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Osteoclastogenesis in fibrous dysplasia of bone: in situ and in vitro analysis of IL-6 expression

M. Riminucci,[a,b] S. A. Kuznetsov,[c] N. Cherman,[c] A. Corsi,[a,d] P. Bianco,[b,c,d] and P. Gehron Robey[c,*]

[a] *Dipartimento di Medicina Sperimentale, Università dell' Aquila, L' Aquila 67100, Italy*
[b] *Parco Scientifico Biomedico San Raffaele, Rome 00100, Italy*
[c] *Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, USA*
[d] *Dipartimento di Medicina Sperimentale e Patologia, Università "La Sapienza," Rome 00161, Italy*

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Abstract

Fibrous dysplasia of bone (FD) is caused by somatic mutations of the *GNAS1* gene, which lead to constitutive activation of adenylyl cyclase and overproduction of cAMP in osteogenic cells. Previous in vitro studies using nonclonal, heterogeneous strains of FD-derived cells suggested that IL-6 might play a critical role in promoting excess osteoclastogenesis in FD. In this study, we investigated IL-6 expression in FD in situ and its relationship to the actual patterns of osteoclastogenesis within the abnormal tissue. We found that osteoclastogenesis is not spatially restricted to bone surfaces in FD but occurs to a large extent ectopicly in the fibrous tissue, where stromal cells diffusely express IL-6 mRNA and exhibit a characteristic cell morphology. We also observed specific expression of IL-6 mRNA in a proportion of osteoclasts, suggesting that an autocrine/paracrine loop may contribute to osteoclastogenesis in vivo in FD, as in some other bone diseases, including Paget's disease. We also generated homogeneous, clonally derived strains of wild-type and *GNAS1*-mutated stromal cells from the same individual, parent FD lesions. In this way, we could show that mutated stromal cells produce IL-6 at a basal magnitude and rate that are significantly higher than in the cognate wild-type cells. Conversely, wild-type cells respond to db-cAMP with a severalfold increase in magnitude and rate of IL-6 production, whereas mutant strains remain essentially unresponsive. Our data establish a direct link between *GNAS1* mutations in stromal cells and IL-6 production but also define the complexity of the role of IL-6 in regulating osteoclastogenesis in FD in vivo. Here, patterns of osteoclastogenesis and bone resorption reflect not only the cell-autonomous effects of *GNAS1* mutations in osteogenic cells (including IL-6 production) but also the local and systemic context to which non-osteogenic cells, local proportions of wild-type vs mutated cells, and systemic hormones contribute.

Keywords: Bone disease; Bone histology; cAMP; Osteoclasts; Stromal cells

Introduction

Fibrous dysplasia of bone (FD; OMIM #174800) is caused by activating missense mutations of the *GNAS1* gene that codes for the alpha subunit of the stimulatory G protein, Gs-alpha [1,2]. These activating mutations occur postzygotically such that the affected individuals are somatic mosaics and represent a heterogeneous patient population depending on the pattern of distribution of mutated cells throughout the body. FD can occur as an isolated monostotic or polyostotic disease or as a part of the McCune-Albright Syndrome (MAS), which also includes skin hyperpigmentation and one or more endocrinopathies. The causative mutations inhibit the intrinsic GTPase activity of Gs-alpha such that it remains active in stimulating adenylyl cyclase and leads to the over-production of cAMP [3]. The result of these Gs-alpha mutations in the skeleton is that abnormal, mechanically unsound bone and a fibrous tis-

* Corresponding author. CSDB/NIDCR/NIH/DHHS, 30 Convent Drive MSC 4320, Bethesda, MD 20892. Fax: +1-301-402-0824.
E-mail address: probey@dir.nidcr.nih.gov (P. Gehron Robey).

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sue that is devoid of normal marrow elements (hematopoietic cells and adipocytes) focally replace normal bone and marrow. This fibrotic tissue is composed of preosteogenic cells that mature into osteoblasts and, as do normal osteoblasts in developing bone, up-regulate the *GNAS1* gene [4]. In osteoblastic cells, the overproduction of cAMP caused by the mutation leads to cell retraction from the surface of the forming bone. Furthermore, there is deposition of woven bone that is abnormal in both its composition and organization, with collagen fibers oriented perpendicular (as opposed to parallel) to the forming surface, thus resulting in the formation of Sharpey fibers [4,5].

The natural history of a fibrous dysplastic lesion, from its initiation through its progression with age, has not been well characterized. However, it has been noted that there are often unusually high numbers of osteoclasts and evidence of increased bone resorption [5,6], although this is not a universal feature of fibrous dysplastic lesions [5,7,8]. Consequently, it is thought that increased bone resorption plays a role in the establishment and growth of some FD lesions and has prompted the use of bisphosphonates as a potential therapy for FD/MAS patients [9,10].

IL-6 is one of several cytokines that have been implicated in increased bone resorption [11,12]. Its expression is up-regulated by PTH, which acts via Gs-alpha and the cAMP-mediated signaling pathway, and is associated with increased bone resorption seen in hyperparathyroidism [13,14]. IL-6, along with other cytokines that mediate their effects through the signal transducer gp130 is also involved in bone resorption induced by myeloma cells [15]. Since cAMP is a known stimulant of IL-6 gene activity [16], it has been hypothesized that the increase in osteoclastogenesis seen in some FD lesions is due to overexpression of IL-6. Increased levels of IL-6 secretion have been shown to be induced by transfecting MC3T3 cells with Gs-alpha constructs that bear the same mutation as in FD/MAS [17]. In addition, cells isolated from some, but not all, fibrous dysplastic lesions were found to secrete increased levels of IL-6 [6,18].

In this study, we have more closely examined fibrous dysplastic lesions with high numbers of osteoclasts with respect to patterns of osteoclastogenesis and IL-6 production in situ and in vitro.

Materials and methods

Patients

Fresh pathological tissues were obtained from four patients undergoing corrective surgeries or biopsy under NIH IRB approved protocols (97-D-0055, 98-D-0145). The characteristics of the FD patients are indicated in Table 1.

Table 1
Patient characteristics

Patient	Gender	Age	Site	Mutation
1	F	10	Mandible	R201H
2	M	8	Femur	R201H
3	M	25	Cranium	R201C
4	F	10	Femur	R201C

Histological analysis

Tissues used for histology were fixed with 4% neutral buffered formaldehyde overnight at 4°C. After fixation, samples were decalcified with 10% EDTA in phosphate buffer, pH 7, embedded in paraffin, and 5-μm sections were used for H & E staining and other analyses. Tartrate-resistant acid phosphatase (TRAP) was detected using a commercially available kit according to the manufacturer's instructions (387-A; Sigma, St. Louis, MO). For in situ localization of IL-6 mRNA, a 153-bp sequence (exon 4) of the IL-6 gene was amplified from genomic DNA in a standard PCR reaction using the following primers: 5′-TAGATGCAATAACCACCCCTG-3′ and 5′-TTTGCCGAAGAGCCCTCAG-3′ (GenBank accession no. NM_000600.1, bases 544–564, bases 678–696) and inserted into the pCR-Script Amp SK(+) cloning vector (Invitrogen Life Technologies, Carlsbad, CA). Using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), the vector was linearized with *Not*1 (New England Biolabs, Beverly, MA) and transcribed with T7 RNA polymerase for the antisense probe, whereas *Eco*RI (New England Biolabs) and T3 RNA polymerase were used for transcription of the sense probe. After removal of paraffin, sections were prepared and hybridized at 55°C, and hybridization was detected as described previously [4].

Isolation of bone marrow stromal cells (BMSCs)

Marrow stromal cell cultures were established from tissue removed from the FD patients under IRB-approved protocols (97-DK-0055, 98-D-0145) as previously described [19]. Briefly, a single cell suspension of fibrous dysplastic marrow was prepared by scraping fresh bone marrow tissue into medium, repeated pipeting, and serial passage through needles at decreasing diameter and a 70-μm cell sieve (Falcon Labware, Franklin Lakes, NJ). Cells were plated at low density (0.007–3.5 $\times$ 10^3 nucleated cells/cm^2) to generate single colony derived strains; that is, the progeny of a single colony forming unit-fibroblast (CFU-F) in α-MEM (Invitrogen Life Technologies) plus 20% FBS from selected lots (Equitech-Bio, Kerrville, TX), penicillin, streptomycin, and glutamine (Biofluids, Rockville, MD). After 13 to 16 days, individual colonies were isolated using cloning cylinders and trypsin release, and expanded in cell number by placing them in vessels of increasing size until at least 6 $\times$ 10^6 cells were available [20].

Mutation analysis

Direct DNA sequencing was used in these experiments to detect the *GNAS1* mutation. Genomic DNA was isolated from cells using the DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The extracted DNA (200–500 ng) was amplified in a 100-μl reaction by standard PCR with primers that generate a 270-bp product spanning the mutation. For each reaction, 10 μmol each of the primers 5′-TGACTATGTGC-CGAGCGA-3′ (forward, exon 7), and 5′-AACCAT-GATCTCTGTTATATAA-3′ (reverse, intron G) (GenBank accession no. M21142.1, bases 272–289, and 521–542, respectively) were added along with reagents from a commercially available kit and 2.5 units of Ampli *Taq* Gold polymerase (Perkin Elmer, Boston, MA). The samples were heated to 94°C for 15 min, cycled 35 times (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) and finished with 7 min at 72°C. The PCR products were purified and sequenced as previously described [19].

Generation of wild-type and mutant BMSC strains

It is known that fibrous dysplastic lesions are composed of both normal and mutant stromal cells in varying ratios [19]. Consequently, it was necessary to separate normal and mutated stromal cells by cloning in order to determine the direct effect of mutation on IL-6 expression. Since a marrow stromal cell colony arises from a single CFU-F and the Gs-alpha mutation is heterozygous, the determination of mutant clonal strains was based on equal levels of normal and mutant bases in direct sequencing of genomic DNA. After determining the genotype of the single colony derived strains isolated from fibrous dysplastic cultures, defined here as wild-type (−/−) and mutant (−/+), multicolony derived strains were made by combining five clonal strains for each genotype, thus generating patient-specific wild-type (normal) and mutant strains of BMSCs. Following combination, the cells were further expanded in culture, and mutation was again analyzed by direct DNA sequencing of genomic DNA to verify the genotype.

RT–PCR analysis of IL-6 expression

Total RNA was extracted from cell cultures by RNA-STAT 60 according to the manufacturer's instructions (Tel-Test, Friendswood, TX), and digested with deoxyribonuclease I (Roche Diagnostics, Mannheim, Germany) for 10 min at 37°C. cDNA was generated using the SuperScript First-Strand Synthesis System using oligo-dT according to the manufacturer's instructions (Invitrogen Life Technologies). A 153-bp sequence of exon 4 of the IL-6 gene was amplified in a standard PCR reaction containing the forward primer 5′-TAGATGCAATAACCACCCCTG-3′ and the reverse primer 5′-TTTGCCGAAGACCCTCAG-3′ (GenBank accession no. NM_000600, bases 544–564, bases 676–696), reagents from a commercial kit, and 2.5 units of Platinum *Taq* polymerase (Perkin Elmer). After 15 min at 94°C, the target cDNA was amplified in 25 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The final extension was at 72°C for 5 min. For comparison, a 816-bp sequence of human GAPDH was amplified using the forward primer 5′-AGCCGCATCTTCTTTTGCGTC-3′ and the reverse primer 5′-TCATATTTGGCAGGTTTTTCT-3′ (GenBank accession no. M33197, bases 12–32, bases 807–827) by running the reaction for 15 min at 94°C and then 35 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min and a final extension at 72°C for 7 min. The amplification products were separated by electrophoresis in acrylamide gels and were verified by DNA sequencing.

Stimulation with cAMP

Wild-type and mutant BMSCs from fibrous dysplastic tissue were plated in triplicate at a density of 40,000 cells/cm^2 in six-well tissue culture plates and allowed to grow for 2 days. The cells were washed extensively with serum-free medium, incubated for 30 min at 37°C, and washed again. Medium containing 1 mM dibutyryl cAMP (Sigma) was added and the cells were incubated for 2 h or 16 h. Conditioned medium was collected for determination of IL-6 levels, and cells were harvested by trypsin release to determine the cell number using a cell counter (Coulter, Hialeah, FL) at the end of the experiment.

Quantitation of IL-6 level secretion

Conditioned medium was centrifuged to remove particulate matter and stored at −20°C until analyzed. Quantitation of IL-6 secreted into the medium at 2 and 16 h was determined using a quantitative sandwich ELISA for human IL-6 according to the manufacturer's instructions (D6050; R&D Systems, Minneapolis, MN). Values for the conditioned medium samples were determined by comparison to a standard curve and corrected for cell number. IL-6 secretion was analyzed in wild-type and mutant strains from two different patients.

Results

Osteoclastogenesis in fibrous dysplastic lesions

The samples used in this study were all characterized by a large number of osteoclasts. Analysis of sections cytochemically stained for TRAP readily demonstrated usual and unusual patterns of distribution of osteoclasts in the FD tissue. In addition to osteoclastic resorption taking place at the surface of FD trabeculae (Fig. 1A), tunneling resorption, a pattern of bone resorption characteristic of both hyperparathyroidism and FD [7,21], was noted (Fig. 1B). Furthermore, a striking finding was the detection of large numbers of mono and multinuclear TRAP-positive cells (sometimes forming large "ectopic" clusters) away from bone surfaces, within areas of fibrous tissue devoid of bone

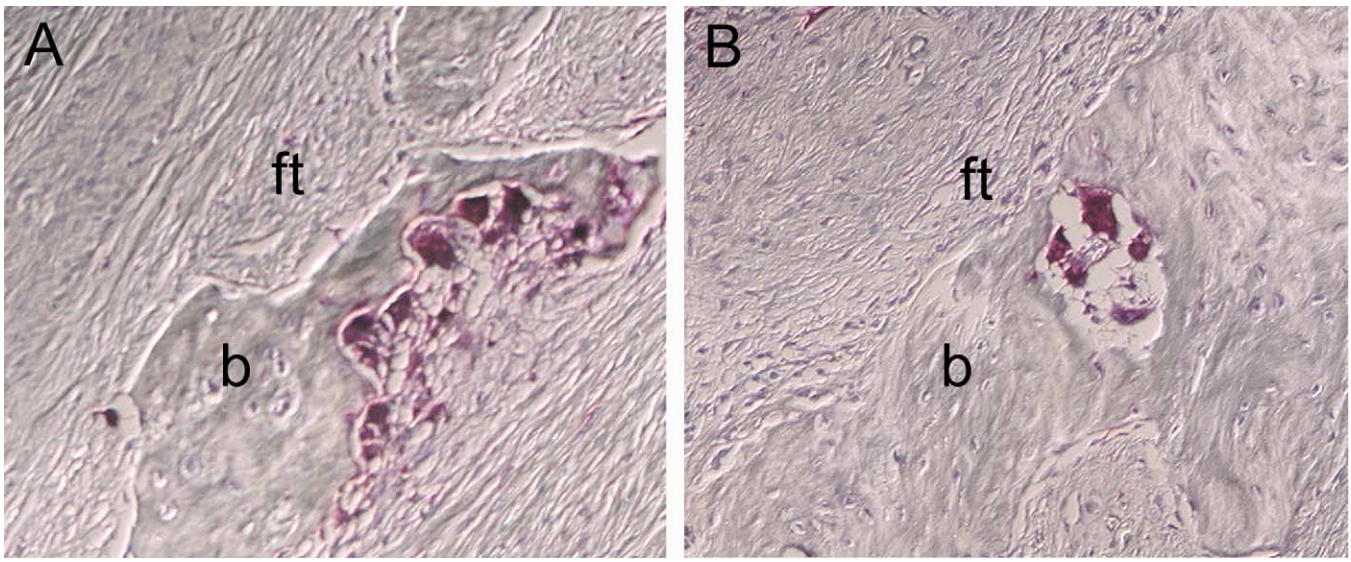

Fig. 1. Bone resorption and orthotopic osteoclastogenesis in FD. (A) Intense osteoclastic activity on the surface of an FD trabecula (b). (B) Tunneling resorption within an FD trabecula (b). TRAP reaction, Nomarski optics. ft, fibrous tissue.

(Fig. 2A–D). Individual TRAP-positive cells within the fibrous tissue, particularly mononuclear cells, were physically closely associated with a meshwork of cell processes emanating from retracted, stellate stromal cells (Fig. 2B).

IL-6 mRNA expression in fibrous dysplastic lesions

In situ hybridization for IL-6 mRNA revealed high levels of expression of the IL-6 gene within the fibrous dysplastic

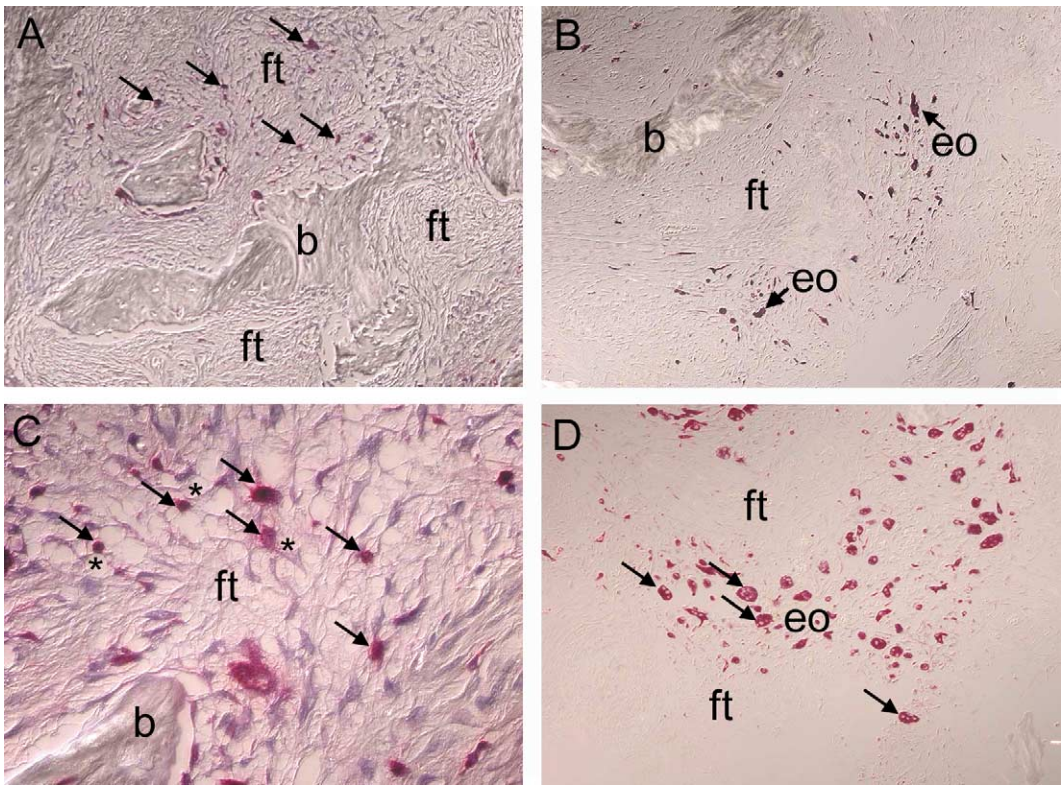

Fig. 2. Ectopic osteoclastogenesis in FD. (A) Most of the TRAP-positive cells (arrows) in the field reside away from bone surfaces (b), fully within the fibrous tissue (ft). (c) Detail of some ectopic TRAP-positive cells in the fibrous tissue shown in A. A sprinkling of mononuclear TRAP-positive cells (arrows) is closely associated with a meshwork of cell processes emanating from retracted, stellate stromal cells (asterisks). (A and C) counterstained with hemalum. (B and D) Large clusters of ectopic osteoclasts (eo) residing within the fibrous tissue. Some multinuclear, TRAP-positive cells are indicated by arrows in D. TRAP reaction, Nomarski optics.

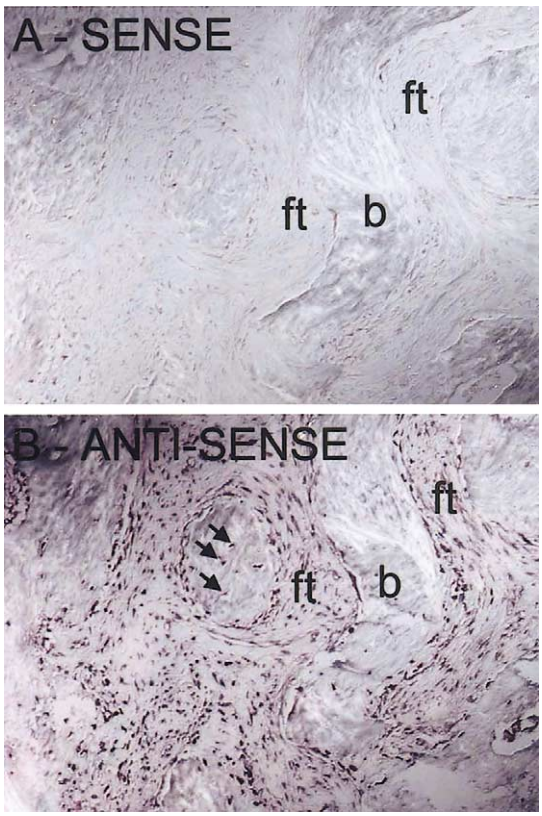

Fig. 3. Localization of IL-6 mRNA in FD tissue. Overview of FD tissue hybridized with sense (control, A) and antisense (B) digoxygenin-labeled RNA probes specific for IL-6. Strong specific signal is seen across the fibrous tissue (ft) separating abnormal fibrous dysplastic bone trabeculae (b). Note also the expression of IL-6 mRNA in osteocytes within FD bone (B, arrows).

tissue (Fig. 3). The hybridization signal was localized in "fibroblastic" cells in the fibrotic marrow spaces, but also to retracted osteoblasts associated with the surfaces of FD bone trabeculae (Fig. 4B and D) and to osteocytes residing therein (Fig. 3B and Fig. 4B). A strong signal was observed in the retracted, stellate-shaped stromal cells in the fibrous tissue (Fig. 4B and D). Interestingly, variable levels of expression of the IL-6 mRNA were also observed in some, but not all (approximately 50%) osteoclasts (Fig. 4F).

IL-6 expression by stromal cells in vitro

The specific effect of *GNAS1* mutation on IL-6 expression was evaluated by creating strains of wild-type and mutant BMSCs derived from the same FD lesion. Individual colonies, arising from a single CFU-F, were isolated and genotyped by direct DNA sequencing, and combined according to genotype. After further expansion, the genotype of the wild-type and mutant strains was again verified (Fig. 5A). Total RNA was extracted from wild-type and mutant BMSCs cultured under basal conditions, and IL-6 mRNA was identified by RT–PCR, with a higher level of amplification being noted in the mutant cells (Fig. 5B).

The amount of IL-6 secreted by wild-type and mutant BMSC stains was then measured at 2 h and 16 h using a human IL-6-specific ELISA. In basal conditions, the amount of IL-6 secreted by mutant cells was threefold higher than in wild-type cells (Fig. 6A). Because activating GNAS1 mutations brings about a sustained increase in cAMP, and cAMP mediates the induction of IL-6 in bone cells in response to PTH, we also analyzed the effects of db-cAMP on wild-type and mutant strains of FD-derived stromal cells. Treatment with db-cAMP

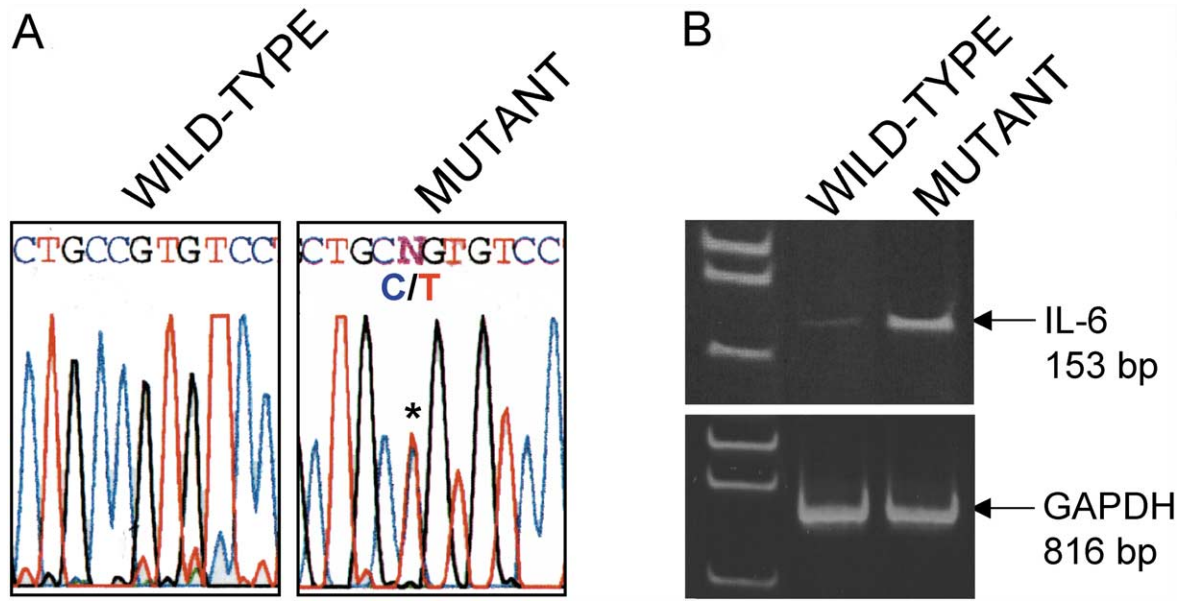

Fig. 5. IL-6 expression in osteogenic cells as assessed by RT–PCR. (A) Wild-type and mutant strains were generated from clonal cells derived from the same FD lesion. Their genotypes were verified by direct DNA sequencing (strains from patient 4 with an R201C mutation shown here as an example), as indicated by equal amounts of C and T (asterisk). (B) mRNA was isolated from wild-type and mutant bone marrow stromal cells derived from fibrous dysplastic tissue. Using an IL-6 specific primer set, a 153-bp product was amplified, and the amount of the amplification product was higher in mutant cells than in wild-type cells.

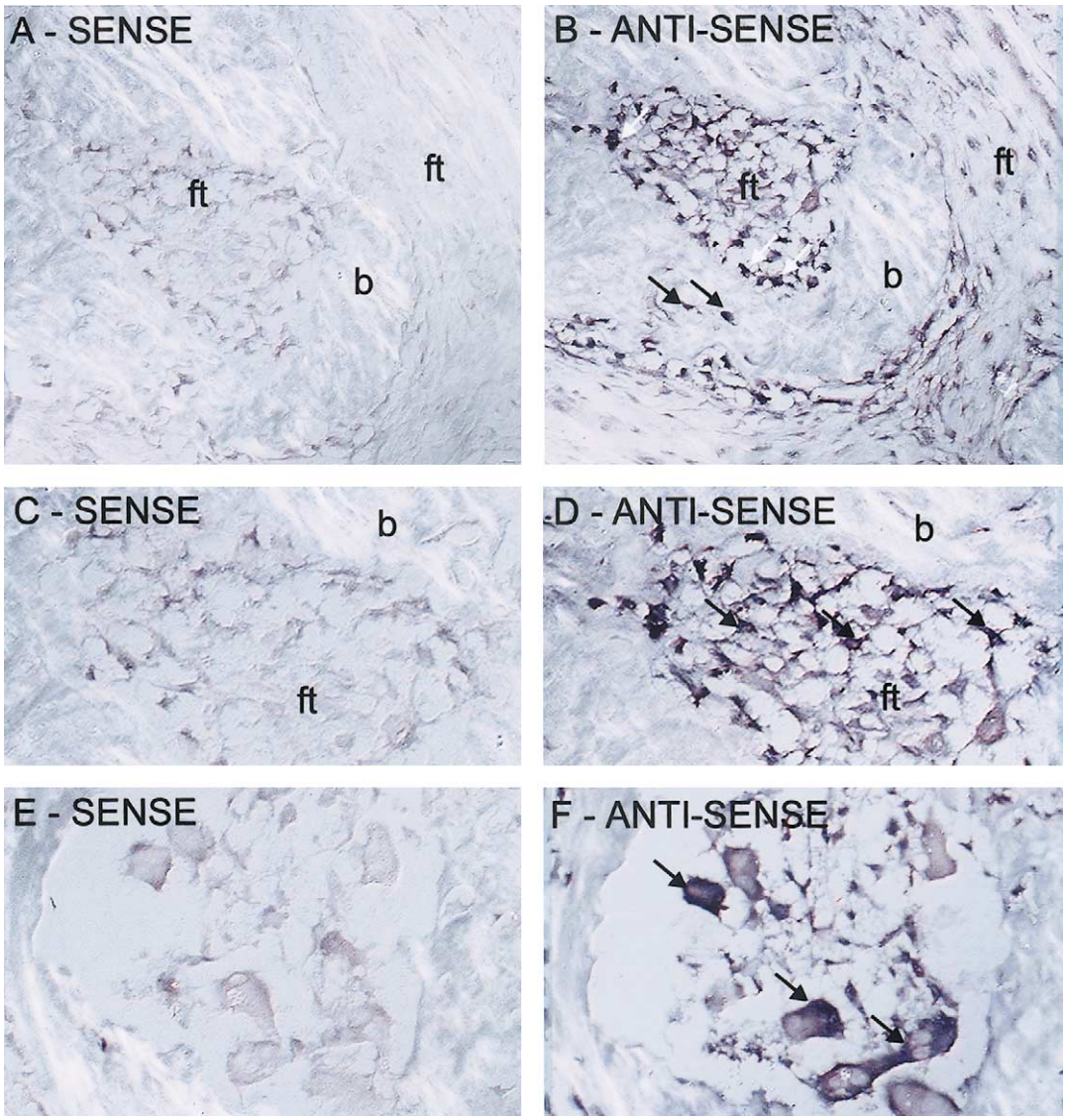

Fig. 4. Localization of IL-6 mRNA to different cell types in FD bone. (A, C, and E) Sections hybridized with sense (control) probes. (B, D, and F) Serial sections of those in A, C, and E hybridized with antisense probe. Specific signal is observed in fibrous tissue (ft) (B), in retracted cells associated with the surface of FD bone (B, white arrows), and in osteocytes residing within FD bone (B, black arrows). A strong signal is observed in retracted, stellate stromal cells within the fibrous tissue (D, arrows). Some osteoclasts (F, arrows) also express high levels of IL-6 mRNA.

for 2 h did not alter the amount of IL-6 secretion in either wild-type or mutant BMSCs. After 16 h in the presence of db-cAMP, there was a threefold stimulation in wild-type strains, but again, no stimulation in mutant strains. Under these conditions, the amount of IL-6 produced by db-cAMP stimulated, wild-type BMSCs essentially equated the amount produced by either untreated or db-cAMP-treated mutant strains (Fig. 6A). Using the two time points that were harvested, the rate of IL-6 secretion exhibited by wild-type and mutant strains on a per cell basis was also calculated ($T_{16\ h} - T_{2\ h}$/14 h) (Fig. 6B). When the data were analyzed in this fashion, the basal rate of IL-6 secretion by mutant strains was threefold higher than that of the wild-type strains. When the rate of secretion was measured after treatment with db-cAMP, the rate was threefold higher in wild-type strains and equivalent to the rate in mutant strains. This was in sharp contrast to the fact that the rate of IL-6 secretion in mutant strains, like the total amount of IL-6 produced, was unaffected by db-cAMP treatment. In addition to stimulating IL-6 secretion by wild-type cells, treatment with db-cAMP affected their morphology, inducing a shape change from fibroblastic to retracted and stellate. Interestingly, the same cell shape was spontaneously exhibited by mutant strains under serum-free conditions, regardless of stimulation with db-cAMP (Fig. 6C), suggesting its dependence upon endogenously high levels of cAMP caused by the *GNAS1* mutation.

Discussion

A pathogenetic role for IL-6 in FD has been inferred from in vitro studies suggesting that enhanced production of IL-6 within FD lesions would be conducive for enhanced

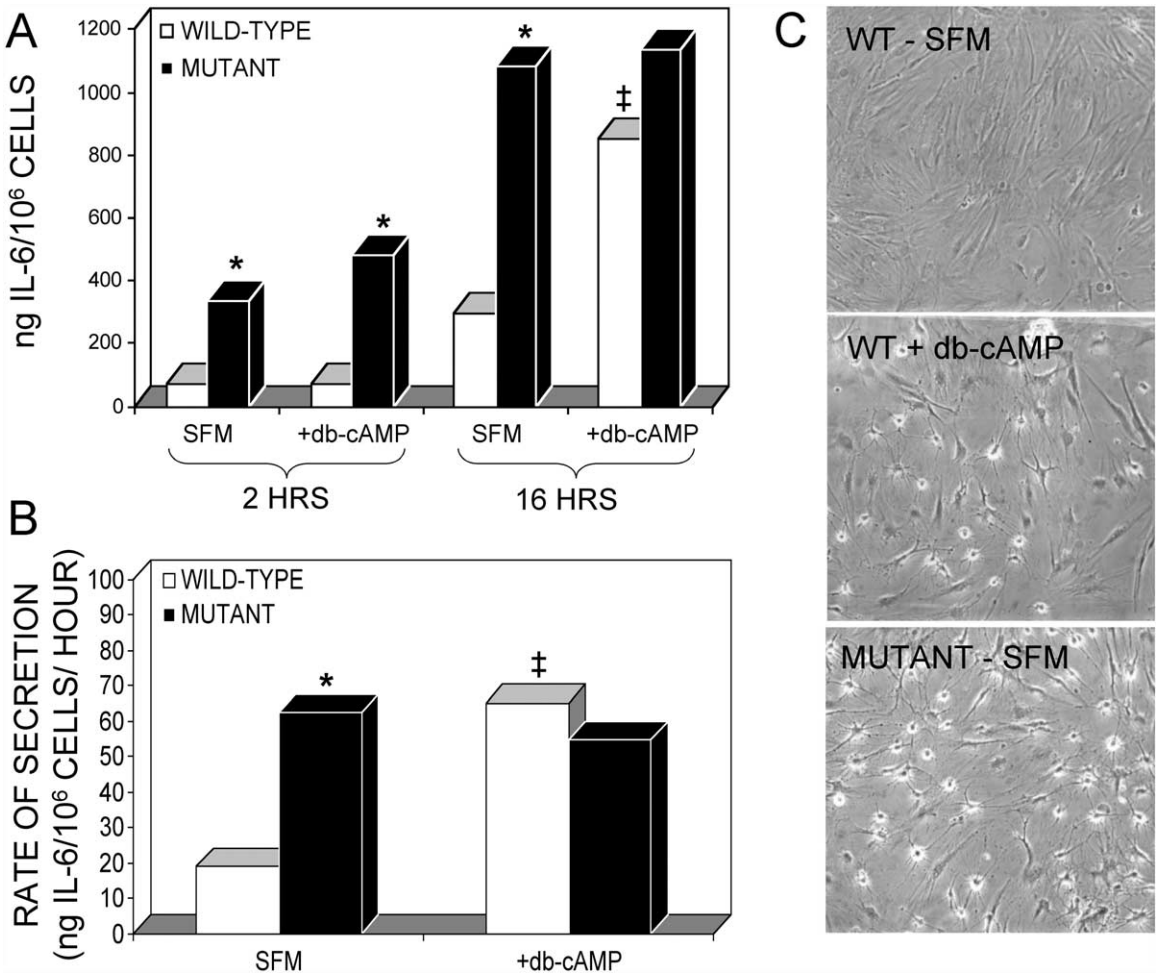

Fig. 6. Effect of db-cAMP on IL-6 production and morphology in wild-type and mutant BMSCs. (A) In serum-free medium (SFM), the basal level of IL-6 secretion was threefold higher in mutant cells compared to wild-type cells (* $p < 0.05$). Treatment with db-cAMP for 16 h increased IL-6 secretion threefold in wild-type cells compared to untreated cells (‡ $p < 0.05$), but had no effect on mutant cells. There was no statistically significant difference in the amount of IL-6 produced by db-cAMP-stimulated wild-type cells and unstimulated mutant cells. (B) The rate of IL-6 secretion was determined per hour ($T_{16\ h} - T_{2\ h}$/14 h). In SFM, the rate of secretion by mutant cells was threefold higher than in wild-type cells (* $p < 0.05$). When treated with db-cAMP, the rate was threefold higher in wild-type cells compared to untreated wild-type cells (‡ $p < 0.05$) and was not statistically different from the rate of mutant cells, which was unaltered by db-cAMP. All values are the mean of triplicate samples. (C) Untreated wild-type cells (WT) exhibited a fibroblastic morphology but underwent a marked shape change (retraction) upon treatment with db-cAMP in SFM. Mutant cells underwent the same type of shape change in SFM, regardless of stimulation with db-cAMP.

osteoclastogenesis and bone resorption, which is often thought of as a characteristic feature of FD. In previous studies, either mixed populations of cells released by collagenase [6] or nonclonal populations of stromal cells [18] were established in culture from FD tissues. Neither approach allows direct evaluation of the impact of *GNAS1* mutations on specific cell types of the bone/bone marrow microenvironment. FD lesions are composed of variable proportions of mutated and nonmutated cells of osteogenic lineage [19], and culture systems employing these two methods result not only in variable proportions of more or less differentiated osteogenic cells (collagenase release), but also in variable proportions of mutated cells (nonclonal stromal cell cultures). These circumstances are reflected in the variable results obtained by measuring IL-6 production. In a previous study, IL-6 production was not higher in stromal cell cultures from certain patients compared to normal controls [18].

In an attempt to overcome these limitations and to derive a better insight into whether and how IL-6 production represents a direct downstream effect of *GNAS1* mutation in osteogenic cells on the one hand and a direct effector of osteoclastogenesis in FD on the other, we looked at the specific cell types producing IL-6 in FD lesions by in situ hybridization and analyzed the production of IL-6 in vitro by purified populations of wild-type and *GNAS1*-mutated stromal cells.

Preliminary to the in situ hybridization analysis, we surveyed the pattern of osteoclastogenesis within FD tissue as revealed by TRAP cytochemistry. This disclosed an unusual abundance of osteoclasts and their mononuclear precursors within the fibrous tissue proper, away from bone surfaces.

At variance with normal trabecular bone, where late stages of osteoclastogenesis detected by TRAP cytochemistry only occur on trabecular surfaces, osteoclastogenesis is not spatially restricted to bone surfaces in FD. Hence, an osteoclastogenic milieu must be generated and maintained throughout the FD tissue. Consistent with this hypothesis, IL-6 mRNA was found to be expressed both in cells residing on trabecular surfaces and across the "fibroblastic" cell population filling the space between FD trabeculae.

In both locations, osteogenic and stromal cells expressing IL-6 were noted for a peculiar retracted/stellate shape, previously characterized as a typical feature of osteogenic cells in FD tissue in vivo and of osteogenic cells exposed to excess cAMP in vitro [4]. Interestingly, mononuclear osteoclast precursors populating the fibrous tissue were in close physical association with cell processes of retracted/stellate stromal cells, and cells with this morphology were noted for abundant expression of IL-6 in vivo. In vitro, exposure of wild-type stromal cells to db-cAMP resulted in a comparable shape change, which occurred in mutant cells upon simple withdrawal of serum, with no need for db-cAMP addition. Taken together, these observations indicate that osteoclastogenesis in FD occurs in physical association with stromal cells expressing IL-6 and bearing a morphological hallmark of excess cAMP production.

Our in vitro data conclusively establish a link between *GNAS1*-mutated osteogenic cells and excess IL-6 production in FD, which was surmised, but not proven, based on the results of previous studies. We have previously shown that FD lesions are somatic mosaics themselves, hence the actual production of IL-6 by mutated cells proper can only by probed by using pure populations of mutant cells. Capitalizing on the clonogenic property of stromal cells, we were able to generate strains of wild-type and mutant cells from the same individual FD lesions. This enabled us to show that the magnitude and rate of IL-6 production by mutated stromal cells was three to fivefold higher than in wild-type stromal cells derived from the same parent lesion. Conversely, wild-type stromal cells responded to db-cAMP stimulation with a threefold increase in magnitude and rate of IL-6 production, whereas mutant cells remained essentially unresponsive.

Our results thus indicate that IL-6 can be produced at levels and rates similar to those of *GNAS1*-mutated cells, by nonmutated cells upon treatment with db-cAMP. When translated into an in vivo scenario, these observations predict that significant variation in local IL-6 production may occur in FD tissue as a sole reflection of the local load of mutated cells, which is known to vary in individual cases, lesions, and specific sites [19]. A high local load of mutated cells predicts high basal levels of IL-6 production and with virtually no change upon hormonal stimulation. Lower proportions of mutated cells, in contrast, predict the contribution of IL-6 derived from normal cells in response to appropriate stimuli, such as PTH, which is known to stimulate IL-6 expression [14,22]. We have recently shown that osteoclast numbers in FD tissue do in fact correlate with serum PTH levels [7]. Thus, although high levels of constitutive IL-6 production appears to be an inherent property of *GNAS1*-mutated stromal cells, the pathophysiological significance of IL-6 with respect to excess bone resorption in FD is modulated by additional determinants, including the degree of mosaicism in a given lesion and at a given microanatomical site within a lesion, and systemic hormonal determinants. Although a mechanistic role for IL-6 in osteoclastogenesis is well established and has been shown for specific pathophysiological circumstances, such as estrogen withdrawal and Paget's disease [11,12,23,24], a direct demonstration of a similar role in FD has been missing. Although we have provided the first direct evidence of physical association of IL-6-expressing cells and maturing osteoclasts within FD, evidence for a dominant role of IL-6 in osteoclastogenesis in FD remains circumstantial. Gaining more direct evidence would require the development of appropriate in vivo models that would reproduce the complexity of the in vivo scenario highlighted by our data. Neither the specific variations in the degree of mosaicism nor the interplay of local and systemic determinants could be easily modeled by current in vitro approaches.

Interestingly, we also observed very high levels of IL-6 mRNA signal in a subset of osteoclasts within FD tissue, indicating that production of IL-6 in FD is neither specifically associated with stromal cells nor with cells in the osteogenic lineage at large, and that at least some osteoclasts participate in creating an osteoclastogenic cytokine milieu. The fact that only about 50% of the osteoclasts express IL-6 mRNA in FD may be seen as an indication of their functional heterogeneity, rooted perhaps in different activation states, associated or unassociated with IL-6 production, respectively. It also could be that a number of osteoclasts are themselves mutant. Mutation has been demonstrated in blood samples from some patients [6,25], suggesting that mutated hematopoietic cells could give rise to mutated osteoclasts. Since osteoclasts also highly express Gs-alpha [4], mutation in these cells may also induce high levels of IL-6 expression. Variable expression of IL-6 in osteoclasts proper has been observed in a variety of skeletal disorders ranging from Paget's disease [23,26] to renal osteodystrophy [27] to giant cell tumors of bone [28,29], and it appears to be significantly higher in osteoclasts within diseased bone than in normal osteoclasts. It has been suggested that osteoclast-derived IL-6 may act as an autocrine/paracrine regulator of osteoclastogenesis, triggering a self-perpetuating loop upon initiation of a resorption phase in certain bone diseases [28]. In the light of this, the finding of large ectopic clusters of multinuclear osteoclasts that we observed within the fibrous component of FD tissue and away from bone surfaces may represent the expression of uncontrolled osteoclastogenesis, but also the morphological remnants of "burned out" bone trabeculae that underwent unarrested, complete resorption instead of a remodeling cycle. In either case, they represent the morphological expression of osteoclastogenic events occurring outside of normal homeostatic control and conventional patterns of

bone remodeling and are a previously unrecognized histological expression of Gs-alpha mutation in the bone microenvironment.

In summary, our in vitro and in vivo analysis of IL-6 expression in FD establishes the notion that mutant stromal cells produce IL-6 constitutively and at the maximal rate, whereas normal stromal cells comprised in an FD lesion may produce IL-6 at comparable levels when triggered by cAMP-mediated stimuli. In vivo, production of IL-6 by normal and mutated stromal cells is inscribed into a complex scenario, in which osteoclasts themselves may participate in regulating osteoclastogenesis by producing IL-6, and local ratios of normal and mutated cells and hormonal stimuli contribute to the final net osteoclastogenic stimulus.

Acknowledgments

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