Expression of type VI, IX and XI collagen genes and alternative splicing of type II collagen transcripts in fracture callus tissue in mice

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Received 22 March 1995

Abstract **The levels of six mRNAs coding for constituent α-chains of three minor collagens of cartilage were analyzed in an experimental fracture model in normal and transgenic Del1 mice harboring a deletion mutation of exon 7 in the type II collagen gene. Reduced and retarded chondrogenesis in Del1 mice was evident in callus samples as reduced mRNA levels for the cartilage specific type IX and XI collagens at days 7 and 9 of fracture healing. Analysis of the calluses for alternative splicing of proα1(II) collagen mRNA also suggested retarded chondrogenesis in Del1 calluses. Another developmentally regulated step in limb development, a switch between alternative promoters of the α1(IX) collagen gene, was also seen during fracture healing but was less obvious in Del1 calluses. Finally, the current data suggest that the abnormality in bone remodelling in Del1 mice involves activation of the genes coding for α1(XI) and α2(VI) collagens.**

Key words: Bone; Cartilage; Collagen; Fracture; Mouse, transgenic; mRNA

1. Introduction

Healing of a fractured bone repeats in an adult organism several of the steps seen during embryonic development of long bones by endochondral ossification [1]. Initially the fracture callus consists of undifferentiated mesenchyme which then undergoes partial chondrogenesis, and is finally replaced by bone. Under optimal conditions the healing process represents true regeneration since bone remodelling results in complete restoration of bone architecture without a scar. We have previously followed the production of the different connective tissue matrices, mesenchyme, cartilage and bone, during fracture healing by determination of mRNA levels for the major fibrillar collagens, types III, II and I, respectively [2–4]. Furthermore we have shown that the mRNA for α2(IX) collagen is coexpressed with the cartilage specific proα1(II) collagen mRNA while the mRNA for α1(X) collagen is expressed somewhat later as expected for this hypertrophic chondrocyte specific collagen [5]. More recently we have shown that the mRNAs for the small proteoglycans, decorin, fibromodulin and biglycan are largely coexpressed with aggrecan, link protein and type II collagen mRNAs [6]. Furthermore the mRNA levels for decorin and biglycan were also increased during the bone remodelling stage of fracture healing.

As an alternative approach to analyze the relative roles of different tissue components in fracture callus we have analyzed the same complex gene expression patterns in transgenic Del1 mice harboring a deletion mutation in the type II collagen gene which results in reduced and retarded chondrogenesis and structural weakening of cartilage [7]. We demonstrated decreased levels of cartilage specific mRNAs in fracture callus tissue of Del1 mice during the first and second week of healing [5]. These studies also revealed an unexplained abnormality in bone remodelling in Del1 mice resulting in osteopenia/osteoporosis particularly in the cancellous bone mass of metaphyses of long bones [5]. Furthermore the mRNAs for decorin, biglycan, osteonectin and TGF-β1 were present in elevated amounts in callus tissue during bone remodelling [5,6].

Availability of new species specific cDNA probes for minor collagens made it possible to extend these gene expression studies on fracture callus tissue of both normal and transgenic Del1 mice. Clearly such information is needed for understanding the complex changes in the extracellular matrix during the fracture healing process. In this study we determined the mRNA levels for all three chains of type IX collagen, for the proα1 chain of type XI collagen found in both cartilage and bone [8], and for the α2 chain of type VI collagen, a ubiquitously expressed microfibrillar collagen [9]. We also analyzed the calluses for the presence of the alternatively processed/transcribed forms of proα1(II) and α1(IX) collagen mRNAs.

2. Materials and methods

2.1. Experimental animals

This study was performed on 33 heterozygous Del1 transgenic mice and on their nontransgenic littermates ($n = 43$) used as controls. The animals were 10–12 weeks of age at the beginning of the study. The study protocol was reviewed and accepted by the institutional animal care committee.

2.2. Experimental model

A closed diaphyseal fracture of the tibia was produced bilaterally in each animal under general anesthesia as described earlier [10]. Briefly, a stainless-steel rod was first introduced into the intramedullary canal of each tibia. A fracture apparatus was then used to produce reproducible fractures in the tibial bones. The animals were allowed unrestricted weight bearing in cages after recovery from anesthesia. The animals were killed at 7, 9, 14 and 28 days postoperatively for analysis of the fracture calluses. Unfractured diaphyseal bones of the nontransgenic animals were used for the isolation of control RNA.

2.3. mRNA analysis

The fracture calluses of each group were pooled for isolation of total RNA at days 7, 9, 14, and 28 [11]. For Northern hybridizations, total RNAs were denatured with glyoxal and DMSO, fractionated on 0.75% agarose gels, and transferred by blotting to Pall Biodyne nylon membranes. The membranes were prehybridized and thereafter hybridized

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SSDI 0014-5793(95)00384-3

with the following cDNA probes: pMCol6α2–1 for mouse proα2(VI) collagen (Säämänen and Vuorio, unpublished), pMCol9α1–1 for mouse α1(IX) collagen [12], pMCol9α2–1 for mouse α2(IX) collagen [13], pMCol9α3–1 for mouse α3(IX) collagen (Perälä and Vuorio, unpublished) and pMCol11α1–1 for mouse proα1(XI) collagen (Perälä and Vuorio, unpublished). Furthermore a cDNA clone pMCol2α1–2 containing the sequence of the alternatively spliced exon 2 of the mouse type II collagen gene was used [14]. Inserts were purified from each plasmid after appropriate restriction digests and labeled with [^{32}P]dCTP to specific activities of approximately 10^8 cpm/mg using the random priming method. After hybridization, the filters were washed and the bound probe was detected by autoradiography and quantified by laser densitometry. These values were corrected for variation in total RNA levels loaded on gels using values obtained by hybridization of the same filters with a probe for 28 S rRNA [15].

3. Results

Total RNAs isolated from fracture callus tissue at four time points of healing were analyzed by Northern hybridization using a panel of mouse specific cDNA probes (Fig. 1). All the probes have been constructed and tested for specificity in the authors' laboratory. The hybridization signals of several exposures of each filter were quantified by laser densitometry (Fig. 2) and corrected for the variation in loading using the level of 28 S rRNA as the reference (data not shown). For comparison the changes in type II collagen mRNAs reflecting the cartilaginous phase of fracture healing in control and Del1 mice are also shown in Fig. 2. When the same hybridization filter was analyzed with a probe containing only exon 2 sequences of the Col2α1 transcript the pattern observed (Figs. 1A and 2C) was clearly different from total type II collagen mRNA.

The three mRNAs for type IX collagen mRNAs exhibited very similar expression patters (Fig. 1). In all cases the mRNA levels in Del1 mouse calluses were reduced when compared with the nontransgenic littermates (Fig. 2). Only low levels of the short form of the α1(IX) collagen mRNA were observed in the sample isolated from the calluses of control animals at days 7 and 9 (Fig. 1).

The mRNAs for proα1(XI) and α2(VI) collagen were present at all time points of fracture healing studied (Fig. 1). The levels of these two mRNAs varied considerably during the process and were low in unfractured bone (Fig. 2). A major difference between calluses of Del1 and control animals was seen during bone remodelling at day 28: the mRNA levels of both proα1(XI) and α2(VI) collagen were markedly elevated in Del1 mice.

4. Discussion

Analysis of vertebrate limb development has lead to the identification of families of regulatory genes whose ordered expression forms complex patterns [16] in the limb mesenchyme prior to chondrogenesis and osteogenesis, i.e. prior to the expression

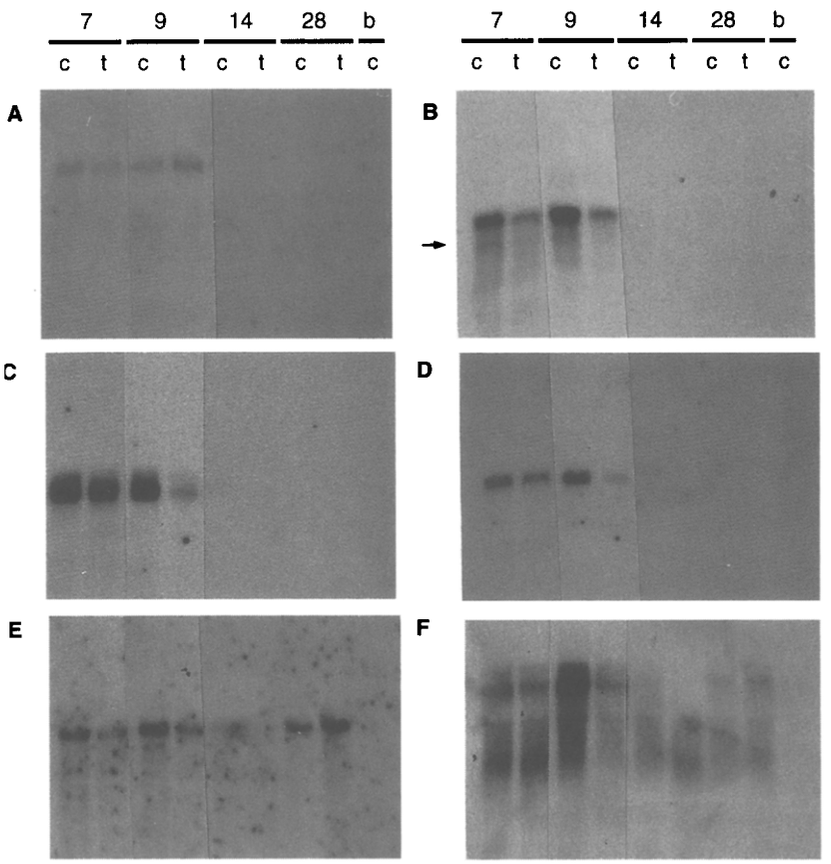

Fig. 1. Northern analysis of callus tissue for mRNA levels of the long form of proα1(II) collagen (A), α1(IX) collagen (B), α2(IX) collagen (C), α3(IX) collagen (D), α2(VI) collagen (E), and proα1(XI) collagen (F). Total RNAs were isolated from callus tissue of normal (c) and transgenic Del1 (t) mice at the time points indicated above each lane, and from unfractured bone (b). The arrow points the location of the short form of the α1(IX) collagen mRNA in panel B. Aliquots of 10 μg were fractionated on 0.75% agarose gels, transferred by blotting onto Pall Biodyne hybridization membranes, and hybridized with ^{32}P-labelled probes.

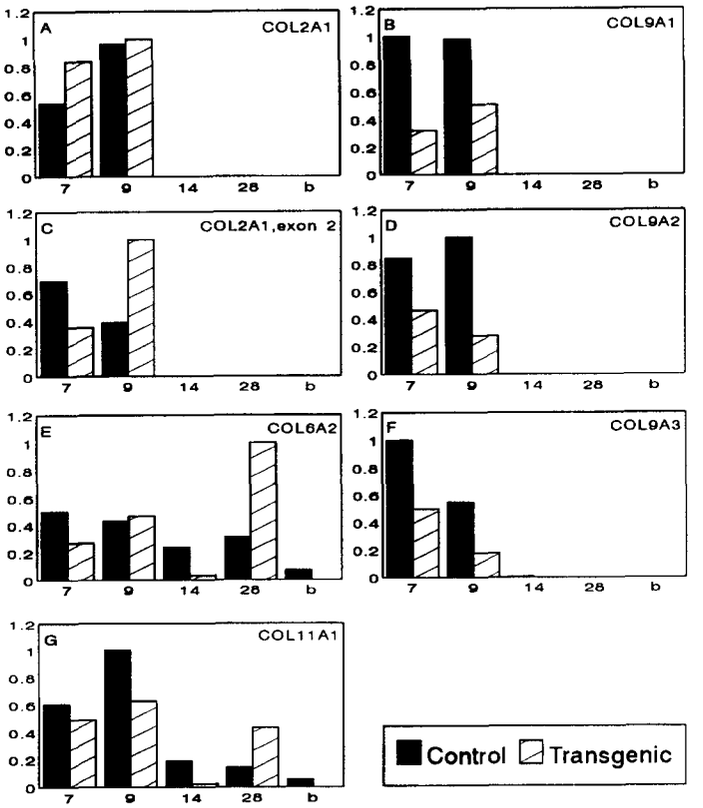

Fig. 2. Quantitation of mRNA levels of the proα1(II) collagen (A, from ref. 5), α1(IX) collagen (B), the long form of proα1(II) collagen (C), α2(IX) collagen (D), α2(VI) collagen (E), α3(IX) collagen (F), proα1(XI) collagen (G), in fracture calluses of normal (black bars) and transgenic Del1 mice (hatched bars) at days shown below the bars. The mRNA levels are given as relative densitometric units. b, unfractured bone.

of genes specific for these tissues. As many of the steps seen during the limb morphogenesis are repeated in an adult organism during the healing of fractured bones [1] it is conceivable that the different families of transcription factors (e.g. Hox-genes and retinoid receptor genes) and signalling molecules (e.g. bone morphogenetic proteins and fibroblast growth factors and their receptors) which apparently regulate morphogenesis and pattern specification during limb development [16] are also expressed during fracture healing. However, it is not known how far back in limb organogenesis dedifferentiation goes before initiation of chondrogenesis during fracture healing. Except for some growth factors, little is known about the production or effects of these factors during fracture healing [17]. We have been studying the gene expression patterns in the murine fracture callus tissue initially by determining the levels of the most abundant mRNAs for the major fibrillar collagens, and proceeded towards less abundant messages [4,5]. In the present study we have focused our interest on minor collagenous components of cartilage matrix and on alternatively processed variants of type II and type IX collagens which have been associated with early steps of chondrogenesis.

The expression of type II collagen gene has been shown to undergo an alternative splicing step of exon 2 coding for a globular domain in the amino-terminal propeptide [14,18]. The longer form of the mRNA is clearly related to prechondrogenic tissue and the short form to mature cartilage [19]. The gene for the α1 chain of type IX collagen has two alternative promoters [20]. Expression of the short form of the α1(IX) mRNA, but not of the protein, has been detected early in developing limb buds in nonchondrogenic regions while the long form of the mRNA and the protein colocalize with type II collagen and its mRNA [21]. Our results demonstrate the presence of the long form of type II collagen mRNA typical for prechondrogenic tissue in fracture callus tissue at days 7 and 9. This shows that the mechanism of de novo chondrogenesis in callus repeats the same steps as during limb chondrogenesis. The results also reveal a difference between Del1 mice and the controls: in the latter the expression of the long form of proα1(II) collagen mRNA peaks at day 7 and in the former at day 9. This is in agreement with our earlier claims of retarded chondrogenesis in Del1 mice [5–7]. A somewhat different picture was seen with the processing variants of type IX collagen: low levels of the short (prechondrogenic) form of α1(IX) collagen mRNA were seen in control calluses at days 7 and 9, but none was detectable in the Del1 samples. This may be explained by a detection problem due to lower overall levels of type IX collagen mRNAs in Del1 calluses. It is also possible that some differences exist in the activation of prechondrogenic mesenchyme between Del1 and control mice. Differential expression of type II and type IX collagen transcripts has been detected in other chondrogenic and nonchondrogenic regions of developing embryos [22]. The expression profiles of the mRNAs coding for the α1, α2, and α3 chains of type IX collagen were quite similar suggesting that the homotrimeric nature of this collagen type is maintained throughout chondrogenesis both in normal and transgenic Del1 calluses.

Type XI collagen mRNA was not only present during the cartilaginous phase of callus healing, but was also activated during bone remodelling stage at day 28 when no other mRNAs for cartilage collagens are present. This is in agreement with earlier data of the proα1(XI) chain being able to associate with type V collagen chains in bone [8,23]. We have previously demonstrated by histomorphometry that the volume of the cartilaginous component in the fracture calluses of Del1 mice is markedly reduced when compared with the control mice [5]. The present data on reduced levels of mRNAs for cartilage collagens of types IX and XI confirms the earlier data and extentend the list of mRNAs which are reduced in fracture calluses of Del1 mice.

No previous data is available on the production of type VI collagen in fracture callus tissue. The present findings on the continuous presence of α2(VI) collagen mRNA in callus suggest that this collagen type is produced throughout the fracture healing process which is in accordance with the suggested ubiquitous expression of type VI collagen in a class of microfibrils [9]. The presence of type VI collagen in cartilage is well documented but the levels have been suggested to be low in bone [24]. Interestingly the highest level of α2(VI) collagen mRNA in the present series was observed in day 28 calluses of Del1 mice. The difference between transgenic Del1 mice and their nontransgenic littermates during the bone remodelling stage is analogous to our recent observation of elevated mRNA levels for decorin, biglycan and TGF-β1 in Del1 mice at this time point [6]. Major differences in the remodelling of cancellous bone have been observed between Del1 mice and their nontransgenic littermates [5]. However, we have no explanation how the deletion mutation in type II collagen transgene in the Del1 mice [7] results in this abnormality. Since decorin has been shown to associate with type VI collagen [25] it is possible that some elements of this widespread derangement of gene expression are interconnected. The present data also suggests that microfibrils may be somehow involved in the remodelling process of bone. Further studies on bone remodelling in Del1 mice may help elucidate underlying mechanism in osteopenia and osteoporosis.

In conclusion, the present results extend our knowledge about the changes in the expression of collagen genes during fracture healing. They confirm that chondrogenesis in the undifferentiated mesenchyme of fracture callus shares similarities with that in the limb mesenchyme. At least the alternative splicing of type II collagen exhibited a pattern typical for prechondrogenic mesenchyme both in normal and transgenic Del1 calluses. Thus the type II collagen mutation in Del1 mice does not seem to disturb the regulation of the alternative splicing. However, based on the current data a switch of alternative promoters of the α1(IX) collagen gene during fracture healing is less obvious in calluses of Del1 mice than in control calluses. Finally the current data suggest that the abnormality in bone remodelling in Del1 mice also involves the genes coding for α1(XI) and α2(VI) collagens.

Acknowledgements: The expert technical assistance of Merja Lakkisto and Tuula Oivanen is gratefully acknowledged. This study was financially supported by grants from the Academy of Finland, the Sigrid Jusélius Foundation and the EU Biotechnology Program.

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