Major fibrillar collagens and fibronectin in an experimental nonunion

An immunohistochemical study

Kalevi Hietaniemi[1], Matti U K Lehto[2] and Pekka Paavolainen[3]

We studied in a rat pseudoarthrosis model the time sequence of expression and distribution of fibronectin and collagens I, II, III and V. Collagens and fibronectin were immunolocalized at the light microscopic level. The major difference from the normal healing pattern was the extension of collagen II and cartilage into the interfragmentary area and at the circumference of the periosteal callus, without any bridging chondral or bony elements in the fracture gap. The formation of a fibrous bond, consisting mostly of collagen III and fibronectin, was observed. This speaks in favor of the failaure of the multipotentional mesenchymal stem cells to change the fracture-healing process towards fibroblast proliferation and the production of tissue unable to mineralize. The decisive zone for mineralization of the callus appeared to be the area of the hypertrophied chondrocytes near the periosteal ossification front.

Departments of [1]Orthopaedics and Traumatology, Helsinki University Central Hospital, Topeliuksenkatu 5, FI-00260 Helsinki. Tel +358 9-4711. Fax -4717350. E-mail: Kalevi.Hietaniemi@Helsinki.fi; [2]Surgery, Tampere University Hospital, [3]Surgery, The Jorvi Hospital, Espoo, Finland
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The collagens constitute a family of proteins that are quantitatively the main element in connective tissues. Cells can synthesize a variety of specific collagens which give the tissues their characteristic physical properties. In normal fracture healing, the synthesis and degradation of certain collagens in a precise time sequence is essential. In delayed union and nonunion, the cascade of participating collagens and other matrix components is poorly understood.

We determined the sites of fibronectin and collagens I, II, III and V in developing nonunions, using immunohistochemical methods in an experimental rat model for pseudoarthrosis (Hietaniemi et al. 1995).

Animals and methods

12 male Wistar rats (median body weight 350 (265-420) g, aged 8–14 weeks were used. They were housed in cages under standard laboratory conditions and fed a standard maintenance diet with a calcium content of 1.0% and a phosphorous content of 0.9%, and they had free access to tap water.

The animals were anesthetized with fentanyl-fluanisone and midazolam solutions given subcutaneously or intraperitoneally. The operation (intramedullary reaming; transverse, manually completed mid-diaphyseal osteotomy of the right femur; and intramedullary nailing with 0.7 mm diameter steel wire allowing unlimited rotation between fracture fragments) was performed, as described previously (Hietaniemi et al. 1995). Unprotected weight bearing was allowed immediately. The rats returned rapidly to normal activity, without postoperative complications.

Anteroposterior radiographs were taken immediately after the operation and weekly during the follow-up period.

The rats were randomly divided into 6 groups of 2 animals each and killed at 1, 3, 5, 7, 9, and 11 weeks postoperatively. The femurs were harvested and fixed in 10% neutrally buffered formalin and embedded in paraffin, after demineralization with 10% EDTA.

5 µm longitudinal sections were cut from the region of osteotomy. After digestion with 0.01% trypsin (2 h at 37 °C), they were stained with collagen antibodies, as follows: 1:100 dilution for anti-collagen I, 1:400 for anti-collagen II, 1:500 for anti-collagen III, and 1:600 for anti-collagen V with peroxidase-antiperoxidase (PAP) (Sternberg 1979). The collagen I antibodies were kindly provided by Professor Jean-Michel Foidart, University of Liège, Belgium. For collagen II, a rabbit antibovine antibody was used (Biodesign®). Antisera against types III and V collagen were raised in rabbits and antibodies were prepared by using the immunization schedule previously described (Lehto et al. 1985). The color reaction was produced

Figure 1. Femoral osteotomy 11 weeks postoperatively. Developing nonunion, with hypertrophic callus formation and without bony bridging.

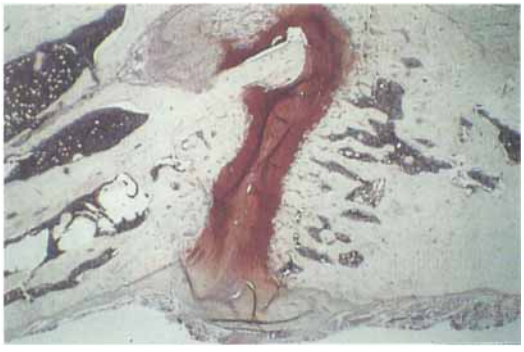


Figure 2. Photomicrograph of nonunion 11 weeks after the osteotomy. Safranin-O stain, ×25.

with 0.03% 3.3-diaminobenzidine tetrachloride. Fibronectin was demonstrated with PAP, after staining the sections with antiserum to fibronectin (Boehringwerke AG, Marburg, Germany) (Burns et al. 1980).

As no counterstaining of the specimens was used, adjacent sections were stained with Safranin O and Alcian blue, to examine the histology of the healing osteotomy.

Results

Macroscopic observations showed in all osteotomies delayed healing and abundant callus formation at both fracture ends, with gross instability and without any bridging bony or chondral elements. Microscopic examinations of the bone specimens showed large amounts of connective tissue at the fracture site.

Radiographically delayed calcification of the fracture was observed. The prominent callus with a clear radiolucent interfragmentary area persisting indicated nonunion (Figure 1).

Immunohistochemical and histological findings

The sequence in which staining appeared in the fracture callus is shown in Table. Histologically, the findings until the third postoperative week appeared to resemble the normal healing pattern, with an organizing interfragmentary hematoma and periosteal proliferation of callus tissue. After 3 weeks, cartilaginous zones were observed at both fragment ends that did not bridge the fracture gap. The cartilage persisted unmineralized in the central interfragmentary area, and was later repeated by fibrous tissue. The histological details of this process have been described earlier (Hietaniemi et al. 1995) (Figure 2).

Collagen I

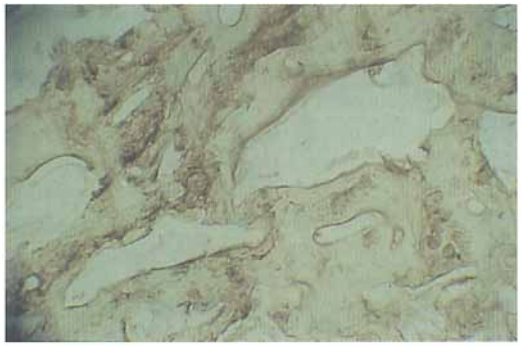
In the 1-week-old specimens, bright staining was seen subperiosteally, showing extensive binding to lamellarly structured new bone under the stripped periosteum. Some staining was present in the vascular canals of the cortical bone as well. On histological examination, loose connective tissue was noted close to the fracture gap. The cartilage in the interfragmentary zone showed no staining at 3 weeks (Figure 3). Later, intensive binding to the newly formed lamellar periosteal bone was observed and the endochondral ossification front was also brightly stained. After 5 weeks, an intensely stained area between the lamellar bone and fibrous callus sleeve could be seen. After this, the remodelling phase had begun, and the staining intensity for collagen I was diminishing. The fibrous callus sleeve remained brightly positive until week 9, but

The intensity of staining in the fracture callus and interfragmentary area (= gap) with antibodies to collagens I, II, III, V and fibronectin in the developing non-union

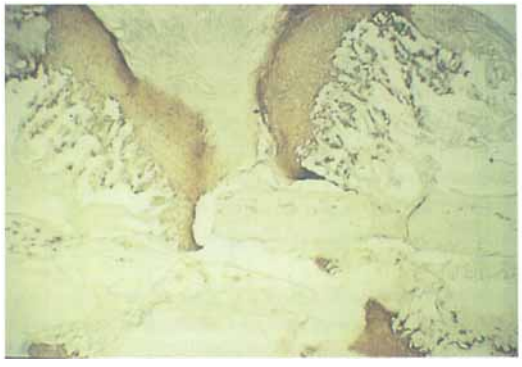
	Collagen I		Collagen II		Collagen III		Collagen V		Fibronectin	
Weeks	Callus	Gap	Callus	Gap	Callus	Gap	Callus	Gap	Callus	Gap
1	+++	0	+	0	+	++	+	++	+	++
3	+++	0	++	++	++	+	++	++	+	++
5	+++	0	++	+++	+++	++	+++	++	+	++
7	++	0	++	++	+++	++	++	++	++	+++
9	++	0	+	+	++	+++	+	+	++	+++
11	+	0	+	0	++	+++	+	+	++	+++

0 no staining, + faint, ++ moderate, +++ intense staining

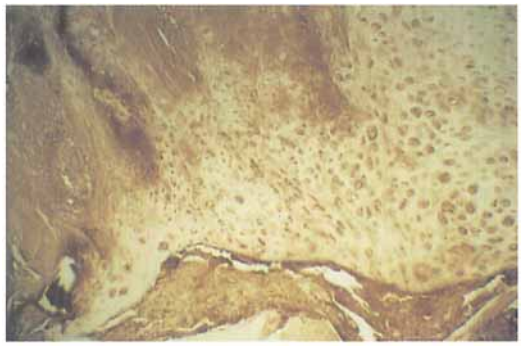
Figure 3. Photomicrographs of immunohistochemical specimens, without counterstaining.



Collagen I at 3 weeks. Strong labeling of the newly formed lamellar bone (arrow), ×250.



Collagen II at 3 weeks. Large chondral elements in both fragment ends, showing intense immunoreactivity (arrows), ×100.



Fibronectin at 9 weeks. Intense staining in the fracture gap. Arrow indicates the area of hypertrophied chondrocytes, ×250.

thereafter the intensity faded and no collagen I antibody binding was seen in the center of the pseudoarthrosis.

Collagen II

During the first week, collagen II labeling was restricted to the area of the ruptured periosteum but, after 3 weeks, immunoreactivity was observed at the ends of both fracture fragments (Figure 3). Labeling at the interfragmentary zone increased until week 5, corresponding to the persisting chondral elements observed in histological specimens. After that, the intensity gradually diminished. After 9–11 weeks, only a small amount of cartilage was present and the area was more faintly stained. The histological appearance was that of soft scar-like connective tissue. No spider-like penetration of osteons into the bone ends was seen, in contrast to the first weeks of healing.

Collagen III

1 week postoperatively, scant labeling of the soft connective tissue and cortex was observed. The granulation tissue was moderately stained collagen III. After 3–7 weeks, the fibrous callus sleeve was intensely stained, and the developing lamellar bone exhibited less staining than the callus. First, the cartilaginous interfragmentary area showed no immunoreactivity for collagen III, but later, when the cartilaginous phase was over and loose connective tissue dominated the area, the fracture gap was labeled for collagen III. At the end of this period, nonhomogeneous areas with faint staining for collagen III were occupied by necrotic cartilaginous tissue. After 9–11 weeks, collagen III showed a distribution similar to that of fibronectin; the central area of the interfragmentary zone and the fibrous callus sleeve exhibited staining.

Collagen V

At 1 week, young granulation tissue, rich in new capillaries, stained for collagen V. Some immunoreactivity was also seen in the matrix of the granulation tissue. After 3–7 weeks, the fibrous callus sleeve and capillaries showed staining. The intensity of the staining of the lamellar bone increased, so that after 5 weeks, large amounts of collagen V had accumulated locally in the lamellar bone area. At week 9, the fibrous callus sleeve had only a pale labeling and, after 11 weeks, immunoreactivity ceased, only the capillaries remaining stained in the center of the interfragmentary area.

Fibronectin

At 1 week, fibroblasts had already produced a fibrillar meshwork in the interfragmentary zone. The whole hematoma showed immunoreactivity with fibronectin, and a marked band was also observed around the nail canal. After 3 weeks, fibronectin with a fibrillar appearance had replaced the disintegrated fibronectin. The central interfragmentary area had the appearance of a non-organized dense connective tissue bar-

rier and was labeled with fibronectin. 5 weeks postoperatively, fibronectin was still visible and had accumulated at the border of the woven bone and along the transverse fracture gap lining the cartilaginous, intensively labeled central area. Immunoreactivity was also observed in the areas of the newly formed capillaries. After 7 weeks, the histological appearance of the fracture gap resembled loose connective tissue, and the total amount of cartilage was diminishing. Fibrillar fibronectin was intensely stained in the interfragmentary area. 9 weeks postoperatively, pericellular staining for fibronectin could be seen (Figure 3), and at 11 weeks, the fibrous callus sleeve had disappeared and the center of the pseudoarthrosis was brightly, but the periphery more faintly, stained for fibronectin.

Discussion

We have developed a previously described experimental model for pseudoarthrosis in rats (Hietaniemi et al. 1995) and studied quantitatively the biochemistry of the nonunion (Hietaniemi et al. 1996). Our experiences with Northern analysis in the same animal model has shown that the regulation of collagen genes is altered, impairing the normal reparative fracture healing cascade (Ekholm et al. 1995). The collagens produced in the matrix of a healing fracture can be quantitated by biochemical methods (Penttinen 1972, Paavolainen et al. 1989). However, these methods cannot localize the proteins, and therefore the distribution of specific collagens during fracture healing was studied by immunohistochemistry (Lane et al. 1986).

The early phases of fracture healing in this experimental model appeared to follow closely a normal repair pattern, as described in previously published immunohistochemical studies using a rat tibial fracture model (Lane et al. 1986, Ashhurst 1990). During the first week of the healing process, loose connective tissue is found at the fracture site, containing mostly collagen III and also collagen I, which is expressed throughout the normal fracture healing process. New periosteum is developing along the periphery of loose connective tissue and contains collagens I, III and V. Collagen V is mainly found in the same tissues as collagens I and III in close contact with blood vessels and is thought to be located in the fibrils of collagens I and III, controlling the diameter of the fibers (Lane et al. 1986, Ashhurst 1990, Liu et al. 1995).

Cartilage was observed after the first week of healing, and the matrix in the interfragmentary area contains collagen II. It is normally the last major collagen synthesized in the endochondral fracture healing cascade and found in the matrix of the interfragmentary zone (Lane et al. 1986, Ashhurst 1990). Collagen II was formed on the fibrous matrix, and type III and type V collagens remained. The synthesis of collagen II depends on the mechanical circumstances under which the fracture is healing. Collagen II production and cartilage formation are abundant only if the healing fracture is mechanically unstable, as in the present model. In the normal endochondral repair process, collagen II is not seen after the third week of healing, if the fracture is mechanically stable (Page et al. 1986). When the cartilaginous bond is fully established, mineralization begins immediately in the areas of chondral cell hypertrophy and degeneration.

In this study, the persistent rotational instability produced mechanical forces, which prevented the formation of bridging callus, and seemed to disturb local and systemic regulatory factors in the repair. An extended chondral phase in the developing callus, with collagen II detection, and a delay in the mineralization process at the site of hypertrophied chondrocytes was observed. This is in accordance with an earlier observation of large amounts of collagen II in the fourth week of repair in unstable fractures (Page et al. 1986).

After 9 weeks, the chondral activity of the developing nonunion ceased and an immature loose connective tissue was synthesized with collagen III and fibronectin. Fibronectin anchors the matrix components and is thought to have a significant role in the differentiation of mesenchymal cells to chondral cells. It has also been observed to play an important role in granulation tissue formation during wound healing and muscle injury repair (Lehto et al. 1985, Mäkisalo et al. 1989). Immunostaining for collagen III was observed not only in the fibrous fracture gap, but also in the newly formed callus tissue, suggesting an active role of osteoblasts participating in the production of collagen III in the nonunion.

Our findings seem to indicate a time-limited healing potency of the fracture, if the repair process is impaired by rotational instability. If mechanical stability is not achieved within a certain time, the normal healing process ceases and the calcification process is delayed. The persisting instability and the delay in the mineralization of the callus tissue seem to switch on a different healing cascade, with production of fibroblasts, collagen III and fibronectin. Bone formation between the fracture fragments is inhibited when the participating cells produce collagens and other matrix components which are intended for healing with scar tissue, leading to nonunion.

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