The articular cartilage of the healthy hip serves to distribute forces across the femoral head and to function as a protective cushion to the underlying bone tissue. Loss of this articular layer in osteoarthritis (OA) may impair load distribution and/or intensity to the underlying structures, which may in turn modulate certain metabolic processes; mechanical loading plays a pivotal role in the maintenance and turnover of bone [1–3]. Since bone remodelling patterns determine both morphological and biochemical characteristics of whole joints, any compromises in the metabolic status of bone during the early stages of cartilage wear may exacerbate the disease process because of changes in certain loading patterns across the femoral head.

Subchondral bone changes have previously been thought to represent an important aetiological element in the pathogenesis of OA. The postulation by Radin et al. [4] that bone tissue is implicated in OA was supported by Seibel et al. [5] who reported elevated excretion of lysyl-pyridinoline, a bone collagen cross-link, in subjects with the disease. Recently, the technetium studies of Dieppe et al. [6] indicated heightened bone tissue activity, which, when coupled with the urine cross-link data [5], supports increased bone collagen catabolism in OA. Furthermore, animal models applied to the investigation of OA have shown morphological changes in the subchondral bone prior to articular cartilage manifestations [7–9]. A more extreme form of bone tissue change in OA is illustrated by the presence of cysts which clearly represent areas of unusual tissue activity and were initially recognized as an important characteristic of the disease by Plewes [10].

Despite these observations, no biochemical investigations of the subchondral bone in OA have been undertaken. In this study, we compared OA trabecular bone tissue from femoral heads with age-matched osteoporotic and healthy individuals for gelatinase A [matrix metalloproteinase (MMP-2)] and B (MMP-9) expression, an index of collagen catabolism, and local alkaline phosphatase expression which is a useful indicator of osteoblast activity and/or number.

**MATERIALS AND METHODS**

**Tissue preparation**

Femoral heads were collected from women undergoing hip replacement surgery at the Bristol Royal Infirmary for both OA ($n = 28$) or osteoporosis ($n = 20$). Normal femoral heads ($n = 6$, kindly provided by Dr Anthony Hollander, Sheffield University) were obtained during post-mortem. Prior to any treatment, all specimens were stored frozen at $−20°C$. Unless otherwise stated, bone tissue was prepared for enzyme extractions within 2 months of surgery. Using a band saw, all femoral heads were sliced through the centre to obtain a 2–3 mm section of tissue. Discs of cancellous bone were isolated using a punch and subsequently processed for enzyme extraction; specimens were snap frozen in liquid nitrogen and pulverized to a fine powder using a stainless steel mill which had been pre-cooled in liquid nitrogen. Powdered specimens (90–150 mg) were dispensed into polypropylene tubes (6 ml Sarstedt) and the enzymes extracted, by stirring, into 1.8 ml of 10 mM triethanolamine (pH 7.5) for 1.5 h at 4°C. This particular extraction procedure was chosen in light of the problems encountered by Eckhout et al. [11] during their extraction of collagenase from bone tissue; heating the material during the extraction had adverse effects on recovery as did the use of high-ionic-strength buffers.
Tubes were then subjected to centrifugation at 10,000 r.p.m. for 10 min and 1.3 ml of each supernatant was dispensed into pre-weighed microcentrifuge tubes, frozen at -80°C, and subsequently lyophilized. The time taken between tissue preparation and the freeze-drying of extractable material was ~4 h. Another aliquot of the extract was reserved for protein and alkaline phosphatase determinations.

Gelatin gel zymography
Freeze-dried extracts were reconstituted into one-tenth of the initial extracted volume using 50 mM CaCl₂, 500 mM NaCl, 50 mM Tris-HCl (pH 7.8) proteolysis buffer. An aliquot of this sample for gelatinase identification was diluted 10-fold in sample buffer as described by Laemmli [12], but containing twice the concentration of sodium dodecyl sulphate (SDS) and no reducing agent. Samples were then heated to 60°C for an hour and electrophoresed in 10% polyacrylamide (Bio-Rad) co-polymerized with 0.5 mg/ml gelatin (bovine skin, Sigma). Sample volumes were loaded in relation to initial tissue mass, with the greatest mass loaded as the least volume. A human MMP-2 standard (Biogenesis) was loaded on each gel to assist in gelatinase quantitation of tissue extracts. Gels were processed for MMP detection by a modified method described by Bailey et al. [13]. Briefly, gels were rinsed three times in 2.5% Triton X-100 over a period of 15 min, washed three times in distilled water and then bathed in 50 ml of proteolysis buffer supplemented with aminophenylmercuric acetate (APMA) at a final concentration of 200 µm and left to incubate at 37°C for 16 h. Following this incubation period, gels were rinsed three times in distilled water and subsequently stained and destained as for conventional SDS–PAGE [12].

Gels were scanned using an Agfa Studioscan II in transmittance mode using Fotolook SA 2.05 set on high resolution (> 200 pp.i.) in grey scale and operated from a Power Macintosh 7100. The images were analysed in NIH Image 1.55 in which absorbances are plotted as a response profile, allowing quantitation of the proteolytically clarified zones, according to their areas and intensities. The figure for each gelatinase zone was expressed as a proportion of the MMP-2 standard. Confirmation that the observed zones were attributable to MMP activity was achieved by incubating identical gels in buffer devoid of CaCl₂ and supplemented with 5 mM EDTA or 10 mM 1,10-phenanthroline. In each case, no proteolytically clarified zones were observed following this procedure. The identification of the MMPs as gelatinases was clarified zones were observed following this procedure.

Alkaline phosphatase and protein determinations
Alkaline phosphatase activities were determined in undiluted aliquots of tissue extracts. The substrate utilized was 4-nitrophenyl phosphate, the product of which (4-nitrophenol) was detected at 405 nm on a Kone specific autoanalyser. Protein in undiluted tissue extracts was analysed on a Cobas Bio (Roche) system and determined using Bradford reagent. Alkaline phosphatase activity from tissue extracts was expressed as units of enzyme activity per milligram of total protein within tissue extracts.

Statistical analysis
Statistical analysis was performed using Minitab Statistical Software (Version 10). The test of choice was an unpaired, two-sided, Student’s t-test, assuming unequal variance. Data had been shown to deviate from a normal distribution by the Ryan–Joiner test of normality. However, variances of the groups were shown to be heterogeneous (Levene’s test), consequently the non-parametric Mann–Whitney test was deemed inappropriate. Differences between groups were assumed to be statistically significant when P < 0.05.

RESULTS
Matrix metalloproteinases
Both pro (P = 0.0002) and active (P = 0.003) MMP-2 were significantly elevated in OA bone tissue when compared to osteoporotic (OP) specimens. In addition, OA samples exhibited increased levels of pro MMP-2 (P = 0.0001) when compared to normal tissue samples. No statistically significant differences were observed between normal and OP subjects for either form of these enzymes. The method of tissue extraction detailed here is both reliable and reproducible: when a number of bone samples were prepared for protein and enzyme extractions, the significant majority (> 85% for protein and gelatinases) of the analytes were extracted in the first step. In addition, duplicate specimens gave comparable extraction data, the observed differences for duplicate preparations were between 2 and 7% for protein extractions and <5% for gelatinase recoveries. Table I summarizes the data obtained for MMP-2 expression in trabecular bone tissue. Gelatinase B (MMP-9) was the major MMP species in bone, but quantification was hampered by the presence of material above and below the main zone. These regions may represent differentially glycosylated species together with aggregates and partially degraded forms [14]. Clarification was not obtained by lower sample loadings.

Alkaline phosphatase
OA bone tissue contained significantly elevated alkaline phosphatase compared to normal (P = 0.0001) and OP (P = 0.0007) samples. There was no statistically significant difference in the expression of the

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal bone</th>
<th>OA</th>
<th>OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro MMP-2</td>
<td>0.063 ± 0.007</td>
<td>0.575 ± 0.110</td>
<td>0.1 ± 0.029</td>
</tr>
<tr>
<td>Active MMP-2</td>
<td>0.055 ± 0.01</td>
<td>0.363 ± 0.069</td>
<td>0.102 ± 0.044</td>
</tr>
</tbody>
</table>

All data are expressed as the mean peak area corrected to the MMP-2 standard ± the s.e.
enzyme between OP and normal tissue. Table II summarizes the data obtained for the expression of alkaline phosphatase in trabecular bone tissue.

DISCUSSION

The expression of pro and active gelatinase A (MMP-2) has been shown to be significantly upregulated in the trabecular bone compartment in OA. This collagenase, in conjunction with MMP-9, has been shown to be instrumental in the resorption of bone tissue in vitro [15, 16]. The recent observation that MMP-2 is an interstitial collagenase, i.e. capable of cleaving native, helical type I collagen [17], further supports the role of MMP-2 in increased bone collagen degradation. Additional evidence for heightened bone collagen degradation in OA was demonstrated by increased excretion of lysyl-pyridinoline [5]. MMP-2 is known to be produced by mature osteoblast phenotypes in vitro [15, 16]. The recent observation that MMP-2 is profoundly affected by parathyroid hormone [19, 20]. Furthermore, preliminary in vitro studies from our laboratory have shown that MMP-2 is a consequence, or precedes, cartilage erosion clearly cannot be determined from these late-stage OA samples. A solution may only stem from studies involving animal models. However, what is clear is that bone is not incidental to the disease process, consequently a greater understanding of bone tissue involvement in OA is warranted. Whatever the significance of these changes, their occurrence in the latter stages of OA is likely to exacerbate the disease process.

**TABLE II**

Expression of alkaline phosphatase in trabecular bone tissue

<table>
<thead>
<tr>
<th>Normal bone</th>
<th>OA</th>
<th>OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.075 ± 0.012</td>
<td>0.179 ± 0.019</td>
<td>0.089 ± 0.015</td>
</tr>
</tbody>
</table>

All data are expressed as the mean units of enzyme activity per milligram of protein ± the s.e.

