Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific

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Abstract

Objectives—Matrix metalloproteinases (MMPs) are thought to be major mediators of cartilage destruction. Osteoarthritis (OA) is characterised by cartilage degradation. This study explores gene expression of three MMPs in articular chondrocytes during the histological development of the cartilage lesion of OA. **Methods**—Biopsy specimens of human normal and OA cartilage, classified into four grades on the basis of histology, were probed for MMPs 1, 3, and 9 using ³⁵S-labelled cDNA probes. The signal was measured at four different depths (zones) using an automated image analyser and compared with signal from sections probed with λ DNA. Rheumatoid synovium was used as a positive control for MMP gene expression.

Results—Rheumatoid tissue contained mRNA for all three MMPs. Expression in chondrocytes varied with the depth of the chondrocyte in the cartilage and the histomorphological extent of the OA changes. There was no detectable mRNA signal for these three MMPs in normal cartilage. In general, in OA, MMP-1 gene expression was greatest in the superficial cartilage in established disease. By contrast mRNAs for MMP-3 and 9 were expressed deeper in the cartilage, MMP-9 early in disease and MMP-3 with a biphasic pattern in early and late stage disease, most pronounced in the latter. This was a consequence of differential expression in single cells and chondrocyte clusters in late disease.

Conclusion—The data indicate that expression of genes for MMPs 1, 3, and 9 is differentially regulated in human articular chondrocytes and, in individual cells, is related to the depth of the chondrocyte below the cartilage surface and the nature and extent of the cartilage lesion.

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Accumulating evidence implicates intra-articular synthesis of matrix metalloproteinases (MMPs) in cartilage damage in joint disease.¹ Many cells within, or associated with, joints manufacture MMPs in vitro, including macrophages, fibroblasts, synovial cells, chondrocytes, and endothelial cells.²⁻⁴ Osteoarthritis (OA), is a major problem of modern societies,⁵ yet little is known of its aetiopathogenesis. OA

is characterised by cartilage damage and MMPs have been implicated in this process.^{6,7} Their source is debated⁸⁻¹¹ but there is evidence indicating chondrocytic chondrolysis (that is, cartilage damage mediated by chondrocytes) is a key event in OA, mediated by MMPs produced by chondrocytes^{12,13} possibly in response to synovial cytokines such as interleukin 1 β .^{14,15}

It has been said that identifying the specific roles of MMPs could provide rational approaches for developing treatments aimed at stopping destruction of the cartilage matrix.¹⁶ However, a number of obstacles inhibit understanding of the role of MMPs in human OA. Most data on MMPs comes from in vitro systems or animal models of joint disease, often with an inflammatory component. Their relevance to human OA in vivo is unclear. Cartilage is a complex tissue and the structure varies considerably between its layers raising the possibility that different processes, perhaps mediated by different MMPs, might be required to initiate damage at different levels in the cartilage. Similarly OA is a complex and progressive disorder and it is not known whether mediators of cartilage breakdown vary as the disease progresses.

In this study we have started to deal with some of these problems. Although there are no ways, yet, for studying in vivo disease mechanisms at the cellular level in cartilage an existing practical alternative is the use of in situ biochemical techniques in tissue sections. We have examined different stages in the progression of the cartilage lesion of human OA for evidence of expression of mRNA for three MMPs (1, 3, and 9), which have been implicated in OA.^{14,17-19} The level of expression has been examined in four different zones of the cartilage at four histologically distinct stages in the progress of the cartilage lesion. The hypothesis being studied is 'In OA, MMPs are produced by chondrocytes and production varies with the position of the chondrocyte and the extent of the cartilage damage'.

Methods

Two adjacent (anterior and posterior) 8 mm vertical core biopsy specimens were taken from the middle third of the medial femoral condyles of 26 patients at the time of knee replacement surgery. This site was chosen because the spectrum of histological cartilage changes in joint replacement specimens is greatest in this site. In addition two identical cores were taken from six age and sex matched

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cadavers within six hours of death. They were selected because routine examination of the articular surfaces showed no evidence of macroscopic OA. The cores consisted of articular cartilage and underlying subchondral bone. Biopsy specimens were fixed in ice cold (+4°C) 10% v/v neutral buffered formalin for exactly 24 hours and decalcified in 20% EDTA (pH 7.2) for approximately 10 days (until decalcified) under radiological control. After decalcification the tissue was processed routinely into paraffin wax and 7 µm serial sections mounted onto Vectorbond (Vector Laboratories, 16 Wulfric Sq, Bretton, Peterborough PE3 8RF) coated slides for *in situ* hybridisation (ISH).

Haematoxylin and eosin stained sections were examined histologically. Each core consisted of a layer of cartilage overlying bone. In this respect the general structure and tissue relations was similar in every specimen. There were, for instance, no areas of eburnation, no osteophytes, and no subchondral fractures. The major differences were found in the cartilage. These cartilage changes were graded according to the following scheme independently by two of us (AJF and JAH). Grade 0—histologically normal cartilage; grade 1—cartilage surface irregularities and loss of metachromasia adjacent to superficial chondrocytes; grade 2—fibrillation of cartilage and the formation of some chondrocyte clusters, with, at the worst, only minor loss of surface cartilage; grade 3—gross cartilage abnormalities including loss of superficial cartilage, extension of fissures close to subchondral bone, and a large number of chondrocyte clusters.

In all, 35 biopsy specimens (9 graded 0, 10 graded 1, 8 graded 2, and 8 graded 3) from 21 patients (mean age 64.3 (range 54–72), men = 9, women = 12), and 10 biopsy specimens from six cadavers (mean age 66.0 (range 41–77), men = 3, women = 3) in which there was exact agreement on the grading, were selected for further study. χ^2 Analysis has failed to show any statistically significant difference between the number of anterior or posterior cores, or of a male/female bias in any grade group. From archival material five biopsy specimens of synovium from rheumatoid arthritis (RA) patients were selected as positive controls, the distribution of MMP mRNA being well described in RA synovium.¹²⁴ The only treatment being received by any of the patients was non-steroidal inflammatory drugs and they were stopped at least 48 hours before surgery.

NON-RADIOACTIVE IN SITU HYBRIDISATION

As is standard practice in this laboratory before probing with a specific probe all sections are probed with a poly d(T) oligonucleotide probe. This probe hybridises with the poly-A tail found on most actively translated mRNA molecules. Screening all biopsy blocks with this probe ensures that mRNA has not been removed during processing or (in the case of cadaveric tissue) in the interval after death. Synthetic 25–30 mer poly d(T) oligonucle-

otides (Pharmacia) were 3' labelled by the addition of digoxigenin (DIG) labelled deoxyuridine triphosphate (dUTP) using the enzyme terminal deoxynucleotidyl transferase (TdT). Sections were dewaxed, rehydrated, and sequentially immersed in: DEPC (diethylpyrocarbonate) water, 0.2 M HCl, 2 × SSC (SSC (standard saline citrate) = 0.15 M NaCl and 0.015 M sodium citrate) and 0.05 M TRIS HCl pH 7.4. The tissue was treated with 10 µg/ml of proteinase K in 0.05 M TRIS HCl, pH 7.4. The sections were then washed with a solution of 10 mg/ml sheared salmon sperm DNA and then hybridised with the poly d(T) probe at a concentration of 0.5% w/v in DEPC water containing 20% polyethylene glycol for 18 hours at 37°C. The sections were then washed in 2 × SSC. Hybridisation was detected using anti-DIG antibodies conjugated with alkaline phosphatase using fast red-naphthol capture system.

RADIOACTIVE IN SITU HYBRIDISATION

The technique was identical to that described previously.^{20 21}

Probes

Human cDNA probes were used for ISH to minimise background. All probes were from British Biotechnology as a personal gift to Dr JA Hoyland. They were designed to be both specific and of similar length so that after labelling the radioactivity carried by the probes would be of similar specific activity. They were: MMP-1 (full length cDNA, 1400 base pairs), MMP-3 (full length cDNA, 1467 base pairs), and MMP-9 (short cDNA, truncated 5' and 3', 1400 base pairs). Each was random prime labelled to a specific activity of approximately 1×10^8 cpm/µg using [³⁵S] α-dCTP with Amersham Megaprime (Amersham international PLC, White Lion Rd, Amersham, Bucks HP7 9LL) DNA labelling system.

Controls

Controls were of three types. The first (later to be used as 'noise') used λ DNA, as a non-specific probe, radiolabelled to the same specific activity as the metalloproteinase probes. In the second control, sections for hybridisation were pretreated with RNase. In the third, ISH was performed but the probe omitted.

Hybridisation

Twenty four randomised serial sections from each biopsy specimen were used for hybridisation (four for each MMP probe and control). The maximum number of sections that can be accommodated on each ISH run is 21 and each took two days to complete. To test the robustness of the technique no run contained more than one section of each biopsy specimen although only one probe was ever used in one run. Controls were included in every run. As a consequence each group of four sections used for signal data generation for each probe consisted of sections probed at four different times over the three month 'staining' period of the study. Sections were dewaxed, rehydrated,

Figure 1 Each diagram shows the distribution and amount of a metalloproteinase mRNA signal in articular cartilage in the various zones and grades of cartilage damage. The $(S-N)/N$ ratio is represented by the density of shading of the box and by the numbers within the box. The numbers represent the mean (SD) ratio for all the sections of all the biopsy specimens in that grade. The shading has been arbitrarily divided into five as shown in the key. It is intended that the figure should aid visual comparison between the different MMPs in terms of signal and distribution.

and sequentially immersed in: DEPC water, 0.2 M HCl, 2 × SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate), and 0.05 M TRIS HCl pH 7.4. The tissue was treated with 10 µg/ml of proteinase K in 0.05 M TRIS HCl, pH 7.4. After proteinase treatment two of the sections were reacted with 1 mg/ml of crude bovine RNase in 0.5 × SSC, to act as a negative control.

Hybridisation with heat denatured ^{35}S labelled probe was followed by a series of high stringency washes.^{20,21} The sections were dehydrated and air dried. Autoradiography was performed with Ilford K5 emulsion diluted 1:1 with distilled water. Slides were exposed for 16

days, developed in Kodak D-19 developer, fixed, and counterstained with Harris's haematoxylin and eosin.

ASSESSMENT OF QUANTITY OF mRNA

Data were recorded over four cartilage zones. Zone 1 was the most superficial and recognised by the lack of polarisable fibres and flattened chondrocytes. Zone 4 was calcified cartilage and zones 2 and 3 represented respectively the upper and lower halves of the residuum of the cartilage.

Quantification of signal was undertaken using automated grain counting. The analysis of signal intensity was carried out by a single

Figure 2 Examples of ISH reaction in articular cartilage. (A) Low power light photomicrograph of zones 2, 3, and 4 grade 3 reacted for MMP-1. The subchondral bone is in the bottom right and the articular surface bearing a small fissure is just visible in the top left. Zones 2 and 4 contain very little reaction product whereas reaction product is visible within zone 3. ISH $\times 50$. (B) Light field photomicrograph of zones 2 and 3 grade 3 reacted for MMP-3. The majority of lacunae containing single cells have little or no overlying reaction product (arrow heads). Almost all the lacunae containing multiple cells (chondrocyte clusters), such as that arrowed, have high signal levels. ISH $\times 150$. (C) Light field photomicrograph of zones 1 and 2 of grade 1 articular changes reacted for MMP-9. There are no grains over the cells in zone 1 and a high grain count over cells in zone 2. ISH $\times 200$. (D) RNase treated section reacted for MMP-1. ISH $\times 150$.

observer (AJF) using the dot counting function of a Magiscan 600 image analyser (Leica (UK), Cambridge). This is a 'state of the art' image analyser with the ability to discriminate overlapping grains. 'Signal' for each MMP was recorded as the mean number of grains over cells in individual zones in the test sections minus the number of grains over cells in serial RNase pre-treated sections. 'Noise' was defined as the mean number of grains over cells in serial sections probed with λ DNA minus the signal over RNase treated serial sections. Data were expressed as (signal-noise)/noise ratios. (In this way, if signal to equal noise, the result would be 0). A minimum of 100 (median = 218, range 100–357) chondrocytes were analysed in each zone of each section and the ratio

(S-N)/N was calculated from the average grain number over cells in four serial sections.

STATISTICS

All data analyses were carried out using two tailed χ^2 tests.

Results

NON-RADIOACTIVE ISH

The cells in all the sections selected for study reacted for the presence of poly-A tailed mRNA. Signal was seen within the cytoplasm of cells in all four zones in all grades of histological change, including all the grade 0 biopsy specimens and the calcified cartilage (zone 4).

RADIOACTIVE ISH

In both RNase treated and λ probed sections the number of silver grains (that is, hybridisation signal) was much lower than sections treated with specific probe. The ratios of λ , RNase, and specific signal form the basis of the signal/noise ratios (see later). In addition the signal over λ probed sections was generally greater than RNase treated sections (mean λ /mRNase signal ratio = 1.76; range 1–2.83) although this did not reach statistical significance.

RA SYNOVIAL EXPRESSION OF METALLOPROTEINASE mRNA

MMP-1—Signal was localised to synoviocytes (S-N/N = 4.3 ± 1.6). **MMP-3**—Weak signal was seen over synoviocytes and interstitial fibroblasts ((S-N)/N = 1.5 ± 0.7). **MMP-9**—Intensity of the signal was variable for this probe, but was found to be localised to synoviocytes and infiltrating lymphocytes ((S-N)/N = 3.9 ± 2.8).

EXPRESSION OF METALLOPROTEINASE mRNA IN ARTICULAR CHONDROCYTES

In this study there are four variables—the MMP under investigation, the reaction product density, zone of the cartilage, and the grading of the cartilage changes. With any one probe the measured signal varied between: the different grades of cartilage damage; the zone; the four sections from each case; and the cases within each grade group.

To assess the reproducibility of the ISH technique the signal in the four serial sections was compared for each probe in every zone. First the mean signal for each probe in each zone was calculated. Then the average of the four means was taken. The greatest deviation of the four means from the average was then expressed as a percentage of the average. The smallest deviation was 3.6% (MMP-9 in grade 1, zone 3) and the largest 8.9% (λ DNA grade 3, zone 2). There was a mean deviation for all the probes in all the zones of 6.2%—an acceptable degree of reproducibility of the in situ technique.

The data for the mean (S-N)/N ratios for each probe, in each zone and each grade of cartilage lesion are detailed in figure 1 and examples of the reaction product in figure 2. (No data are available for zone 1 in grade 3

disease because this zone is uniformly lost during the progression of the cartilage lesion of OA).

Chondrocyte clusters reacted in a similar way to single cells for MMPs 1 and 9. For MMP-3 the density of grains was much more complex as regards its relation to single cells and clusters. The data are shown in figure 1 and illustrated in figure 2. At their simplest they showed greatest expression of MMP-3 mRNA over single chondrocytes in grade 1, decreasing to grade 3. The grain density was uniform over chondrocyte clusters. These structures were not present in grade 1 lesions or grade 2 lesions in zone 3, and of greatest number in grade 3. The expression in single cells and clusters and their relative numbers gives an apparent biphasic pattern when the two are summated.

No signal reproducibly exceeded noise (that is, $(S-N)/N=0$) in the calcified zone (zone 4), or within normal cartilage (either cadaveric or the histologically normal cartilage from patients undergoing joint replacement).

In general terms, in OA cartilage, expression of MMP-1 mRNA was greatest in the superficial zones (1 and 2), whereas that for the other MMPs studied was greatest in deeper zones (2 and 3). MMP-9 showed the greatest mRNA expression in grades 1 and 2, and MMP-1 in stage 2. MMP-3 seemed to have a biphasic pattern of expression with greatest signal overall being detected in grades 1 and 3. As discussed above this is a consequence of differential signal over single cells and cell clusters.

When signal was related to structures such as fissures there was no specific relation between cells expressing signal and such morphological features.

Discussion

Cartilage integrity relies on coordinated matrix remodelling by chondrocytes. In OA, a disorder characterised histologically by focal cartilage degradation and loss, the mechanism of cartilage damage is incompletely understood. A current working hypothesis is that in OA, cartilage breakdown^{14,22} is caused by up regulation of MMP production by articular chondrocytes^{10,23,24} in response to abnormal synthesis of synovial cytokines (notably interleukin 1) and growth factors.^{22,25}

The evidence indicates that articular chondrocytes normally produce balanced basal values of both MMPs and their inhibitors, TIMPs, but in OA there is increased MMP production without a corresponding rise in TIMPs.^{7,8,13,26} Changed chondrocyte MMP synthesis, therefore, seems to be a pivotal event in cartilage damage in OA. Because cartilage does not have a uniform chemical composition^{27–29} and the lesion propagates downwards and laterally^{30,31} it is possible that in OA there may well be complex spatial and temporal variation in the production of a number of MMPs, with differing and specific functions, in the developing cartilage lesion.¹⁶ It is this we have started to examine in this study by following gene expression for three of the MMPs implicated in the cartilage damage

in OA—interstitial collagenase (MMP-1)^{14,19,32} stromelysin (MMP-3),^{14,15,19,33–35} and the 92 kDa gelatinase (MMP-9).^{8,36,37}

The hypothesis that chondrocytic chondrolysis involves a medley of site specific enzymes that change as the cartilage lesion progresses is very difficult to test. In vitro studies of chondrocytes, generally, important for understanding cellular biosynthesis and its regulation,^{2,7,12,27,38–40} remove the cells from their complex physical and soluble chemical environment with profound effects on synthesis of MMPs.²⁸ In vivo there are generally relatively few chondrocytes and the hypothesis implies that changed MMP synthesis may be so localised that it is difficult to examine by conventional biochemical techniques. However a few sophisticated studies have successfully used these techniques to study MMPs extracted from OA cartilage. They showed that levels of neutral metallo collagenolytic (NMCOL) and proteoglycan (NMPg) degrading activity increased with the macroscopic severity of the cartilage lesion in OA,⁴¹ and in another levels of acid and neutral metalloproteinases were greatest closest to the area of maximum cartilage damage.⁸ Careful dissection of cartilage has enabled these same investigators to show by comparison with weight bearing areas increased expression of NMPg, but not NMCOL, activity in non-weightbearing areas of the tibial plateau in OA, and grade and zone dependent NMPg activity.⁴¹ Our two studies are not directly comparable as neither the zones, the grades nor the enzymes are exactly the same (although NMPg incorporates MMP3 activity and NMCOL, MMP-1 activity), however the pattern of change in enzyme activity and enzyme expression throughout the full thickness of the cartilage is similar in both studies.

It is important that the two approaches are seen as complementary, each contributing to an improved understanding of chondrocytic chondrolysis in OA. The strength of the biochemical approach is that active, uninhibited MMPs can be measured; and that of the in situ techniques, precise cellular localisation of MMP mRNA or immunodetectable protein and the ability to define relations between their expression and local changes in the matrix. Before data derived from in situ techniques can be fully interpreted their limitations need to be recognised.

There are problems using IHC to detect relative changes in MMPs: (a) most MMP antibodies react with both the pro- and active form of the MMP and staining should not necessarily be seen as evidence of the presence of active enzyme; (b) most MMPs are exported; as such, the amount that can be detected in the cell is a balance between rate of production and export, thus cytoplasmic reaction product density may not correspond to synthesis; (c) antibody binding is usually disclosed by enzyme histochemistry, which produces a coloured product in a reaction that is usually allowed to go to completion, consequently the density of reaction product is not a reliable measure of the number of bound antibody

molecules. All these factors make it very difficult to compare MMP synthesis using immunohistochemistry.

ISH does not tackle the issue of enzyme activity and indeed, because mRNA has to be translated into protein it could be argued that its use introduces a further element of complexity into interpreting the data. Fortunately there is evidence that the production of MMPs is regulated at the level of gene transcription thus changes in mRNA levels probably reflect changing synthesis.^{29,38} Many ISH techniques are disclosed using enzyme histochemistry and the same problems arise with quantifying the reaction product as with immunohistochemistry. We routinely use autoradiography for disclosing radioactively labelled probe. There is no steric hindrance from large molecular tags and, in a previous study, we have shown, except at the very extremes of autoradiographic grain density, a direct relation between the amount of mRNA and signal.⁴²

A concern of comparative ISH on processed tissue is that of variable mRNA stability. mRNA is a comparatively unstable molecule, potentially susceptible to breakdown by endogenous RNases and physical and chemical insults. The reproducibility of our data from this and other studies⁴³⁻⁴⁵ shows that careful, consistent, and informed handling of the tissue largely eliminate this concern. For instance in one study we showed that formalin fixation at 4°C, for 24 hours, combined with EDTA (but not acid) decalcification, and subsequent paraffin wax embedding using standardised regimens (as used in this study) results in only a small and predictable loss of signal and minimal variation between specimens.⁴⁶ Taking all the available information into account we have shown that mRNA for three MMPs implicated in OA (MMP-1, MMP-3, and MMP-9), is expressed by chondrocytes in lesional OA cartilage and that expression varies with the zone of cartilage, type of MMP, and degree of cartilage degradation. Although this cannot prove that the enzymes are active in these sites we have discussed the evidence that indicates why this might be so.

Previous studies have attempted to localise MMPs to human cartilage using ISH and immunohistochemistry.^{11,47-50} (M. Stahle-Bechdahl, *et al.* Meeting of the American Association of Cancer Research, Florida, 1966). Many have been conducted on normal articular cartilage, growth plate or cartilage in inflammatory joint disease. There are fewer studies that have localised MMPs by ISH and immunohistochemistry to OA cartilage^{9,50,51} and fewer still that have used immunohistochemistry or ISH in human OA cartilage.^{35,52}

Okada *et al.*³⁵ experienced difficulty in demonstrating MMP-3 in human OA cartilage without pretreating explants with 2 µM monensin in 10% fetal calf serum. After this treatment they identified MMP-3 in chondrocytes in an area equivalent to our zone 1 and upper zone 2 and in cells beside fissures. Using a Mankin scoring system⁵³ they failed to demonstrate MMP-3 in grade 0 (normal) cartilage

and found a pattern of increasing numbers of reactive cells with increasing histological grade up to grade 12 (equivalent to our grade 3). They found no evidence of the decrease in grade 2 zone 3 we describe, with this exception our results and theirs are in accordance with the biochemical data that show acid MMP/MMP-3 enzyme activity increasing with the grade of the cartilage lesion.^{8,10}

Nguyen *et al.*⁵² showed mRNA by ISH for proMMPs 1 and 3 in normal cartilage in the area equivalent to our zone 1 but describe as surprising their finding of a decrease in MMP mRNA in chondrocytes in the lesional tissue in OA, wondering if this was a reflection of anti-inflammatory drug treatment being taken at the time of surgery. Our patients had no treatment for at least 48 hours before surgery. Their results differ from the biochemical findings and our ISH data.

In general our data correlate reasonably well with the results of *in vivo* enzyme extraction studies and some of the *in situ* data from human OA cartilage. There are also similarities between our results and animal models of OA, notably the Pond-Nuki dog model.⁵⁰ The data presented here also give a different perspective to data on MMP synthesis derived in other ways. One obvious example is that it has been suggested that the superficial layers of cartilage are responsible for most MMP production^{9,54} perhaps through stimulation of MMPs by soluble cytokines, produced by synovium, diffusing into the cartilage.⁵⁵ We have shown that the genes for some MMPs are expressed most in deeper layers and there is variation between the activity in single cells and adjacent chondrocyte clusters. If the production of chondrocyte MMPs is stimulated by diffusion of cytokines these findings beg the question of how and why deep chondrocytes should be affected when more superficial ones, closer to the source of the cytokines, are not; and, in particular raises the intriguing possibility of factors other than cytokines alone being responsible for gene transcription in these cells.

If the inhibition of MMPs is a realistic goal in the treatment of progressive cartilage loss in OA as some suggest,¹⁶ there is a need to extend this type of *in vivo* study to examine the expression and distribution of other MMPs implicated in OA. Particular candidates for early further study include MMP-8⁵⁶ and MMP-13,⁵⁷ which have also recently been shown to be present in OA cartilage and which preliminary data indicate have the potential to play a significant part in cartilage breakdown.

In summary the results presented here show that chondrocytes in human cartilage have the potential to actively up and down regulate transcription of different metalloproteinase genes during the development of the cartilage lesion of OA. They also show how complex is the expression of mRNA for only three of the potential large number of MMPs that might be involved in the cartilage lesion of OA, and how for individual MMPs expression is often focal and frequently occurs deep in the cartilage, without corresponding detectable expression

in the superficial zones. The data have important implications for understanding the biology of disturbed chondrocyte function in OA and the methodology offers a system for examining, in detail, gene expression for all MMPs in OA cartilage during the progression of the cartilage lesion.

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Figure 1 Popliteal cyst. The volume of the cysts is widely variable: from that of a pin's head to that of a hen's egg. Sometimes there are a large number of cysts, similar to a bunch of grapes.

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