Quantitative Cytochemical Evidence for Local Increases in Bone Turnover at the Acromial Enthesis of the Human Coracoacromial Ligament

ANDREW CHAMBLER, SIMON RAWLINSON, ROGER EMERY, and ANDREW PITSILLIDES

ABSTRACT. ***Objective.*** Enthesophytic bone outgrowths are found at many ligament attachment sites, and while their incidence is associated with many pathologies, the mechanism by which they form remains controversial. We hypothesized that changes in local cell behavior, provoked by mechanical alterations within the coracoacromial ligament (CAL), lead to acromial enthesophyte formation. We investigated whether cell behavior at acromial entheses is consistent with this.

Methods. We used quantitative enzyme cytochemistry to measure glucose 6-phosphate dehydrogenase (G6PD), alkaline phosphatase (ALP; osteoblastic activity), and tartrate-resistant acid phosphatase (TRAP; osteoclastic phenotype) activities in cells of the acromial attachment into the CAL in patients with rotator cuff tears.

Results. (1) Resident osteoblasts on the acromion's inferior aspect express elevated activity of G6PD and ALP, indicative of increases in osteogenic potential. (2) These activities are selectively raised at the "leading edge" of acromial bone CAL enthesis. (3) In contrast, distribution of TRAP-positive cells does not exhibit a spatial correlation with enthesis architecture. We also found that cells situated close to the CAL attachment into the acromion exhibited elevated levels of G6PD and ALP activity, but intriguingly, also showed higher TRAP activity than neighboring cells distant from entheses.

Conclusion. These results suggest that the acromion in these patients undergoes bone accretion at the inferior attachment of the CAL, and that enthesial ligament cells close to the bone express characteristics consistent with enthesophyte formation at the leading edge of this bony spur's extension into the ligament. (J Rheumatol 2004;31:2216–25)

Key Indexing Terms:
LIGAMENT ENTHESIS OSTEOBLAST
ALKALINE PHOSPHATASE CYTOCHEMISTRY
GLUCOS -6-PHOSPHATE DEHYDROGENASE

Enthesophytic bone outgrowths are found at many sites of ligament attachment and are a common feature of bone architecture[1]. It is well recognized that their incidence is associated with many forms of skeletal pathology; however, there remains much controversy regarding the mechanism by which they form. It has been proposed that enthesophytes at the acromial end of the coracoacromial ligament (CAL) may be either genetically determined or an acquired phenomenon[2,3]. Yet since the local environment is likely to contribute to the behavior of cells in entheses, it is also pertinent that the control of bone remodeling at such sites remains the subject of speculation. Thus, despite an understanding of the material and structural properties of these entheses, the specific characteristics expressed by resident cells and their role, if any, in local matrix remodeling is relatively undefined.

The human shoulder provides an example in which an enthesophyte is associated with a particular form of clinical pathology[4]. The coracoacromial arch is a unique environment in which the CAL spans 2 processes of the same bone, and the acromial attachment of the CAL is a well established site where enthesophytes are found. It has been proposed that the presence of such bony spurs is closely associated with rotator cuff tendon tears[5]. However, there is debate as to the etiology of these spurs and their precise role in impingement and rotator cuff disease. Indeed, Bigliani, *et al*, described a correlation between particular acromial morphology and the occurrence of rotator cuff tears, and suggested that this supports "Neer's theory" for an extrinsic cause of rotator cuff tears[6]. Ultrastructural analyses of the coracoacromial arch have, however, shown that changes in the CAL are unlikely to be a primary initiating factor during impingement and tearing of the rotator cuff tendon[7,8], sup-

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porting an alternative "intrinsic" etiology of cuff tears[3]. It is clear that the pathogenesis and pathophysiology of this syndrome are enigmatic.

There are many opinions regarding the function of the CAL, including: a putative role as a counterbalance, acting against the scapular muscle insertions; a tertiary restraint upon glenohumeral superior migration; or even a rudimentary vestigial structure with some undefined alternative function in tetrapods. Regardless, it is clear that the CAL is likely to serve a mechanical role during shoulder movement or for stabilization. Indeed, if local mechanics contribute to enthesophyte development, consideration of these mechanical roles of the CAL is likely to provide an etiological basis for understanding their morphology. Recent evidence suggests that the CAL exists in a state of constant tensile strain. Further, studies using cadaver specimens have shown that the CAL is forced upwards during impinging movements of the shoulder, adding a dynamic component to this tension experienced by the CAL attachment[10]. Lesions apparently produced by local attrition on the undersurface of the coracoacromial arch are seen in patients with rotator cuff tears[11], and this may also cause an alteration in mechanical tension engendered by the ligament at its acromial enthesis.

Adaptive changes in the structural properties of both bone and ligament in response to alterations in the mechanical environment are well documented. Investigations by Lanyon and Rubin[12] using an avian model have shown that dynamic loading of bone leads to enhanced bone formation, whereas Goodship, *et al*[13] showed ossification of a ligament with changes in its static tension. It is possible that bursal inflammation in the subacromial space, cuff tendonitis, or tear may influence static and dynamic load applied to the coracoacromial arch during shoulder movements. We hypothesized that alterations in tension/compression (static or dynamic) engendered within the coracoacromial arch, and particularly increases in tension along the length of the CAL, will provoke changes in local cell behavior that lead to formation of acromial enthesophytes.

To establish whether the behavior of cells at the acromial enthesis is spatially consistent with this hypothesis and with the proposed secondary development of the acromial spur, we employed quantitative enzyme cytochemical techniques to examine the activity and distribution of selected enzymes within cells in both sagittal and coronal planes of the acromial spur attachment site into the CAL in patients with rotator cuff tears. For this purpose we measured glucose 6-phosphate dehydrogenase activity (G6PD; pentose phosphate pathway), alkaline phosphatase activity, (ALP; osteoblastic activity) and tartrate-resistant acid phosphatase activity (TRAP; osteoclastic phenotype) in individual bone and ligament cells in sections of the enthesis using quantitative cytochemical enzyme assays[14]. This would provide an assessment of the spatial distribution and the level of activity of these selected marker enzymes within individual cells at defined histological locations within the CAL/acromial enthesis.

MATERIALS AND METHODS

Patients. Acromial samples were taken from 15 consecutive patients undergoing modified open acromioplasty and rotator cuff repair. All had medium-grade cuff tears, and radiographs showed no upward translation of the humerus. Patients' mean age was 62.2 ± 1.75 years, with a male to female ratio of 11:4. Ethical committee approval was obtained before the study and informed consent was given preoperatively for collection of suitable acromial samples. No patient refused to participate in this study.

Sample collection and preparation. The CAL was identified on the anterior and inferior surface of the acromion extending to the coracoid process. The ligament was released near its coracoid attachment, leaving the acromial enthesis intact, and a "Neer" acromioplasty was performed using an oscillating saw. This sample contained the anterior part of the acromion, the spur, and the attachment of the remaining CAL. Positioning of suture material and markings made by a marker pen on the CAL *in situ* were used to facilitate sample orientation (Figure 1). Unwanted soft tissue was removed and the sample immediately immersed for 1–3 min in aqueous 5% solution of polyvinyl alcohol (GO4/140). The sample was then snap-chilled by precipitate immersion in *n*-hexane (BDH: low in aromatic hydrocarbons) at –70°C, placed in precooled containers, and stored at –70°C for up to 14 days[14,16]. Subsequently, triplicate 10 µm sections were cut using a tungsten-tipped knife on a heavy-duty microtome in a Bright's cryostat, with a cabinet temperature of –25°C to –35°C.

To allow comparison between the superior and inferior surfaces of the ligamentous enthesis, 15 specimens were mounted separately in an orientation that would allow lateromedial sections in the sagittal plane to be collected. In 5 cases, anterior to posterior sectioning in the coronal plane allowed comparison between the "tip" of the bony sample and its descending surfaces on either side. The positioning of suture material and markings (see above) allowed the site from which sections were collected to be accurately determined.

Assessment of G6PD activity. To assay G6PD activity, fresh unfixed undecalcified sagittal and coronal 10 µm sections of the "acromial spur" insertion were incubated in medium containing 3.3 mM glucose 6-phosphate (disodium salt), 3 mM NADP in 40% polypep (5115) in 0.05 M glycylglycine buffer, 3.0 mM nitro blue tetrazolium, pH 8.0, 37°C. Just before use, medium was deoxygenated by bubbling with humidified nitrogen, and 0.7 mM phenazine methosulfate, an intermediate hydrogen acceptor, was added. Perspex rings were placed around the sections to retain the reaction medium and sections incubated at 37°C for 20 min. Control sections were incubated in reaction medium from which NADP was omitted. After the reaction, sections were washed in water, allowed to dry, and mounted in Aquamount[16,17].

Assessment of ALP activity. To assay ALP activity, the reaction medium consisted of 2% barbitone-sodium, 0.16 mM α-naphthyl acid phosphate, and 10% magnesium chloride adjusted to pH 9.2 at 37°C. The coupler, 1.0 mg/ml Fast Blue RR, was stirred into the medium and filtered immediately into Coplin jars containing the slides. Control sections were reacted in medium lacking α-naphthyl acid phosphate. At the end of the reaction time (4 min) the slides were washed in distilled water and placed in 1% acetic acid for 1 min, then slides were rinsed in distilled water, allowed to dry, and mounted in Aquamount[18,19].

Assessment of tartrate-resistant acid phosphatase. TRAP activity was assayed using medium containing 1.4 mg/ml naphthol AS-TR phosphate, 5 µl/ml N-N-dimethylformamide, 0.1 M sodium acetate, and sodium tartrate adjusted to pH 5.2 with acetic acid. Fast Red TR (1.4 mg/ml) was added and the medium was filtered immediately into jars containing the slides. Reaction specificity was confirmed by incubating sections in medium containing 0.5 mM levamisole (Sigma Chemical, Dorset, UK). After reaction (5 min), slides were washed in distilled water, allowed to dry, and mounted in Aquamount[20].

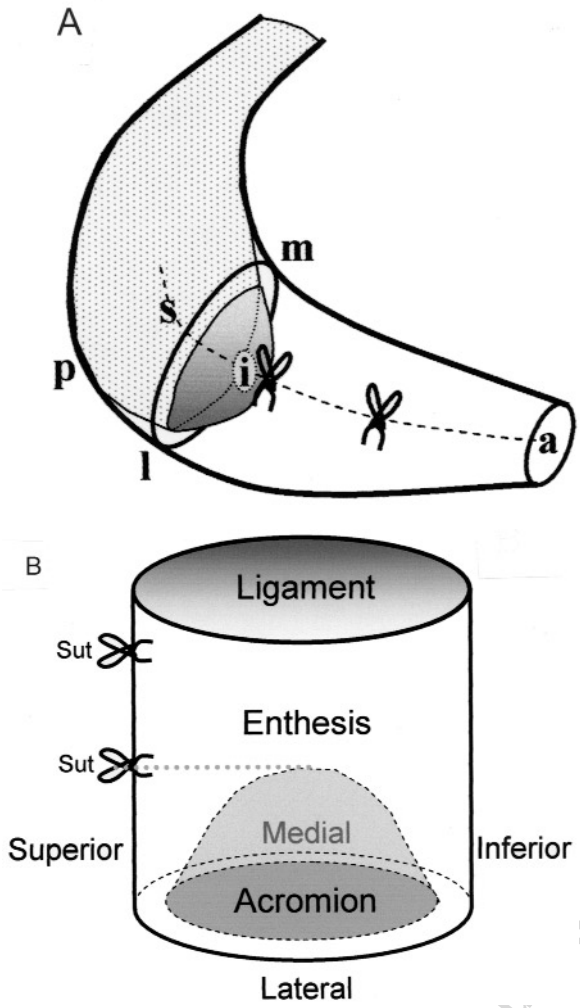

Figure 1. The acromial:coracoacromial ligament (CAL) attachment. A. Viewed from the superior (s) aspect to show the acromion (shaded), its bony spur (grey) extension into the CAL enthesis, its orientation [posterior (p), anterior (a), lateral (l), medial (m), and inferior (i, grey fill to show its "deep" position) aspects], and the position along the superior aspect of a pair of sutures used for orientation. B. Simplified diagram viewed from the lateral aspect showing position of the sutures for orientation and for localizing the extreme projection of the bony enthesis into the ligament (the tip).

Microdensitometry. The amount of reaction product, a stoichiometric measure of G6PD (rate-limiting in the pentose phosphate pathway), ALP (osteoblast marker), and TRAP (osteoclast marker) activity per cell, was measured in bone cells in histologically defined zones in trabecular bone of entheses or on their outermost junction with the ligament, and in enthesial ligament cells. This was achieved using a Vickers M85A scanning and integrating microdensitometer, which measures optical density of a colored product in a defined area of material, such as an individual cell[21]. Monochromatic light (λ = 585 nm for G6PD and ALP, and 550 nm for TRAP activity) is used to scan an area enclosed within a masked field. In practice, a ×40 objective and a scanning spot 0.4 µm in diameter in the plane of the section was used. Values were calculated as mean integrated extinction (MIE) per cell in at least 10 cells per section, and 5 sections per patient were used for each area in each sample. Thus, the corresponding values of enzyme activity per cell within each location for each patient represented an average of at least 50 cells[22].

RESULTS

Glucose-6-phosphate dehydrogenase activity. Acromial entheses have been described as having one of several distinct morphologies[23]. Our hypothesis would predict that these are, at least partly, acquired by mechanisms dependent on the local mechanical milieu and that the activity of G6PD in resident cells should exhibit a specific pattern of inter-site variation related to changes in local bone architecture. Examination of G6PD activity in enthesial osteoblasts and adjacent ligament cells showed that osteoblasts on both trabecular bone surfaces within the enthesis and on the enthesophytic surface at the bone-ligament junction contained significant levels of G6PD activity. However, a small fraction of cells within the ligament itself also expressed significant G6PD activity, and these were only present near the attachment site and not in the body of the ligament itself (Figure 2). Previous studies concluded that these cells are produced by local chondrocyte proliferation and they will be referred to as enthesial ligament cells (ELC) here.

Measurements of G6PD activity were therefore made both in the trabecular bone osteoblasts and in ELC in bone and ligament of the enthesophyte, respectively. These measurements revealed heterogeneity in the level of G6PD activity in both resident ELC and osteoblast cells in samples from different patients (Figure 3A). Further analysis disclosed that the level of G6PD activity in trabecular bone osteoblasts and ELC within each patient exhibited a significant correlation (Figure 3B; $r^2 = 0.92$ and p value < 0.0004).

To examine whether there were local differences in ELC and osteoblast activity on the surface of entheses in the sagittal plane, differences in G6PD activity in osteoblasts on its inferior and superior aspects were measured. The mean G6PD activity in osteoblasts on the inferior aspect of the tip of the acromial bone enthesis (mean ± SEM 0.92 ± 0.04; Figure 4A) was significantly higher ($p < 0.001$, paired t-test)

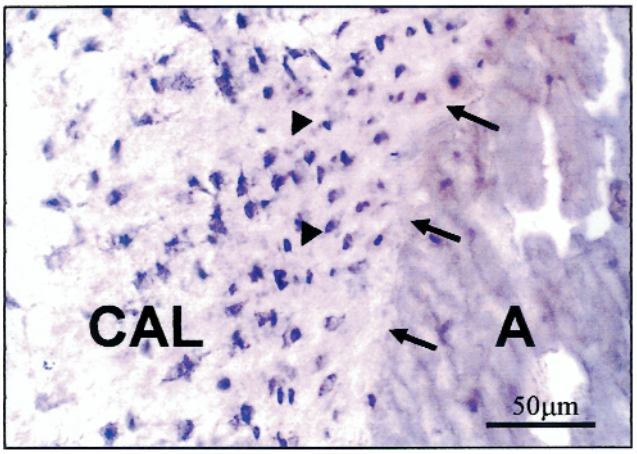

Figure 2. G6PD activity at the acromial attachment of the coracoacromial ligament (CAL). Photomicrograph shows section of the attachment of CAL into the acromion (A) reacted for G6PD activity, illustrating high levels of G6PD activity in enthesial ligament cells (arrowheads) close to the interface between the acromion and ligament (arrows).

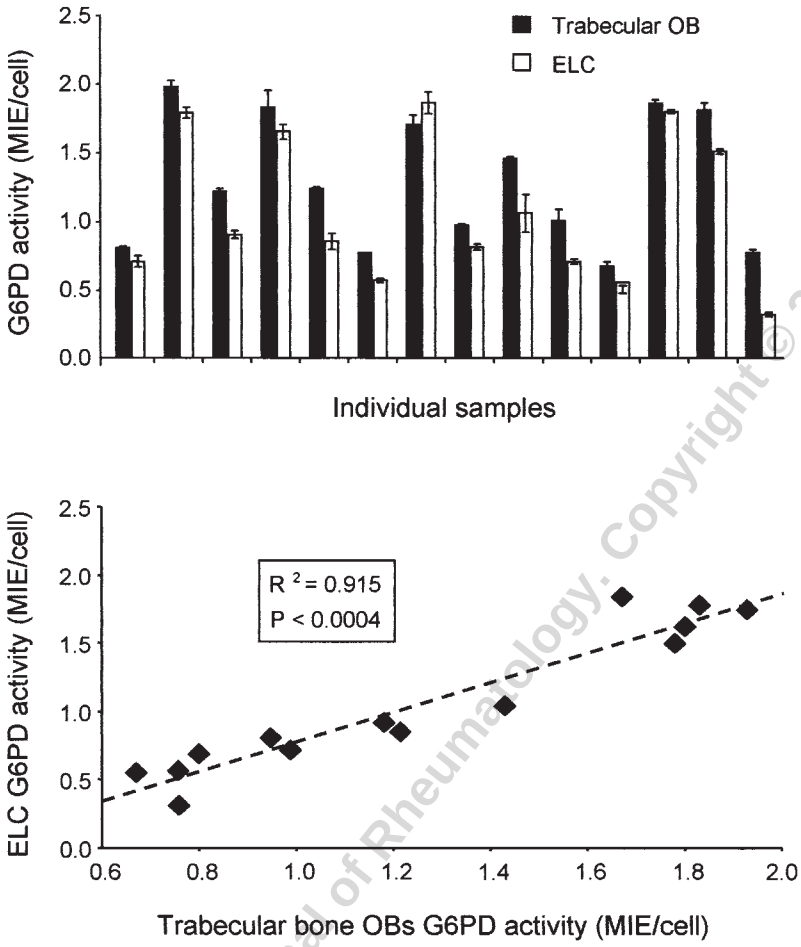

Figure 3. G6PD activity in trabecular bone osteoblasts (OB) and enthesial ligament cells (ELC) at the CAL-acromial enthesis. A. G6PD activity (MIE/cell) in these 2 cell types within individual patients with rotator cuff tears. B. Significant correlation ($p < 0.0004$, $R^2 = 0.915$) between the G6PD activities in these 2 cell types.

than on the superior aspect (0.80 ± 0.03; Figure 4A). Moreover, when the activity of these 2 sites (inferior and superior) was directly compared, this increased level of G6PD activity in osteoblastic cells on the inferior aspect of the acromion was also evident within any single patient (Figure 4A), and this was also reflected in a significant direct correlation between G6PD activity in resident cells on inferior and superior surfaces in each sample ($r^2 = 0.863$, $p < 0.001$; Figure 4B).

To determine whether osteoblasts and ELC displayed differential G6PD activities nearer the extreme projection of the bony enthesis into the ligament (the "tip") we also examined resident osteoblasts and ELC at the junction with the ligament, at 3 sites (pre-tip, post-tip, and tip), in coronal sections of the CAL enthesis from 5 patients. We found that in 4 of the 5 cases, G6PD activity/cell was higher in both periosteal osteoblasts and ELC at the most extreme projection (tip) than on either side (pre- and post-tip) of the acromial enthesis (Figures 5A, 5B).

Alkaline phosphatase activity. G6PD activity generates NADPH and ribose sugars and is directly linked with various biosynthetic processes, flow of calcium, vitamin K cycle activity and mineralization, and protein synthesis and cell proliferation[25]. It is therefore clear that an increase in G6PD activity in resident cells is not necessarily a direct measure of bone formation. ALP activity, on the other hand, is a robust marker of the osteoblast phenotype, and the level of ALP activity per cell has been directly correlated with local bone formation rate in human bone sections[19]. We therefore examined ALP activity in sections of CAL attachment, and found that ALP activity in resident bone cells on the inferior aspect was significantly higher than on the superior aspect of the acromion, with the mean ALP activity of 1.02 ± 0.03 on the inferior and 0.89 ± 0.04 on the superior aspects of the acromion ($p < 0.001$; Figure 6A). Further analysis showed that inter-site variability in osteoblast ALP

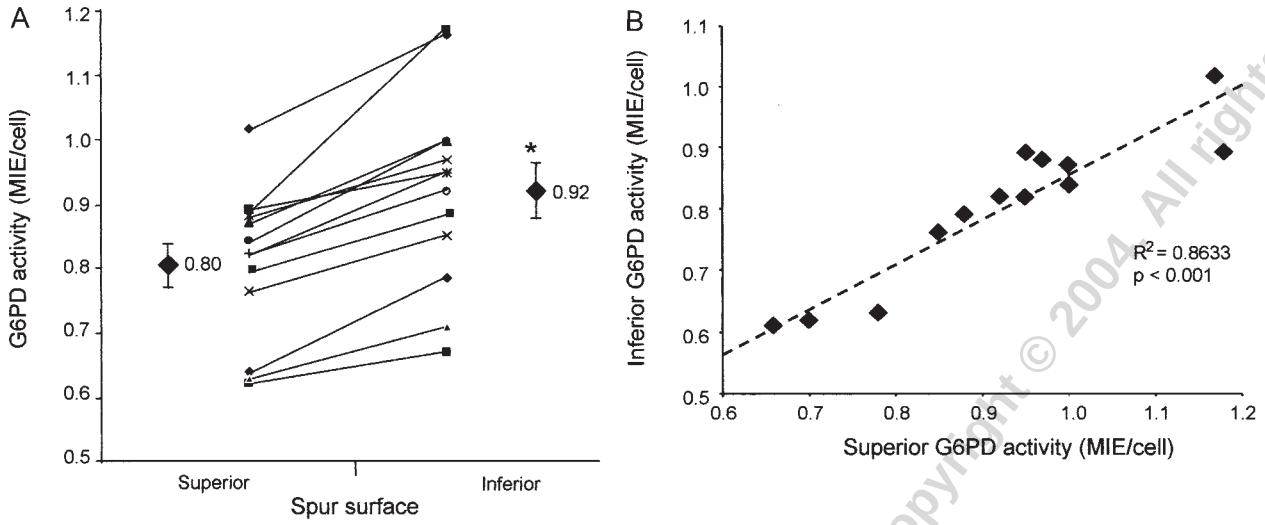

Figure 4. G6PD activity in osteoblasts at the inferior and superior surface of the acromial "spur" enthesis. A. There are increases in osteoblast G6PD activity (MIE/cell) at the inferior aspect of the enthesis in patients with rotator cuff tears. Black diamonds show mean ± SEM (*p < 0.001). B. Significant correlation (p < 0.001, R^2 = 0.863) between G6PD activity in bone cells at these 2 sites.

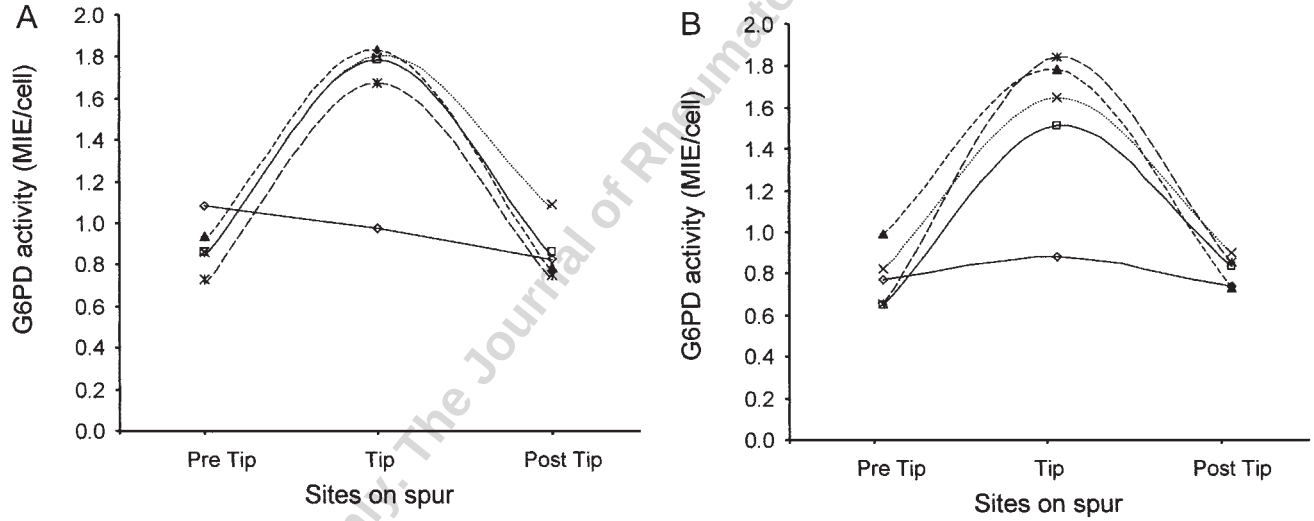

Figure 5. G6PD activity (MIE/cell) in trabecular bone osteoblasts (A) and enthesial ligament cells (B) at sites either side of (pre-tip and post-tip) and at the extreme projection of the bony enthesis into the ligament (tip) in coronal sections of the CAL enthesis from 5 patients with rotator cuff tears.

activity exhibited a direct and statistically significant correlation (r^2 = 0.856, p < 0.05; Figure 6B).

In all patients, ALP activity was also identified within the ELC, but not in cells within the CAL away from its acromial enthesis. In accord with our previous results, ALP activity was markedly elevated in ELC at the inferior compared to the superior surface of the enthesis. However, in marked contrast to the trends in periosteal osteoblasts, there was no correlation between the activities found in ELC at the inferior and the superior aspects within individual samples (r^2 = 0.064, NS; not shown). When the ALP activity within ELC and osteoblasts on corresponding acromial surfaces was compared, a direct correlation between ALP activities in these cells was seen on the inferior aspect, while in contrast no such correlation was evident on the enthesophyte superior surface (r^2 = 0.69, NS; not shown).

Our results show that G6PD activity is higher at the extreme projection of enthesial spur insertion into the ligament, suggesting local increases in biosynthetic potential in these cells; we therefore examined whether this was also associated with localized increases in ALP activity. We found that, unlike the association between increased G6PD and ALP activity in osteoblasts and ELC observed at the inferior aspect of the acromion, no such association was evident for osteoblast or ELC ALP activity at the extreme bony tip projection into the ligament. Similar activities were evi-

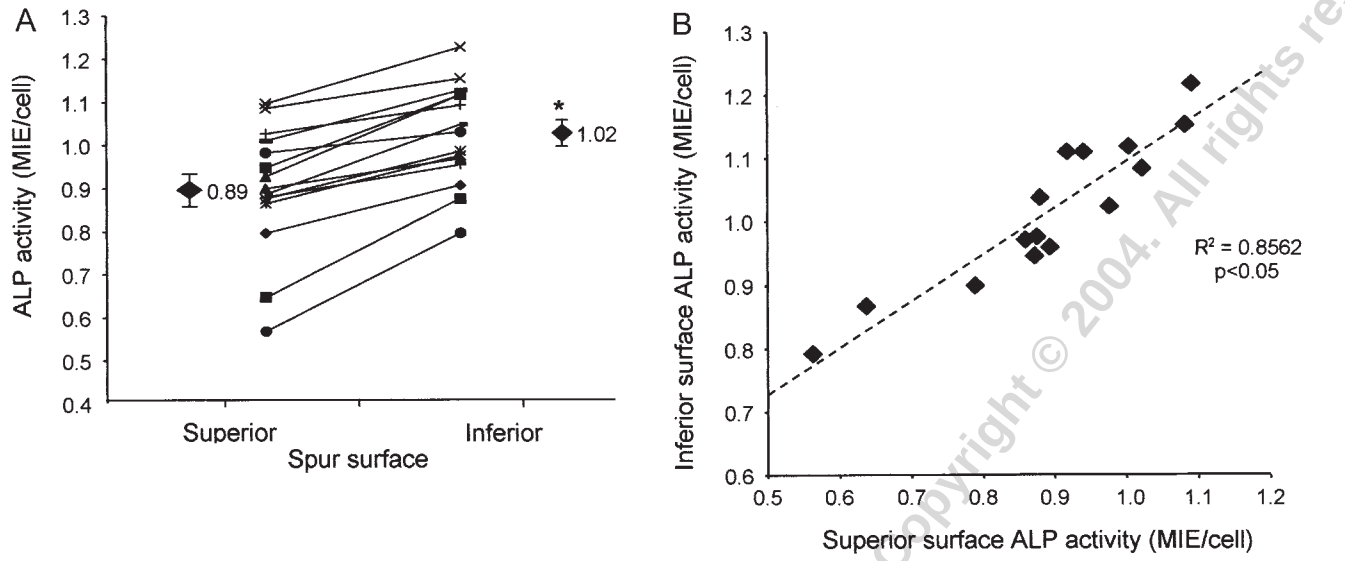

Figure 6. ALP activity in osteoblasts at the inferior and superior surface of the acromial "spur" enthesis. A. There is an increase in osteoblast ALP activity (MIE/cell) at the inferior aspect of the enthesis compared to the superior aspect in individual patients with rotator cuff tears (black diamonds show mean ± SEM; *$p < 0.001$). B. Significant correlation ($p < 0.05$, $R^2 = 0.856$) between ALP activity in bone cells at these 2 (inferior and superior) sites.

dent in the tip, pre-tip, and post-tip regions of coronal sections (Figure 7). Together, these results suggest that acromial periosteal osteoblasts and ligament cells (only ELC close to attachment) show greater bone-forming capacity on the inferior aspect of the enthesis. It remains possible, however, that these are sites of rapid bone turnover and that local indices of bone resorption might show similar distribution.

Tartrate-resistant acid phosphatase activity. TRAP activity is a marker of the bone-resorbing osteoclast[20]. We investigated patterns of TRAP activity to determine whether sites of localized increases in ALP and G6PD activity also exhibit changes in numbers of cells with TRAP activity. Assessment of TRAP-positive cell distribution did not show a consistent pattern, with marked heterogeneity evident throughout the sections. It was observed, however, that trabecular bone surfaces and not the enthesis-ligament junction most commonly contained TRAP-positive cells (Figure 8A).

Intriguingly, ELC also expressed high levels of TRAP activity (Figure 8B). This was an unexpected finding and it was noted that ELC found only near the attachment site, but not those in the body of the ligament, were positive for TRAP activity. In serial sections, similar mononuclear cells also possessed high levels of G6PD and ALP activity. Quantitative and spatial analysis was performed on 4 samples, in which we measured TRAP activity/cell in trabecular bone osteoclasts and ELC at the tip of the spur and on either side of this some distance from the tip (in the coronal plane). This showed that TRAP activity/cell was greater in resident trabecular bone osteoclasts than in ELC (Figure 8C), and that there was no evident spatial predominance in TRAP activity on the inferior or superior aspect of the bone of the enthesis (not shown).

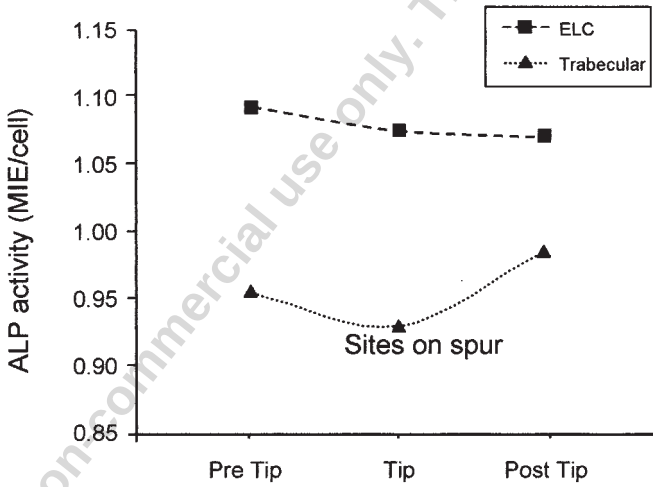

Figure 7. ALP activity (MIE/cell) in trabecular bone osteoblasts and enthesial ligament cells (ELC) at sites either side (pre-tip and post-tip) and at the extreme projection of the bony enthesis into the ligament (tip) in coronal sections from 5 patients with rotator cuff tears (mean ± SEM).

DISCUSSION

Our studies have shown site-selective elevations in G6PD activity in bone cells within and on periosteal surfaces of the inferior aspect of the acromial CAL enthesis, and that these are accompanied by higher levels of ALP activity, but not by differences in TRAP activity at these sites. We have also described the presence of cells in a location close to the attachment of the ligament into the acromion that exhibit high levels of G6PD and ALP activity, but which intriguingly also contain higher TRAP activity than neighboring

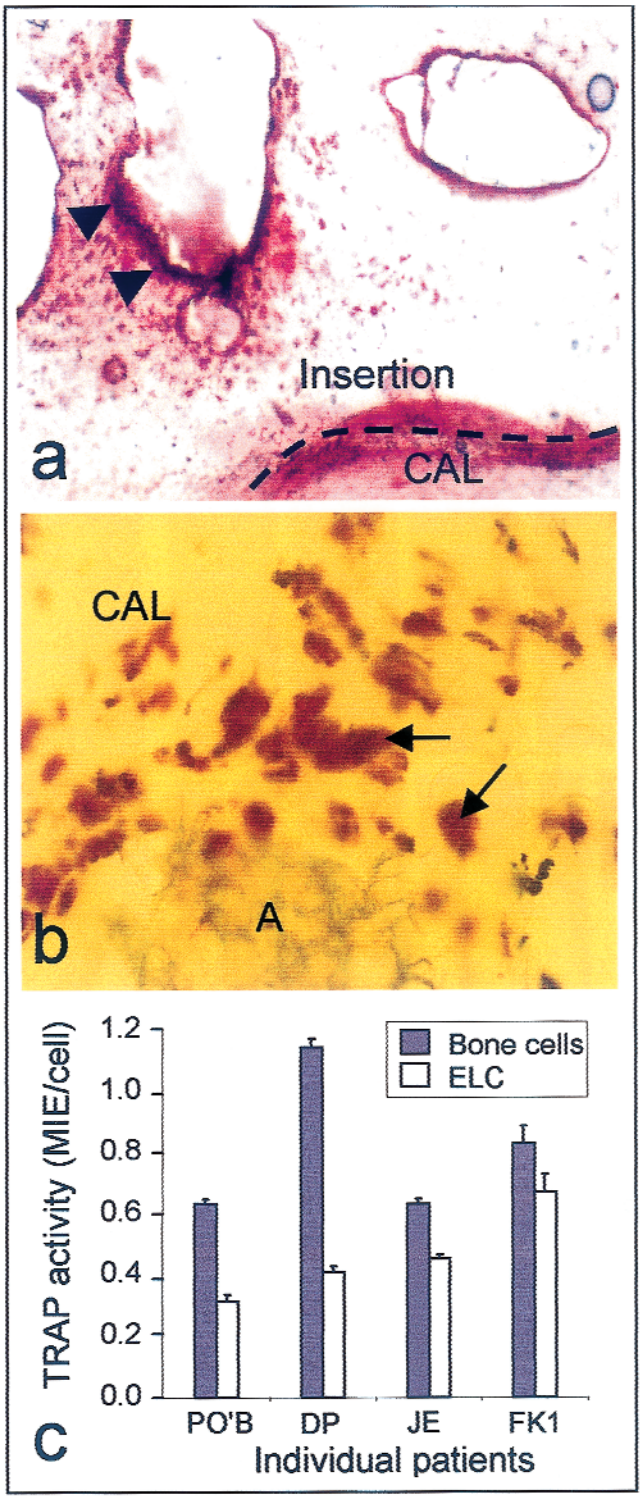

Figure 8. TRAP activity in cells within the acromial spur and in the acromial enthesis of human coracoacromial ligament (CAL). A. Photomicrograph of a section reacted for TRAP activity, showing high levels of TRAP activity (arrowheads) in osteoclasts at surfaces within the bone spur of the acromion close to the attachment (broken line) of the acromion with the CAL (see Figure 1). B. Higher magnification of a section reacted for TRAP activity, showing high levels of TRAP activity (arrows) in enthesial ligament cells close to the attachment of the CAL with the acromion (A; see Figure 1). C. TRAP activity in trabecular bone osteoclasts and enthesial ligament cells (ELC) at the extreme projection of the bony acromial enthesis into the CAL (tip) in individual patients with rotator cuff tears, showing consistently higher TRAP activity (MIE/cell) in cells within the acromial spur compared to ELC (*$p < 0.05$).

cells distant from the enthesis. These results agree with the notion that the acromion in these patients undergoes bone accretion at the inferior attachment of the CAL and that enthesial ligament cells close to the bone express characteristics that support a role in local remodeling.

It has recently been emphasized that the obvious role of the extracellular matrix in determining the physical properties of ligament entheses has for a long time meant that the importance of the resident cells is commonly overlooked[1]. Studies contributing to this view have speculated that an elaborate 3-dimensional network of ligament cell processes connected via gap junctions creates a mechano-sensory system that, much like that proposed for bone osteocytes, may regulate ligament tissue remodeling[26,27]. Ultrastructural analyses of the CAL from patients with chronic impingement found increased cytoplasmic organelle content in cells at attachment sites, indicative of raised metabolic activity[8]. These authors concluded that such changes were an acquired product of chronic stress placed upon the ligament by increased subacromial soft tissue volume as well as attrition of some of its fibers, particularly at its insertion[8]. It is possible that changes in local strain patterns in the ligament could produce such increases in "cell activity." Our studies provide the first direct evidence regarding specific changes in cell biochemistry and function that might support this.

In cadavers, the application of distraction forces (30 kg) is capable of producing a separation (15 mm) between the acromion and coracoid processes[28]. Although the reason for this is unclear, it appears that the CAL is therefore under continuous tension between these 2 processes and experiences corresponding compression across its width. The existence of an inherent tension in the CAL is supported by studies showing (1) that forces between 50 and 100 N through the scapular muscles produced greater acromial distortion after the CAL was cut, (2) that it is surprisingly difficult to reattach the CAL after acromioplasty and, (3) that there is direct arthroscopic observation supporting changes in CAL length during impingement[29-31]. Thus, although CAL function remains enigmatic, it nonetheless retains an intimate involvement with shoulder movement[10]. We hypothesize that changes in inherent CAL tension induce an osteogenic adaptive response at the acromial insertion. Evidence that CAL from patients with rotator cuff tear are shorter, with lower elastic modulus than normal, and that the CAL exhibits constant tension, provides further support for this hypothesis[9,32].

Our analyses providing a quantitative measure of activity per cell can be directly related to tissue histology[21]. G6PD is rate-limiting in the pentose-phosphate shunt, the significance of which is best evaluated by looking more closely at the products of the pathway[25]. An important product is ribose, a constituent of coenzymes such as NAD and ATP and also of RNA nucleosides. The pathway also yields cytosolic NADPH that is vital for many biosynthetic mechanisms. The level of G6PD activity in a given cell may provide a direct indication of its biosynthetic capacity. We found that bone and ligament cells in the inferior aspect of the CAL attachment to the acromion show higher G6PD activity than those in the superior aspect, suggesting an elevated biosynthetic potential at this inferior aspect. It is important that this aspect is in contact with the subacromial space and that bursal thickening, which exists with impingement and rotator cuff tears, may increase pressure exerted against the coracoacromial arch[33]. Thus, G6PD activity may be increased in cells at this site in response to changes in the mechanical environment or may be related to the inflammatory processes occurring nearby. Indeed, studies have shown, for instance, that synovial lining cells exhibit increased G6PD activity in inflamed joints compared to noninflamed normal joints[16,34]. This does not, however, preclude the direct involvement of mechanical factors; the finding that cells at the tip of the acromial spur also have greater G6PD activity is likely to support the mechanical regulation of G6PD activity. This is further supported by finite element analysis of the femoral insertion of the medial collateral ligament, which describes local mechanical stress concentration in ligaments at sites closest to their bone attachment[35]. If a similar argument applies for the CAL enthesis, then it is clear that both osteoblasts and enthesial ligament cells experience increased G6PD activities close to those sites at which greatest mechanical stress would be predicted. This is consistent with findings in which osteoblasts in bones subjected to a short period of mechanical loading show elevated G6PD activities for at least 24 hours after loads were applied[18,36,37]. Prolonged increases in osteoblast G6PD activity may reflect the contribution these cells make to the elevated rates of bone formation that are induced by such loading. It is therefore tempting to speculate that the localized increases in G6PD activity in CAL entheses also support local osteogenesis.

The finding that ELC close to their attachment on the inferior aspect of the acromion express elevated G6PD activity levels is less easy to interpret. This is an entirely novel finding that may reflect normal patterns of behavior at such sites, and only examination of entheses from normal shoulders will address this possibility. Nonetheless, coordinated upregulation of G6PD and ALP activities in cells at the inferior aspect suggests a greater potential for local osteogenesis compared to cells in the body of the ligament. Association between ALP activity, mineralization, and increased bone turnover is well supported. ALP apparently has many roles in mineralization, including hydrolysis of organic phosphate esters to produce high local phosphate concentration; facilitating precipitation of calcium phosphate; destruction of physiological crystal growth inhibitors such as inorganic pyrophosphate and ATP through its hydrolase activity; and action as a phosphate transporter and active transport of Ca^{2+} or phosphate through its ATPase activity[38,39]. Indeed, ALP raises local phosphate ion concentration to initiate calcification. Moreover, the extracellular matrix-binding domain of ALP directs matrix vesicle migration along collagen fibers during mineralization[39]. It appears that increased ALP activity in individual acromial osteoblasts, ELC on the inferior acromial aspect, and osteoblasts close to the most prominent enthesial projection into the ligament (the tip) is an indicator of bone-forming capacity at this enthesis. Indeed, this possibility is supported by the studies of Bradbeer, *et al*, in which osteoblast ALP activity was found to be directly related to rates of local bone formation[19].

Unless the acromion's inferior aspect exhibits fundamentally distinct behavior from the superior aspect, it appears that the former is more likely to be actively involved in bone accretion. The CAL's acromial enthesis is known to undergo attrition by encroachment of the underlying structures (subacromial bursa and supraspinatus) and the resultant reduction in insertion area, without presumably any alteration in the distractive forces acting within the coracoacromial arch, may account for the observed differences in local ALP activity between the 2 surfaces. It is not clear, however, if the upregulation of G6PD and ALP activities at the enthesis reflects a positive bone formation balance, and to clarify this we also examined osteoclastic activity. It is known that osteoclasts resorb bone by creating a subcellular low pH compartment in which proteolytic enzymes such as lysosomal TRAP[20] facilitate degradation of the organic phase. TRAP activity therefore detects bone-resorbing activity of osteoclasts *in vivo*. The random distribution of TRAP-positive cells in the trabecular bone of the enthesis suggests that this is not spatially regulated in the same manner as G6PD and ALP activity. On the other hand, the elevated TRAP activity in ELC, specifically at its acromial spur insertion, was unexpected. The definitive characterization of these cells remains the subject of current investigations.

There are, however, limitations restricting the conclusions that can be made based on this study alone. One major limitation is that only samples from patients with rotator cuff tears were evaluated. This makes it difficult to discern whether our observations reflect a physiological process or are features that are secondary to the associated pathology. A lack of normal control cases due to the limitations concerning retention and use of post-mortem material in the UK has meant that we did not examine control samples. Thus it remains unclear whether these findings represent a physio-

logical or pathological process, or are indeed an epiphenomenon related to increases in stress from rotator cuff insufficiency, disuse due to pain, or some other factor related to tearing of the rotator cuff. Firmer conclusions regarding our findings will be possible when the biomechanics of the coracoacromial ligament in both normal and rotator cuff tear specimens is fully described and when similar studies have been performed in samples from normal shoulders.

We found (1) that resident bone cells on the inferior aspect of the acromion possess elevated levels of G6PD and ALP activities, both of which have previously been associated with localized increases in osteogenic potential; and (2) that these activities are also selectively raised at the "leading edge" of the bone in the acromial enthesis of the CAL. In addition, we found that cells of the ligament occupying sites close to the acromial enthesis also express similar patterns of G6PD and ALP activity, but that these cells surprisingly also express pronounced levels of TRAP reactivity. These results suggest that spatially localized osteogenic events in both bone cells and within cells in the region of the ligament closest to the bone, at these sites, promote acquisition of the acromial enthesophyte. It is tempting to suggest that cells within the ligament actively contribute to tissue remodeling at the leading edge of this bony spur's extension into the ligamentous enthesis.

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