cell types including fibroblasts [14]. Cell-attachment mediating properties have also been ascribed to a fusion protein containing the collagenous domain of the α3(VI) chain [15]. Furthermore, recent studies have shown that the globular domains of type VI collagen contain motifs similar to type A domains of von Willebrand factor, which have been shown to bind to extra-
cellular matrix such as type I collagen, implying a possible bridging role of type VI collagen with other matrix proteins in connective tissues [15–17]. All of these findings suggest that type VI collagen may play an important role in the organization of a coordinate matrix deposition as is required for wound healing of primary intent. Therefore, the present study was designed to characterize the expression of collagen VI mRNA species in comparison to that of the interstitial collagens type I and III in wound healing.

MATERIALS AND METHODS

Preparation of Tissue Samples  The study was performed on excision biopsy specimens obtained from patients 8, 11, 17, 21, 24, and 34 d after initial wounding for removal of a malignant melanoma. All wounds were healing by primary intention. Normal control skin was obtained from healthy volunteers. All these full-thickness biopsies were obtained under local anesthesia after informed consent. These tissue specimens were immediately snap frozen in liquid nitrogen and stored at -80°C until used.

RNA Extraction and Analysis by Northern and Dot-Blot Hybridization  Frozen biopsies were cut on dry ice into tiny sections and homogenized in 4 M guanidine-isothiocyanate containing 0.1 M 2-mercaptoethanol. After centrifugation at 14,000 rpm at 4°C for 15 min, supernatants were extracted with phenol (Serva, Heidelberg, Germany) and chloroform (J.T. Baker Chemicals B.V., Deventer, the Netherlands). RNA was then separated by ultracentrifugation through a 5.7 M cesium chloride cushion according to standard protocols [18]. For dot-blot analysis, serial dilutions of total RNA (2, 1, 0.5, and 0 μg) were applied to nylon membranes (GeneScreen, DuPont, Boston) using a dot blot apparatus. For Northern blot analysis, total RNA [2 μg per lane for α1(I), α1(III), and α1(VI) and 20 μg per lane for α3(VI)] was separated by gel electrophoresis on 1% agarose gels under denaturing conditions and transferred onto nylon membranes by capillary blotting. After crosslinking of RNA to the filters by exposure to UV light they were pre-hybridized in 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 5 × sodium chloride, sodium citrate buffer (SSC), 5 × Denhardt’s solution, and sonicated herring sperm DNA (100μg/ml). Hybridization was carried out with cDNA probes that had been labeled with 32P-dATP by random priming to specific activities of 2–5 × 10⁶ cpm/μg (Amersham, UK) for 12 h at 42°C in the buffer described above. After hybridization filters were washed twice in 2 × SSC, 0.1% SDS at room temperature for 10 min, followed by stringent washing in 0.1% SSC, 0.1% SDS at 42°C or 65°C for 15 min. Filters were then exposed to radiosensitive film (Kodak, X-Omat AR) at -80°C. Relative amounts of RNA were measured by densitometry (Hirshmann Elscript 400). Before rehybridization, filters were stripped in 0.1 × SSC, 0.1 × SDS at 90°C for 20 min.

Inserts isolated from the plasmids Hf-677 with a 1.4-kb insert specific for the α1(I) collagen chain [19], PIII-33 with a 0.9-kb insert for the α1(III) collagen chain [20], P18 with a 1.8-kb insert for the α1(VI) collagen chain, P24 with a 1.8-kb insert for α3(VI) collagen [21], and D8-1 with a 264-bp insert for ß-tubulin [22] were used as hybridization probes.

Preparation of cRNA Probes  To generate cRNA probes for the detection of α1 and α3-type VI collagen chain mRNA species by in situ hybridization, fragments of the original cDNA clones P18 for α1(VI) and P24 for α3(VI) [21] were subcloned into Gem 3 vector (Promega Biotec, Madison, USA) containing SP6 and T7 RNA polymerase promoters on either side of the multiple cloning site. In brief, a 1.8-kb (1795-bp) fragment of the original cDNA clone P18 was excised by EcoRI, purified, and subsequently digested with KpnI. A resulting 461-bp fragment from the 5’ end of the original cDNA clone P18 corresponding to the sequence coding for a major portion of the α1(VI) triple-helical domain was subcloned into Gem 3 vector that had been prepared by linearization with KpnI followed by digestion with EcoRI. Similarly, a 1.8-kb (1787-bp) EcoRI fragment of the original cDNA clone P24 was purified and submitted to digestion with SmaI. A resulting 726-bp fragment from the 3' end of the original cDNA clone P24, complementary to the sequence coding for a large portion of the C-terminal globular domain of the α3(VI) chain, was subcloned into the Smal/Gem vector site of Gem 3 vector (Fig 1).

The hybridization specificity of the probes thus generated using these subcloned cDNA fragments as templates to α1(VI) and α3(VI) collagen mRNA species was verified by Northern blot analysis (data not shown). cRNA probes for detection of mRNA for α1(I) and α1(III) collagen chain were described previously [3]. Following linearization with appropriate restriction enzymes, in vitro transcription was carried out for 90 min at 37°C in the presence of 1 μg DNA template, 10 mM dithiothreitol (DTT) (Sigma, St. Louis, MO), 20 U RNasin (Promega Biotec), 500 μmol each ATP, GTP, CTP (Boehringer, Mannheim, FRG), 10 μmol of unlabelled UTP, 50 μCi of 35S-UTP (specific activity 1282 Ci/mmolar, Amersham, UK), and 1 μl (15–20 units) of SP6 or T7 polymerase (Promega Biotec). After digestion of the DNA templates with 2U of DNase I (Promega Biotec), RNA probes were extracted with phenol and chloroform, precipitated from 0.3 M sodium acetate with ethanol, and redissolved in 20 μl of water.

In Situ Hybridization  The protocol for in situ hybridization was described elsewhere [3]. Briefly, 5-μm frozen sections mounted on slides were hybridized with α1(VI), α3(VI), α1(III), and α1(I) anti-sense riboprobes. After hybridization, the slides were washed and treated with RNase to remove non-specific binding, washed again, and dried. Subsequently, slides were dipped into NTB2 emulsion (Kodak) and exposed for 1 to 2 weeks at 4°C. Slides were developed in D19 (Kodak) and sections were stained with hematoxylin. To check the integrity of tissue RNA and the specificity of the hybridization, a cRNA probe for human suprabasal keratin [23] was used as positive control, and sense cRNA probes for α1(VI), α3(VI) collagen were used as negative controls.

Combined Use of In Situ Hybridization and Immunohistochemistry  For use of in situ hybridization and immunostaining on the same section, the slides were treated after in situ hybridization as follows: after RNase treatment, the slides were washed in PBS and dried for 30 min at room temperature. The slides were then incubated for 1.5 h at room temperature with mouse antibodies diluted in Dulbeccos modified (DMEM) supplemented with 10% fetal bovine serum (FBS) against vimentin (1:10) [24,25], desmin (1:50) [24,25], von Willebrand factor (1:25) [26] (Dakopats, Denmark), and α-smooth muscle actin (1:50) (kindly provided by Prof. G. Gabbiani) [27]. Slides were then incubated with peroxi-
enzyme-conjugated rabbit anti-mouse immunoglobulin antibodies (1:100, Dakopatts Denmark) for 45 min at room temperature. Control experiments were performed using PBS instead of primary antibodies. The color reaction was produced using 3-amino-9-ethylcarbazole in a final concentration of 0.008% in PBS for 40 min. The slides were dipped two times into distilled water, air dried, and submitted to autoradiography as described above.

**Quantitative Evaluation of mRNA Expression by In Situ Hybridization** To chronologically compare the relative proportions of fibroblasts displaying positive hybridization signals, six biopsies (obtained on days 8, 11, 17, 21, and 24 after initial operations, and normal control skin) were cut and mounted on the same slides. These slides were hybridized with α1(I), α1(III), α1(VI), and α3(VI) collagen cRNA probes respectively and exposed in parallel under identical conditions (1.5 X 10^6 cpm riboprobe/section for 1 week). Five fields in each of the samples were randomly selected and all of the fibroblasts in each field were examined for the presence or absence of positive hybridization signals. Only fibroblasts with signals that were clearly above background were considered positive.

**RESULTS**

To identify the presence of specific mRNA species for the α1(I), α1(III), α1(VI), and α3(VI) collagen chains in scar tissue, Northern blot analysis was performed. Total RNA was isolated from wound biopsy specimens and from normal control skin, and separated by agarose gel electrophoresis as described, confirming that the RNA was intact. Subsequent hybridization with the corresponding labeled cDNA probes revealed the presence of characteristic mRNA species for α1(I), α1(III), α1(VI), as well as α3(VI) collagen chains (Fig 2).

In an attempt to quantify the chronologic development of mRNA expression for collagen VI in comparison to that of collagens I and III during wound healing, dot blot analysis was performed of total RNA isolated from normal control skin and specimens obtained 8, 11, 14, and 34 d after initial insult (Fig 3). The expression of all mRNA species tested α1(I), α3(III), α1(VI) and α3(VI) was increased during wound healing, reaching its maximum 2 weeks after the initial insult. After 14 d mRNA expression for the α1(III) collagen and α3(VI) collagen chains started to decline. In contrast, mRNA expression for the α1(I) and α1(VI) collagen chains was still upregulated on day 34 after insult.

Attempts to quantify specific mRNAs by dot blot analysis of total tissue RNA have the disadvantage of not allowing an estimation of the proportions of normal skin to actual wounded skin in the specimen, thus not allowing a fair representation of changes in mRNA levels at the actual site of the lesion. Therefore, we also tried to quantify localized collagen mRNA expression by in situ hybridization as described. This allowed to assess mRNA expression at the level of individual cells, directly at the site of the lesion. The expression of mRNAs for type VI collagen was increased in the early phase and reached a plateau 11 d after initial insult. This was paralleled by a similar increase in collagen I and III mRNA expression. In the later phase there was clear evidence for a discoordinate regulation of α1(VI) and α3(VI) mRNA expression. The expression of α3(VI) mRNA started to decline after day 17. In contrast, no reduction from the plateau level of α1(VI) mRNA expression could be observed until day 24. In comparison, the number of cells displaying a positive signal for α1(III) mRNA also started to decline after day 17, whereas that with a positive α1(I) mRNA signal remained at almost maximal levels throughout day 24 (Fig 4).

In situ hybridization also allowed determination of the localization of cells expressing collagen VI mRNA during the different phases of wound healing (data not shown). Until day 17, a similar distribution of cells yielding positive signals for α1(I) and α3(VI), as well as α1(I) and α1(III), mRNA was observed throughout the entire dermis at the incision site. Later on day 24, when α1(III) and α3(VI) mRNA were down regulated again, fibroblast-like cells expressing α1(I), α1(III), and α1(VI) mRNA were concentrated more in the papillary dermis. The number of cells displaying a positive
signal for \( \alpha3(VI) \) mRNA was lower than that expressing the other three mRNA species. At this stage \( \alpha3(VI) \) mRNA expressing cells were mainly seen in the vicinity of newly formed blood vessels.

It has been hypothesized that collagen-producing cells in the early phase of wound healing have characteristics of myofibroblasts (positive immunostaining for \( \alpha \)-smooth muscle actin), a feature lost in the late stages of wound healing [28]. Therefore, we determined the phenotype of the cells expressing type VI collagen, by combined application of immunostaining and in situ hybridization on the same section. Our findings are summarized in Table I and representative examples shown in Fig 5. Myoepithelial cells around eccrine glands stained positively with antibodies directed against \( \alpha \)-smooth muscle actin. However, they did not express detectable levels of mRNA for collagen VI chains (Fig 5a). Smooth muscle cells in the dermis were positive for desmin, but did not show positive mRNA signals for the \( \alpha1 \) or \( \alpha3(VI) \) collagen chain either (Fig 5b). Positive \( \alpha1 \) and \( \alpha3(VI) \) mRNA signals could only be detected in cells staining with antibodies directed against vimentin (Fig 5c), identifying these cells as normal fibroblasts. However, vimentin positive fibroblasts displaying positive mRNA signals for the \( \alpha1(VI) \) and the \( \alpha3(VI) \) collagen chains were also found in close proximity to smooth muscle cells and nearby eccrine glands.

Because endothelial cells have been reported to produce type VI collagen [29], we also performed immunostaining with an antibody against von Willebrand factor. Indeed, positive mRNA signals for collagen VI could be detected in endothelial cells of newly formed vessels in the healing wound (Fig 5d).

**DISCUSSION**

In this study we were able to demonstrate that type VI collagen gene expression is increased in the early phase of wound healing in humans and that the expression of mRNA for different constituent chains appears to be regulated coordinately. Type VI collagen mRNA species are expressed by fibroblast-like mesenchymal cells, but not myofibroblasts. In addition, endothelial cells of newly formed vessels express mRNA for type VI collagen.

Our data show that mRNA species for the collagen VI chains are abundantly expressed in the early phase of wound healing in a manner similar to that of the interstitial collagens I and III. The increased gene expression of the latter two collagen chains in the early phase of wound healing observed here confirms previous reports on primary wound healing in humans and rats [2-5]. The time-dependent regional distribution of \( \alpha1 \) and \( \alpha1(III) \) mRNA expressing cells could also be confirmed. Both the time-dependent, increased type VI collagen gene expression as well as the regional distribution of mRNA expressing cells appeared to be analogous to that of the interstitial collagens.

We could demonstrate that collagen VI mRNA is mainly expressed by fibroblastic cells. However, endothelial cells of newly formed vessels also yielded positive type VI mRNA signals. These data are in agreement with studies describing increased collagen VI gene expression in keloid [30] and neurofibroma [29], as well as with the fact that collagen VI was first isolated from the vessel wall [31]. They also corroborate in vitro findings that fibroblasts and endothelial cells in monolayer culture produce collagen VI [32,33]. Collagen VI synthesis has also been demonstrated in cultured smooth muscle cells isolated from chick embryo and adult human vessels [34,35]. However, no collagen VI mRNA signal could be detected in smooth muscle cells of the dermis, neither within nor around the wound. This discrepancy could be explained by the low sensitivity of the assay or by the fact that cells may rapidly alter their phenotype under culture conditions [36-38].

In addition to collagen VI gene expression endothelial cells of newly formed vessels, fibroblast-like cells expressing collagen VI mRNA were observed in immediate vicinity of these blood vessels. This observation implies the possibility that both endothelial cells as well as surrounding fibroblasts contribute to the deposition of collagen VI, which has been observed by immunoelectronmicroscopy near the basement membrane of vessel walls [13].

The finding that \( \alpha3(VI) \) mRNA is down regulated during the later phase of wound healing whereas \( \alpha1(VI) \) is still up regulated may be a reflection of the different chromosomal location of the genes for the \( \alpha1(VI) \) chain and the \( \alpha3(VI) \) chain [11]. It is also in accordance with previous in vitro studies from our laboratory revealing a similar discordant regulation of the different collagen VI \( \alpha \)-chains in response to IFN-\( \gamma \) treatment of cells [12]. In that particular study, the gene expression for the \( \alpha3(VI) \) chain appeared to be the rate-limiting step for the overall collagen VI synthesis. In analogy, it may be postulated that the observed down regulation of the \( \alpha3(VI) \) mRNA in the later stage of wound healing may be sufficient for a down regulation of total collagen VI protein synthesis.

Recently, it has been reported that the \( \alpha3(VI) \) chain binds to collagen I [15] and that collagen VI has important cell-attachment properties [14], indicating a potential bridging function of collagen VI with interstitial collagen fibers during the organization of con-
Figure 5. Identification of the cell type expressing collagen VI mRNA by combined application of immunohistochemistry and in situ hybridization. 

(a) shows a tissue specimen from day 11 after injury that was stained for α-smooth muscle actin and hybridized with the α1(VI) cRNA probe. Myoepithelial cells surrounding eccrine glands are identified by positive staining for α-smooth muscle actin (open arrow). α1(VI) mRNA signal is present over fibroblast-like cells in the vicinity of eccrine glands (solid arrow), but not over α-smooth muscle actin positive cells. 

(b) shows another section from the same specimen stained for desmin and hybridized with the α1(VI) cRNA probe. Smooth muscle cells are clearly identified by positive staining for desmin (open arrow). Fibroblast-like cells generating a positive mRNA signal are detected in the surroundings of smooth muscle cells (solid arrow), but the smooth cells themselves do not generate detectable signals for α1(VI) mRNA. 

(c) shows the identification of fibroblasts beneath the epidermis (E) by staining for vimentin. An abundant autoradiographic signal for α1(VI) mRNA is detected overlapping the majority of vimentin positive cells. 

(d) shows a specimen obtained on day 17 after insult, stained with an antibody against von Willebrand factor and hybridized with a α1(VI) cRNA probe. Positive α1(VI) mRNA signals were seen not only over fibroblast-like cells (open arrow), but also over von Willebrand factor positive endothelial cells (solid arrow) of newly formed vessels (V). 

(Bar, 100 μm.)

nective tissues [15]. Observations that collagen VI gene expression is increased in active fibrotic syndromes such as the eosinophilia myalgia syndrome [39] or systemic sclerosis [40], in addition to our observations of increased expression of collagen VI in the early phase of wound healing with subsequent down regulation during the later phase, provide further support for the hypothesis that this molecule may play a key role in matrix organization that merits further investigation.

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