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Altered Relative Expression of BMPs and BMP Inhibitors in Cartilaginous Areas of Human Fractures Progressing towards Nonunion

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Abstract

The present study was conducted to evaluate the hypothesis that an imbalance in the local production of bone morphogenetic proteins (BMPs) and BMP inhibitors exists within the cartilaginous intermediate of nonhealing fractures. Biopsies were recovered intraoperatively from human fractures that, upon follow-up, were found to heal normally or become nonunions. The samples were examined by immunohistochemistry to determine the expression of BMP-2, BMP-14, and the BMP inhibitors noggin and chordin. Expression was determined semiquantitatively based on the area of positive staining per area of cartilage and by determining the number of positively staining cells and the intensity of staining. There was a significant reduction in BMP-2 and BMP-14 expression in cartilaginous areas of nonhealing fractures compared to healing fractures. However, there was no difference in the expression of the BMP inhibitors between the two groups of fractures. This imbalance in the expression of BMPs and BMP inhibitors within cartilaginous areas of developing nonunions may account for their reduced bone forming ability. These data suggest strategies for preventing the development of nonunions by altering levels of BMPs and their inhibitors within fracture sites.

Keywords

Fracture; nonunion; BMP; noggin; chordin; cartilage

INTRODUCTION

Fracture healing is normally an efficient process resulting in newly formed bone, similar in quality to the original tissue. However, in a significant proportion of cases, the regenerative process is impaired and fracture nonunion can result.[1,2] Although the clinical risk factors for fracture nonunion are well known to the trauma surgeon,[3] the intermediate pathological processes leading to fracture nonunion remain ill-defined. When the diagnosis of nonunion

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is made radiographically, all reparative activity has ceased and there is no potential for repair between the bone ends. Thus, to gain pathophysiologically relevant information on the bio- logical alterations that lead to nonunion, it is better to study fractures that are in the process of becoming nonunions. For this reason, we have studied biopsies recovered intraoperatively from human bone fracture sites that were determined, on follow-up, to have healed normally or to have become nonunions.

New bone formation in fracture repair is mainly attributable to endochondral ossification, in which cartilage formation is an important intermediate step. Common histological findings in end-stage human fracture nonunion are an abnormally high content of fibrous tissue and the absence or minimal amount of bone formation.[4,5] In animal models of fracture nonunion, cartilage formation is followed by little or no bone formation.[6–8] This is true whether the cause of nonunion is periosteal cauterization, rotational instability, or ischemia.[6–8] In these different models of fracture non-union, cartilage formation is therefore not followed by efficient endochondral ossification; fibrous tissue forms instead. The transition from cartilage to bone is a processwhich is regulated bylocally produced growth factors.[9,10] Whether cartilage formation is followed by matrix degradation and the formation of bone (fracture union) or not (nonhealing fractures), may be due to differences in the molecular signaling within the cartilaginous areas. Very little data exist on any possible phenotypic differences between the chondrocytes within the cartilage of fractures that eventually heal and those that do not.

With the critical role played by bone morphogenetic proteins (BMPs) in fracture repair, it is possible that there is an alteration in the biological activity of BMPs in the pathogenesis of fracture nonunions. This hypothesis is derived from preliminary observations which suggest that some adverse clinical factors leading to fracture nonunion, mediate changes in the biology of fracture repair by affecting BMP production. For example, mechanical forces can affect the differentiation of progenitor cells by altering their endogenous expression of BMP.[11 – 13] The critical importance of endogenous BMP-2 production in the early phases of fracture repair was demonstrated in genetically modified mice, where BMP-2 knockout animals were unable to initiate fracture repair.[14] Mice lacking BMP-4, however, were able to heal fractures normally.[15] BMP-2 is among the most osteoinductive members of the family, with biological activity throughout most of the stages of fracture repair.[16] A lesser known member of the BMP family is BMP-14, also known as Growth and Differentiation Factor-5 and Cartilage-Derived Morphogenetic-Protein-1. BMP-14 influences endochondral bone growth[17,18] and its ectopic implantation intramuscularly induces the formation of cartilage and bone.[19] BMP-14 deficiency inhibits long bone fracture healing, secondary to a delay in cellular recruitment and chondrocyte differentiation.[20]

The effects of BMPs can be modulated by a group of extracellular binding proteins that prevent the association of BMPs with their receptors on the cell surface. Extracellular BMP antagonists of this type include noggin, chordin, follistatin, ventroptin, twisted gastrulation, and the Dan/Cerberus family of genes which is comprised of Dan, gremlin, and sclerostin.[21] Studies of BMP inhibitors in fracture repair have focused on noggin and, to a lesser extent, chordin.[22 – 25]

We have previously identified the cartilaginous areas within fractures as sites of high expression for BMP-2, BMP-14, and the BMP inhibitors chordin and noggin.[39] The present study is based upon the hypothesis that, there is a difference in the relative expression of BMPs and BMP inhibitors in fractures with different outcomes.

MATERIALS AND METHODS

Human Fracture Specimens

All tissue samples used in this study were obtained with the approval of the Local Research Ethics Committee, and informed consent was obtained from the patients. The fractures were chosen from an archive of human fracture specimens previously used to localize the expression of Insulin-like Growth Factor, Platelet Derived Growth Factor, and Transforming Growth Factor-b in healing fractures.[26 – 28] Using the same archive, the unusual phenotype of osteoblasts in nonhealing fracture calluses was also demonstrated.[29,30] The fractures in this study were selected, based on the detection of cartilaginous areas in histological sections stained with hematoxylin and eosin (H&E).

The two groups of fractures compared in this study were called “healing fractures” and “nonhealing fractures.” The “healing fractures” group consisted of fracture biopsies taken at surgery for treatment of malalignment or failure of fixation, as well as acute fractures that were operated upon in a delayed fashion. Specimens were taken from the fracture site of eight closed fractures. Patients were aged between 18 and 87 years, and were otherwise fit. These fractures were all biopsied between 1 and 6 weeks after the initial injury. All fractures were found to have healed on subsequent follow-up.

“Nonhealing” specimens of human fracture callus were taken from patients who were subsequently diagnosed with fracture nonunion within the 1 year follow-up period, or were diagnosed with fracture nonunion at the time of surgery. A nonunion was defined as the absence of osseous healing9 months after the operative or nonoperative treatment of the fracture. The “nonhealing” fractures therefore consisted of a group of fractures which were in various stages of progression towards nonunion. The “nonhealing” specimens, eventually selected for this study, were recruited from seven patients with extraarticular fractures between 1 and 48 months after the initial injury. No patient had evidence ofan active infection as determined on the basis of radiographic, physical, laboratory, and intraoperative findings.

The biopsy specimens were fixed in 10% neutral buffered formalin and decalcified in 20% EDTA (pH 7.2) until radiologically complete (2 – 4 weeks). The specimens were then embedded in paraffin wax and sectioned at 5 mm. One slide per patient was stained with H&E for routine evaluation of the stage of fracture repair and other slides used for immunohistochemical analysis, with one slide per patient used for the immunohistochemical stain ofeach protein.

Immunohistochemical Analysis

All chemicals used were from Sigma-Aldrich (Dorset, UK), unless stated otherwise. Goat and rabbit (noggin only) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA)

were used for the immunohistochemical detection of the four proteins under investigation. Santa Cruz Biotechnology references for the antibodies are as follows: GDF-5 (BMP-14): sc-6901; chordin: sc-18265; noggin: sc-25656; and BMP-2: sc-6895. The sections were prepared for immunohistochemistry by first dewaxing and rehydrating. After washing in deionized water, sections were exposed to their respective antigen retrieval system at 378C. BMP-2 antigen retrieval consisted of exposure to 0.4% pepsin in 0.01 M hydrochloric acid for 20 min. BMP-14 antigen retrieval was performed by 20-min treatment with 0.01% chymotrypsin 0.1% CaCl 2 dissolved in Tris buffered saline (TBS). Noggin and chordin antigen retrieval was performed with exposure to 1 mg/ml of trypsin for 15 min. The endogenous peroxidase enzyme activity was blocked using methanol containing 3% hydrogen peroxide for 30 min at room temperature. Following washing, nonspecific binding was inhibited by the following incubation for 30 min at room temperature in a humid chamber: 10% swine serum/1% bovine serum albumin (BSA) for noggin; 10% donkey serum/2% BSA for chordin, 10% donkey serum/1% BSA for BMP-2, and 10% donkey serum/1% BSA for BMP-14.

Immunostaining was then carried out by applying the appropriate concentration of antibody to each sample (BMP-2: 1:10; BMP-14: 1:100; noggin 1:200; chordin 1:100) and incubating overnight at 48C. As a negative control, normal IgG at the equivalent concentration was used in place of the primary antibodies. Following incubation, the samples were washed in TBS and incubated with the biotinylated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The avidin-biotin-peroxidase complex (Elite ABC Reagent, Vector Laboratories, Burlingame, CA) was then added for 30 min at room temperature. The color reaction was developed with use of3, 3-diaminobenzidine tetrahydrochloride solution. Counter-staining was performed with use of Mayer's Haematoxylin (Raymond A Lamb, East Sussex, UK), dehydrated, and a coverslip applied with Pertex (Histolab, Goteborg, Sweden).

Image and Statistical Analysis

All slides were visualized using a Leica RMDB research microscope (Leica Camera Limited, Knowlhill, Milton Keynes, UK) and images captured using a digital camera and Delta Pix Viewer Software (Delta Pix, Maalov, Denmark). Following immunohistochemistry, positive and negative controls were examined to check that the intensity of immunoreactivity was similar in all experiments.

Semiquantitative expression of the protein in each specimen was assessed using two different methods, to increase the validity of the findings. First, each sample was scored according to a previously described method: (a) for the percentage of labeled cells (0 ¼ absence of labeling over cells; 1 ¼ less than 30% of cells labeled; 2 ¼ 30% – 60%; and 3 ¼ more than 60%); and (b) and for the intensity of immunostaining (0 ¼ no staining; 1 ¼ weak; 2 ¼ mild; and 3 ¼ strong staining).[31] Multiplication of both scores allowed the final scoring of samples, ranging from 0 to 9. Scores were obtained from six random fields per section and the observer was blinded to the type of fractures (healing vs. nonhealing). The score obtained for each section is referred to as the "immunohistochemistry score (visual)."

Secondly, quantitative analysis to determine the area occupied by cells immunoreactive with antibodies against BMP-2, -14, noggin, and chordin was used, using a previously described automated image analysis software.[32] A Leica Quantiment 600 Image Analysis System controlled by QWIN software (Leica Microsystems, Cambridge, UK) was used with the same microscope. Each section was viewed at x10 magnification and an area of cartilage was manually mapped out on the camera picture. The software provided an automated indication of the area stained positive (brown color) and the total area of cartilage mapped out. Six to 10 fields of cartilage were chosen per slide and the average area of staining per unit area of cartilage was calculated. The number of cells in each area of cartilage tested was manually counted, and the number of cells per unit area cartilage was also calculated for each specimen.

Comparison between the corresponding groups was made by the unpaired Student's t-test, using the Graph Pad Prism Software (Graph Pad, San Diego, CA.), with a p value ::;0.05 considered as significant.

RESULTS

Only fractures with areas of cartilage were chosen for this study. Out of 20 nonhealing fractures, 7 were found to have areas of cartilage formation on histology and were therefore selected. Healing fractures all consisted of areas of cartilage and significant woven bone formation. The nonhealing fractures revealed a mixture of different tissue types. In most of the nonhealing fractures, cartilaginous areas were accompanied by the presence of small amount ofwoven bone, but significant fibrous tissue. There were no notable differences in cellular morphology in the cartilaginous areas of the fractures between the healing versus nonhealing group. Specific expression of the proteins under investigation was detected in areas of cartilage formation and, to a lesser extent, in areas of bone formation, but was insignificant in other tissues of the fractures.[39] There was no significant staining when an IgG antibody was used as a negative control (Figs. 1A and 2A).

Semiquantitative evaluation of staining revealed that there was decreased BMP-2 expression in the cartilaginous areas in the nonhealing fractures, compared to the healing fractures [Fig. 1B and 1C; $p < 0.001$ for area stained per cartilage area, and p ¼ 0.05 for the immunohistochemistry score (visual)]. Semiquantitative expres- sion of BMP-14 in the two groups of fractures showed that there was decreased expression of BMP-14 in the nonhealing fractures, compared to the healing fractures [Fig. 1B and 1C; $p < 0.001$ for area stained per cartilage area, and $p < 0.05$ for immunohistochemistry score (visual)]. There was no difference in the number of cells per area of cartilage between the healing and nonhealing groups. This implies that the difference in area of staining per unit area of cartilage, detected by the image analysis software, was not due to a difference in the number of cells within cartilaginous areas between healing and nonhealing fractures. There were no statistical differences in the endogenous expression of noggin and chordin between the healing and nonhealing fractures (Fig. 2).

DISCUSSION

A common observation reported in fracture nonunion is the persistence of cartilage between the bone ends, associated with the formation of fibrous tissues and minimal bone regeneration.[6 – 8,33] In this study, fractures, with histological evidence of areas of cartilage, were chosen from an archive of human fracture specimens. They were divided into two groups, depending on whether the fracture achieved union or not on subsequent clinical follow-up. In healing fractures, these areas of cartilage would undergo endochondral ossification. In nonhealing fractures, the areas of cartilage would not be accompanied by significant bone formation and nonunion would result.

The endogenous expression of BMP-2 and BMP-14 was lower in the nonhealing group of fractures, compared to the healing fractures. There was no difference between the number of cells per area of cartilage between the two groups of fractures. The differences in expression were qualitatively similar using the two independent methods of semiquantitative expression of BMP-2 and -14. These findings disagree with those of Kloen et al., who reported no detectable difference in BMP-2 expression between fracture unions and nonunions.[4,34] This discrepancy from our results may occur because they used a population of nonunions which was more heterogeneous than ours. However, our results are supported by the findings of one animal study, which demonstrate the downregulation of BMP-2 and -14 gene expression in experimental atrophic nonunions, compared to standard healing fractures.[35]

Interestingly, there was no statistical difference in BMP inhibitor expression between our two groups of fractures. This is a novel finding which, together with the BMP-2 and -14 data, suggests that BMP:BMP inhibitor ratio may be lower in fractures with low healing potential. This finding may have implications for understanding the pathophysiological processes leading to fracture nonunion, as well as suggesting novel ways to reduce the incidence of nonunions. These include the local and timed application of BMP-2 or -14 by delivery of the recombinant protein or by gene transfer.[36] Alternatively, BMP antagonists could be blocked with, for instance, neutralizing antibodies or knocked down with siRNA molecules.[25]

Early detection of nonhealing fractures would facilitate such treatments and save the patient significant amounts of time. Despite the different causes of fracture nonunion, there may be common biological pathways which are activated in their development. Identification of these common altered biological processes will contribute to the earlier diagnosis of nonunion. The recent finding of increased expression of matrix metalloproteinases in venous blood in patients with nonunion suggests that this may be the case[37,38] and, if confirmed, could lead to the development of a simple blood test as a predictor of nonunion.

For sound ethical and clinical reasons, the availability of fracture calluses from human is limited. From our archive of human fracture biopsies, the process of endochondral ossification was detectable in healing fractures between 1 and 6 weeks after the original injury. Biopsies from patients with nonunion are usually taken at the time of surgery which, in terms of interval from fracture, is often equivalent to a time when normally healing fractures would have fully healed. Direct comparison between normally healing fractures

and nonunions is therefore not feasible based on the interval between fracture and biopsy. We have therefore compared the biology of reactive cartilage in the two situations. All the nonhealing fractures (including those biopsied between 1 and 6 weeks postinjury) showed a markedly decreased expression of BMPs by chondrocytes to that seen in normally healing fractures.

In summary, this study has shown an alteration in the balance of BMPs and their inhibitors in cartilaginous areas of fractures with different clinical outcomes. This may be an important factor in the impaired transition from cartilage to bone formation during the endochondral ossification stage of fracture repair in fracture nonunion. Further characterization of this cartilaginous intermediate phenotype may have useful implications in the understanding of the pathogenesis of fracture nonunions and the development of improved treatment modalities.

Acknowledgments

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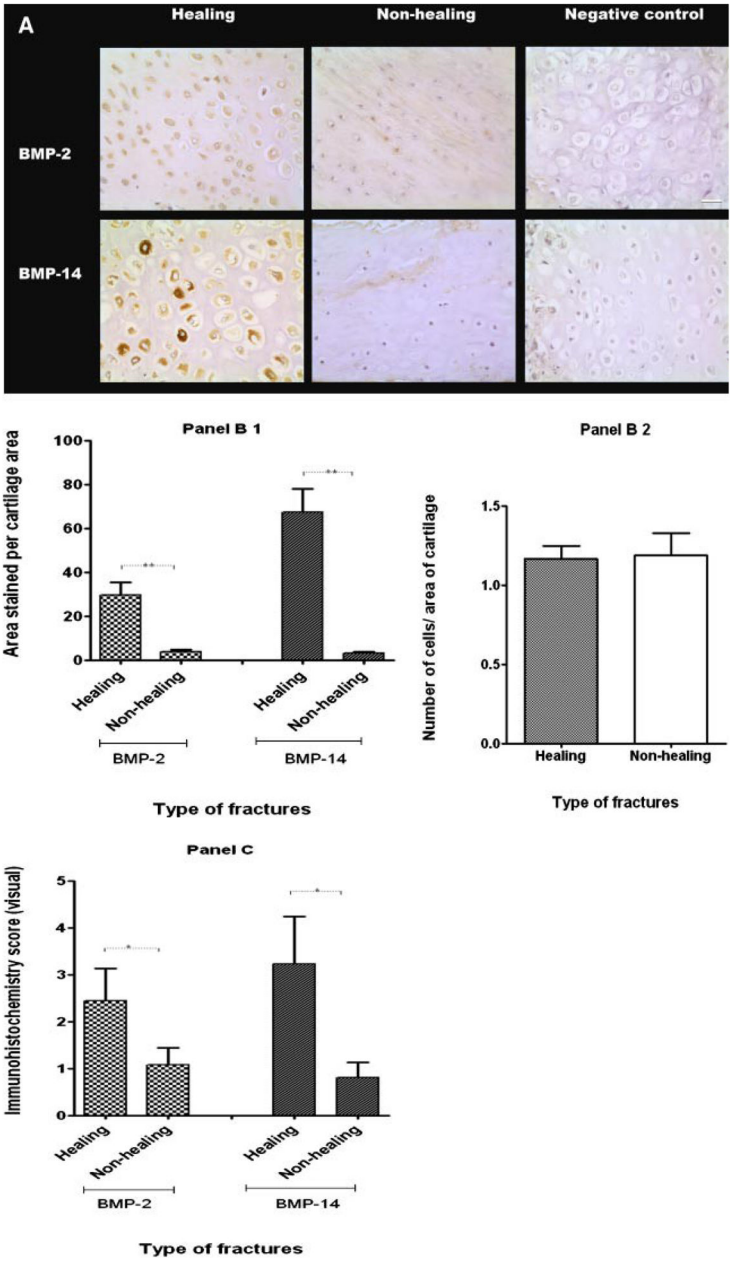

Figure 1.

Expression of BMP-2 and BMP-14 in cartilaginous areas of healing and nonhealing human fractures. (A) Histological appearance of BMP-2 and BMP-14 expression in cartilaginous areas of human fractures. Negative control (IgG) antibody used as primary antibody illustrates background staining in cartilaginous areas of a healing fracture. Scale bar indicates 50 μm. (B) Comparison of BMP-2 and BMP-14 expression between healing (n = 8) and nonhealing fractures (n = 7), by image analysis software. (B1) Area of positive stain per unit area of cartilage. (B2) Comparison of the number of cells per unit area of cartilage between healing and nonhealing fractures. (C) Comparison of immunohistochemistry score (visual) of BMP-2 and BMP-14 between the healing (n = 8) and nonhealing fractures (n =

7). The respective healing and nonhealing groups were compared with a one-tailed Student's t-test; * indicates a $p \leq 0.05$, and ** indicates a $p \leq 0.001$.

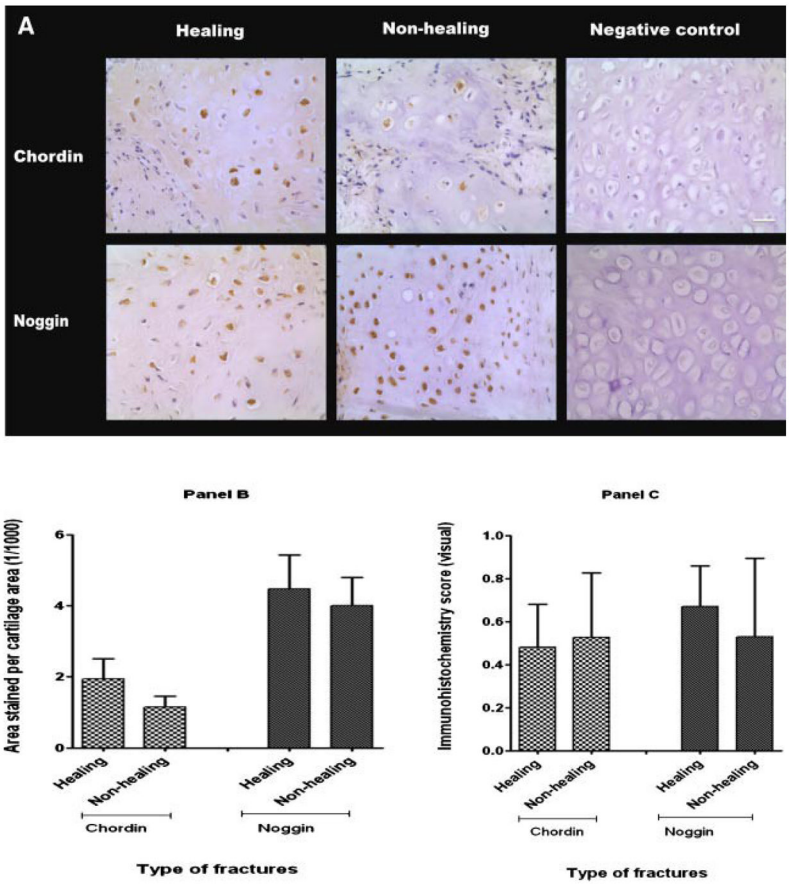

Figure 2.

Expression of noggin and chordin in cartilaginous areas of healing and nonhealing human fractures. (A) Histological appearance of noggin and chordin expression in cartilaginous areas of human fractures. Negative control (IgG) antibody used as primary antibody illustrates background staining in cartilaginous areas of a healing fracture. Scale bar indicates 50 μm. (B) Comparison of chordin and noggin expression in human fractures, according to the image analysis detection software. (C) Comparison of immunohistochemistry score (visual) of noggin and chordin between the healing (n = 8) and nonhealing fractures (n = 7).