

Mature osteoblasts in human non-union fractures express collagen type III

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Abstract

Aims—High levels of collagen type III are biochemically detectable in biopsies of non-uniting fractures, and in the serum of patients suffering from this condition. The aim of this study was to determine whether the expression of collagen type III was limited to fibrous tissue in non-unions, or whether some was present in bone.

Methods—Biopsies from normally healing human fractures and non-unions were examined using in situ hybridisation and immunohistochemistry.

Results—The mesenchymal cell population, which includes fibroblast and osteoblast precursors, expressed mRNA for collagen type III. However, mature osteoblasts on the surface of woven bone varied profoundly between normally healing fractures (in which they were negative or occasionally weakly positive) and non-unions (in which they were strongly positive). Areas of woven bone that had osteoblasts positive for collagen type III mRNA also immunostained positively for the protein.

Conclusions—This study shows that non-union fracture callus osteoblasts on the surfaces of woven bone exhibit an unusual phenotype: they express collagen type III, a molecule characteristic of an earlier stage of osteoblast differentiation, which is not expressed by osteoblasts on woven bone surfaces of bone that develops normally. This finding may be useful in developing an early clinical test for impending non-union.

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Keywords: collagen type III; fracture non-union; mature osteoblast; woven bone osteoid

During their differentiation, osteoblasts express a succession of different matrix molecules.¹⁻³ In normal bone development, collagen type III is expressed early in osteoblast differentiation. It is produced by pre-osteoblasts and fibroblastic cells, and the protein is a constituent of the collagen fibres produced.⁴ Collagen type III mRNA is expressed in fetal, but not adult, long bone.⁵⁻⁸ Later, osteoblasts secrete osteoid, a matrix deficient in collagen type III. With remodelling, the collagen type III containing fibres, and osteoid, that together comprise woven bone, are replaced by lamellar bone. In consequence,

the level of collagen type III in bone falls rapidly as remodelling proceeds. At fracture sites, preliminary biochemical data have indicated that non-unions contain substantially greater amounts of collagen type III than sites of recently healed normal fractures.⁹ Other work has indicated abnormally high serum levels of collagen type III (pIIINP, the amino terminal extension peptide) in patients with slowly healing fractures.^{10,11} We sought to determine whether additional collagen type III was located in non-union fibrous tissue, but found that some collagen type III was expressed by osteoblasts on woven bone.

Material and methods

PATIENTS

Specimens of human external fracture callus from non-unions were taken from the fracture site of 12 patients with extra-articular non-united long bone fractures between four and 48 months after fracture. Biopsies from fractures that were healing normally were taken from 15 closed fractures at operations carried out to treat malreduction that had developed during conservative treatment. Biopsies were obtained up to 23 days after fracture. Patients were aged between 18 and 87 years, and were otherwise fit. On subsequent follow up (up to one year), all the fractures classed as healing normally were found to have united conventionally.

TISSUE PREPARATION

Biopsy specimens were fixed in 10% neutral buffered formalin, decalcified in 20% ethylenediamine tetra-acetic acid (EDTA), pH 7.2, until decalcification was radiologically complete, embedded in paraffin wax, and sectioned at 7 µm.

IN SITU HYBRIDISATION

The probes for in situ hybridisation of human procollagen types I and III mRNAs were those prepared, used as described previously,⁵ and kindly supplied by Dr E Vuorio (University of Turku, Finland). They have minimal homology. Type I: collagen pro 1(I), clone pHCAL1, PvuII-PstI 372 base pair fragment of the 3' C-propeptide region. Type III: collagen pro 1(III), clone pHFS3, PstI-PstI 295 base pair fragment of the 3' telopeptide region.

The method for in situ hybridisation has been described previously.¹²⁻¹⁶ Briefly, dewaxed sections were rehydrated, pretreated with proteinase K, dehydrated, and air dried; RNAase controls were used. Sections were prehybridised for one hour at 37°C in 50%

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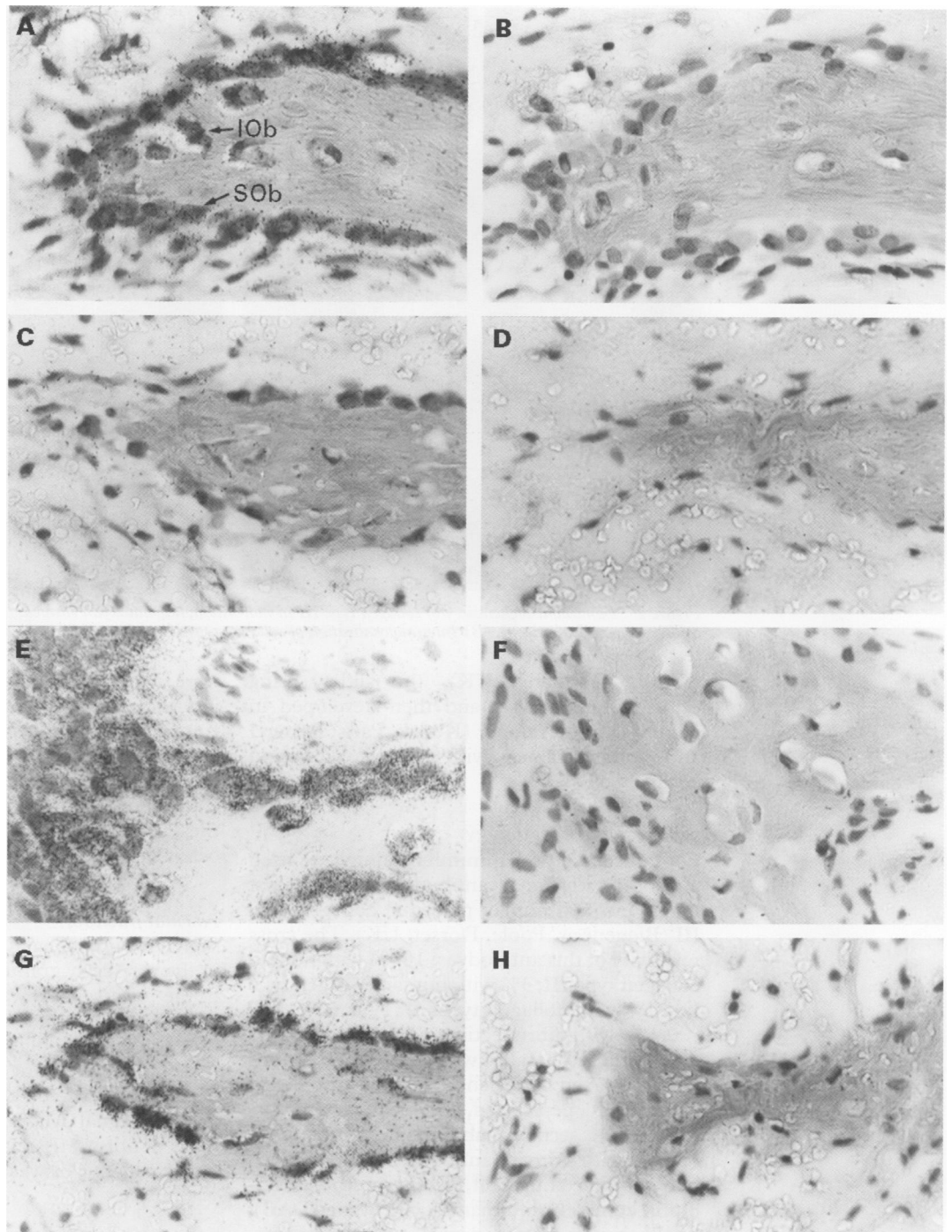


Figure 1 *In situ hybridisation; haematoxylin and eosin stained. (A–D) Collagen type III. (A and B) Non-union. (A) Test; (B) RNAase control. Osteoblasts (included osteoblasts (IOb) and surface osteoblasts (SOB)) and immediately adjacent cells possess a signal for procollagen type mRNA. Other cells are weakly positive or negative. (C and D) Normally healing fracture. (C) Test; (D) RNAase control. Osteoblasts in normally healing fractures are usually negative for procollagen type III mRNA signal. (E–H) Collagen type I. (E and F) Non-union. (E) Test; (F) RNAase control. In non-unions, osteoblasts on the surface of woven bone are positive for procollagen type I mRNA signal. (G and H) Normally healing fracture. (G) Test; (H) RNAase control. Osteoblasts on the surface of woven bone are positive for procollagen type I mRNA signal in normally healing fractures.*

formamide, 1 mg/ml bovine serum albumin, 0.2% (wt/vol) Ficoll, 0.2% (wt/vol) polyvinyl pyrrolidone, 0.6 M NaCl, 0.2 mg/ml sheared salmon sperm DNA, 10 mM Tris (pH 7.4), 0.5 mM EDTA, 10 mM dithiothreitol (DTT), and 10% (wt/vol) dextran sulphate. Hybridisation with heat denatured ^{35}S labelled probe (100 ng/ml prehybridisation mixture) was carried out at 37°C for 16 hours in prehybridisation solution. Aliquots of 50 μm were applied to each slide, which were covered with siliconised coverslips.

After hybridisation, the tissue sections were washed with a series of high stringency washes: twice for five minutes in 0.5 \times saline sodium citrate (SSC) with 1 mM EDTA and 10 mM DTT; twice for five minutes in 0.5 \times SSC with 1 mM EDTA; 15 minutes in 50% formamide, 0.15 M NaCl, 5 mM Tris (pH 7.5), and 0.5 mM EDTA; four times for five minutes in 0.5 \times SSC at 55°C, followed by five minutes at room temperature in 0.5 \times SSC. Sections were then dehydrated and air dried. Autoradiography was performed with K5 emulsion

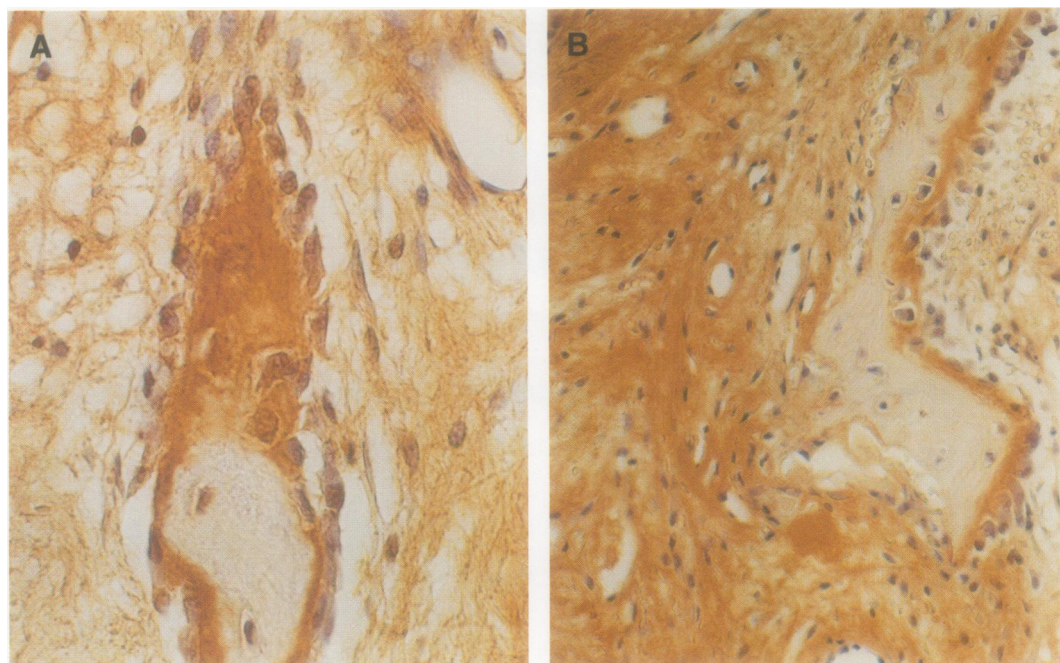


Figure 2 Immunohistochemistry of collagen type III in non-union; haematoxylin stained. (A) Immunolocalisation of collagen type III in osteoid. (B) Immunolocalisation of collagen type III in fibrous tissue in the fracture gap and in osteoid.

(Ilford, Mobberly, UK), the slides being exposed for 14 days and then developed in Kodak D-19 developer (Kodak, Paris, France) and counterstained with haematoxylin and eosin.

IMMUNOHISTOCHEMISTRY

For collagen type III immunostaining we used a rabbit polyclonal antibody raised against highly purified human placental collagen type III (Biogenesis, Poole, Dorset, UK). The cross reactivity of this antibody is: 100% with human collagen type III; 3% with human collagen type I; < 1% with collagen types II, IV, and V; and < 0.1% with fibronectin and laminin. Fixed demineralised paraffin wax embedded sections were dewaxed in xylene, treated with methanolic H₂O₂, pepsinised, and blocked with normal swine serum; subsequently, they were reacted with primary antibody, secondary antibody (swine antirabbit, biotinylated), avidin-biotin complex, and diaminobenzidine (DAB); finally they were stained in Mayer's haematoxylin.

Results

In normally healing fractures, the blood clot was invaded, became granulation tissue, and was transformed into fibrocellular matrix. Within this were foci of intramembranous bone and nodules of cartilage on an indeterminate matrix with variable chondroid and osteoid features. In non-unions, there were areas of old bone, new bone formation, non-union gap (either fibrous, cartilaginous, or both), and an interface between the gap and bony material.

COLLAGEN TYPE III GENE EXPRESSION

Non-unions

In all cases, a population of surface and included osteoblasts in non-unions was strongly positive for the procollagen type III mRNA signal (fig 1A; SOb and IOb); these

were in the zone of new bone formation and the interface zone. Osteoblasts in the old bone zone were usually negative, while the gap zone contained osteoblasts only rarely. In addition, fibroblasts were frequently positive in the gap zone and interface, but signal strength over these cells was usually weaker than that of osteoblasts in the same section.

Normal fractures

Signal for procollagen type III mRNA was seen in the very early granulation tissue, where most of the positive cells were mesenchymal spindle cells, a cell population that includes osteoblast precursors. Osteoblasts in the large majority of these fractures were negative (fig 1C), with occasional scattered osteoblasts possessing low levels of signal. Many sections contained small areas of fibrous tissue in which fibroblasts were either negative or weakly positive.

COLLAGEN TYPE I GENE EXPRESSION

Signal for procollagen type I mRNA over fibroblasts and over osteoblasts on woven bone was uniformly strong in most non-unions (fig 1E) and normal fractures (fig 1G).

IMMUNOHISTOCHEMISTRY

In areas of new bone covered by plump osteoblasts, the matrix was either stained uniformly (golden brown) or in a superficial zone (fig 2) indicating the presence of collagen type III; fibrous tissue in the fracture gap also immunostained positively (fig 2B).

Discussion

The mechanisms controlling osteoblast differentiation in the fracture callus are understood poorly, but are of obvious importance to the clinical management of fractures. Previous investigations of collagen expression in animal

models revealed collagen type III mRNA expression to be restricted to the early, granulation tissue phase of normal fracture healing.¹⁷⁻¹⁹

In abnormally healing fractures, three previous biochemical studies showed elevated levels of collagen type III, consistent with abnormally high levels of production. The first of these demonstrated, in non-union biopsies, approximately double the normal amount of collagen type III (four fractures in total).⁹ Two further serological investigations showed elevated levels of procollagen type III (pIIIINP) in patients with delayed healing of tibial fractures.^{10 11} Although it was suspected initially that abnormally high levels of collagen type III in these circumstances might be the result of fibrous tissue formation⁹ (which is often excessive in non-union), none of these studies established which cells were responsible for the high levels of collagen type III.

The current study, which examined biopsy material from non-unions and normally healing fractures, found expression of collagen type III mRNA and protein in osteoblasts adjacent to the non-union gap, on woven bone surfaces. (In contrast, in normal fractures, collagen type III mRNA expression was seen at an early stage (granulation tissue), but was very rarely seen in osteoblasts.) This apparent prolongation into osteoblast maturity of a phenotype characteristic of an earlier phase is likely to result from the local factors present in the non-union fracture gap, an environment apparently defective and incapable of promoting an effective repair process. The clinical significance of our finding relates to the need for a marker of impending non-union. Such a marker, preferably in serum, is needed, as a matter of some urgency. This is because treatment for non-union (using bone morphogenetic proteins will be available soon but must be administered early and, for reasons of cost, needs to be targeted to fractures selected for risk of failure. To date, no method for making this selection has been discovered. Our finding of "late" expression of collagen type III by osteoblasts gives an indication that it, or factors associated with its synthesis or degradation, or its breakdown products, could form the basis of an early clinical test for impending non-union.

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