THE JOURNAL OF EXPERIMENTAL ZOOLOGY 273:82–86 (1995)

RAPID COMMUNICATION

Calcium, Sulfur, and Zinc Distribution in Normal and Arthritic Articular Equine Cartilage: A Synchrotron Radiation-Induced X-Ray Emission (SRIXE) Study

R. RIZZO, M. GRANDOLFO, C. GODEAS, K.W. JONES, AND F. VITTUR
Dipartimento di Biochimica, B.C.M., Università di Trieste, I-34127 Trieste, Italy (R.R., M.G., C.G., F.V.); Brookhaven National Laboratory, Upton, New York 11973 (K.W.J.)

ABSTRACT Calcium, sulfur, and zinc content in normal and arthritic equine cartilage have been studied by synchrotron radiation-induced X-ray emission (SRIXE). Ranging from the superficial to the columnar zone of the normal tissue, calcium and zinc concentrations are increasingly higher, whereas sulfur is at its highest concentration in the transitional zone. In the arthritic tissue, calcium concentration is at its maximum in the transitional zone, whereas zinc and sulfur distributions are relatively homogeneous. Sulfur concentration in arthritic cartilage is reduced to about one-third with respect to that in normal tissue. The possibility that zinc concentration reflects the distribution of the zinc-containing enzyme alkaline phosphatase is presented.

Senescence and/or traumas can produce osteoarthritic degeneration in articular cartilage. This disease is characterized by the destruction of cartilage and by subchondral bone changes. Neutral and acidic proteases degrade both tissue proteoglycans and collagen, eventually promoting the death of cartilage cells and the erosion of the tissue (Mannik and Gilliland, '83). The metacarpal joints of horses affected by osteoarthritis are a suitable model for the study of this disease. Very recently we have characterized some metabolic aspects of this kind of cartilage from healthy horses (Vittur et al., '94). In the present study, the distribution of three elements of biological relevance (sulfur, calcium, zinc) was determined in normal and arthritic cartilage by means of SRIXE technique. In this methodology, X-rays obtained from a synchrotron light source are collimated (up to 10 μm × 10 μm) and impinged on a tissue section. Depending on the energy of the incident photons, K electrons of atoms can be ripped out; contemporarily, external electrons move to the empty K level while emitting energy in the form of X-ray fluorescence. The energy of the emitted radiation leads to the detection of the kind of atom, whilst the evaluation of the number of the emitted photons permits quantitative results. By scanning the sample it is possible to obtain one- or two-dimensional maps of element distribution.

SRIXE is an emerging technique which only recently is being used for the study of growing cartilage (Vittur et al., '92), and only few examples can be quoted of its use for the investigation of biological samples (for example Bockman et al., '90; Ali et al., '92).

MATERIALS AND METHODS

Normal and arthritic horses were identified by a veterinary check before the sacrifice. Samples of articular cartilage were obtained from the zones of the metacarpal bones normally subjected to loading. Cartilage slices were excised, under aseptic conditions, immediately after the sacrifice of the animals (2- to 5-year-old horses) and fixed in 2.5% formaldehyde. They were then dehydrated and paraffin embedded, according to the usual techniques of optical microscopy. Finally, transverse sections, 5 μm thick, containing the superficial, the transitional, and the columnar zones (Ghadially, '78), were cut. For SRIXE analysis, sections were mounted on a slide frame between two perforated acetate cellulose sheets. Care was taken that the part of the section to be analyzed

Received December 14, 1994; revision accepted May 10, 1995.
Address reprint requests to Franco Vittur, Dip. Biochimica B.C.M., Via L. Giorgieri 1, I-34127 Trieste, Italy.

was in correspondence with the hole (5 mm in diameter) in the cellulose acetate sheets.

X-ray microprobe

X-ray microprobe studies were carried out with the X-ray microscope (XRM) facility in operation at beamline X-26, National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (Upton, NY) as reported by Vittur et al. ('92). Emission spectra, obtained by means of a microbeam collimated to 10 μm × 10 μm, were stored and analyzed by using a Digital Equipment Corporation Micro Vax II computer and a Nuclear Data Corporation (ND9900) data acquisition system. Relevant peaks were selected to obtain one-dimensional maps corresponding to the distribution of the trace and minor elements along a transverse section of the tissue.

Quantitative determination of calcium, sulfur, and zinc concentrations in the samples was performed by calibration with standard mixtures of the different elements at known concentration. The concentrations of the elements in cartilage samples were calculated by the following expressions:

$$C_{Ca,unk} = C_{Ca,std}\, R_{Ca}\, \hat{A}_{Ca,unk}/\hat{A}_{Ca,std}$$
$$C_{Z,unk} = C_{Ca,unk} Y_{Z,Ca}\, A_{Z,unk}/A_{Ca,unk}$$

where *std* indicates standard samples and *unk* refers to cartilage samples; $C_{Z,unk}$ indicates the concentration of an element different from calcium (sulfur, zinc), R_{Ca} is the ratio between the calcium fluorescence yield in the standard and that in the cartilage sample. $\hat{A}$ is the area of the fluorescence peak normalized for the total photon flux in the detector, A is the area of the fluorescence peak, and the $Y_{Z,Ca}$ is the relative fluorescence yield for calcium and either sulfur or zinc in the cartilage sample. The relative fluorescence yield (Y) was evaluated from the standard samples through data processing of the number of photons emitted per square radiant by the different elements.

Biochemical essays

Chondrocytes were obtained from the whole normal and arthritic cartilages by collagenase digestion as described by Pollesello et al. ('90). They were isolated from the same cartilage samples used to obtain sections for SRIXE analysis. Alkaline phosphatase activity was measured according to Stagni et al. ('79) on the cells immediately after their isolation. Results were normalized to the DNA content measured according to Labarca and Paigen ('80).

Histology

Staining of sections was performed with alcian blue at pH 1.8 to reveal sulfated glycosaminoglycans.

RESULTS

As a preliminary investigation, spectra were collected from each of the three zones of the cartilage for the identification of the elements present in the tissue. Figure 1 shows a spectrum collected in the transitional zone (those collected from the other zones are similar). The presence of argon in the air produced the major signal, whereas signals relevant to titanium, chromium, and iron were due to the radiation scattering on the experimental set-up, as shown also by their presence when a blank, i.e., a sample of the paraffin used for tissue embedding, was analyzed. Potassium is normally present in the tissue as K^+, a freely diffusible species; its concentration might easily be modified by the procedure of fixation, dehydration, and embedding. In conclusion argon, titanium, chromium, iron, and potassium distributions are not considered in the following discussion. Sulfur, calcium, and zinc are the three elements investigated in the different zones of cartilage.

In the normal tissue (Table 1), calcium concentration rises continuously from the superficial to the columnar zone. On the contrary, in the pathological tissue, the concentration of calcium reaches its maximum value in the transitional and columnar zones.

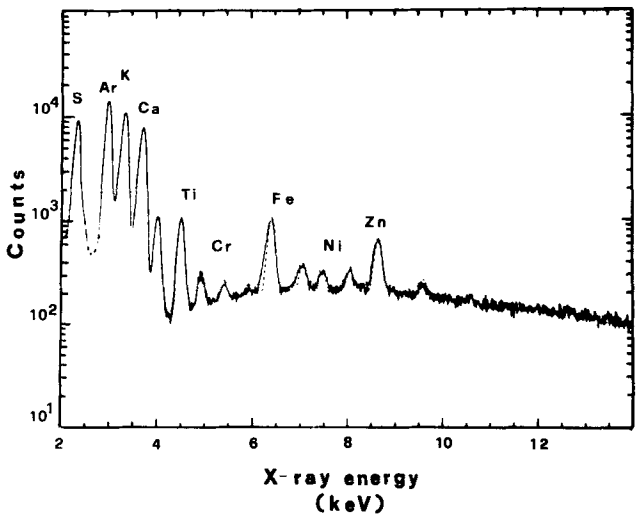

Fig. 1. X-ray emission spectrum. Typical X-ray spectrum obtained over a collection time of 300 s with a beam size of 10 μm × 10 μm from a thin section (5 μm) of articular cartilage (transitional zone).

TABLE 1. Calcium, sulfur, and zinc concentration in three different layers of equine normal and arthritic articular cartilage[1]

	Ca	S	Zn
Normal cartilage			
Superficial	0.16 ± 0.06	5.43 ± 1.58	0.005 ± 0.002***
Transitional	0.92 ± 0.06*	15.72 ± 2.16	0.029 ± 0.005**
Columnar	1.96 ± 0.48	12.65 ± 0.84	0.047 ± 0.015
Arthritic cartilage			
Superficial	0.19 ± 0.12	3.16 ± 0.31	0.043 ± 0.022***
Transitional	1.45 ± 0.39*	5.07 ± 0.96	0.046 ± 0.010**
Columnar	1.50[2]	4.08[2]	0.042[2]

[1]Data ($\mu g/cm^2$) are the mean ± SD of at least four experiments on different cartilage sections.
[2]Data are the mean of two experiments.
*, **, ***The statistical differences were evaluated by unpaired t-test: (*)$P = .03$, (**)$P = .009$, (***)$P = .0001$.

In the transitional zone, sulfur concentration is about three times higher than in the superficial zone of normal cartilage; it is decidedly lower in the columnar zone. The pathological tissue is characterized by a regular distribution of sulfur; in fact, the mean concentration of this element in all three zones is about one-third of that measured in the transitional zone of the normal cartilage.

The distribution of zinc is relatively constant throughout the three zones in pathological tissue. However, its concentration in the superficial and transitional zones of pathological tissue is higher than in the corresponding zone in normal cartilage.

The scanning of the sections along a transverse axis (Fig. 2) from the superficial to the columnar zones confirms the data reported in Table 1, and exhibits better spatial resolution. The data collected in the scanning mode were not normalized to standard samples, so that they are only qualitative; no quantitative comparison can be made either among different elements or among identical elements measured in different sections. It is interesting to note that sulfur distribution, as revealed by sample scanning, is superimposable to that of sulfated glycosaminoglycans as shown by alcian blue staining (Fig. 3).

In order to test a correlation between the zinc concentration and the alkaline phosphatase presence in the cartilage, the alkaline phosphatase-specific activity in chondrocytes isolated from normal and arthritic tissues has been measured. Four samples of both normal and arthritic cartilage obtained from different horses were analyzed. The values obtained are of the

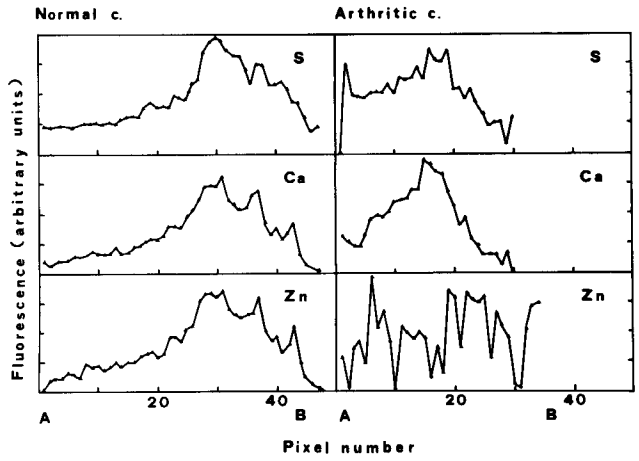

Fig. 2. Calcium, sulfur, and zinc distribution throughout the different layers of normal and arthritic cartilage. Beam size = 10 μm × 10 μm. Pixel size 25 μm × 25 μm, acquisition time per pixel = 30 s. Scanning direction: from **A** (superficial zone) to **B** (columnar zone).

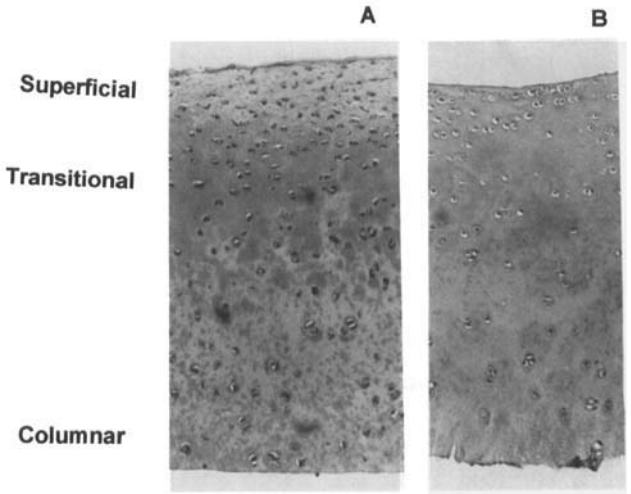

Fig. 3. Distribution of sulfated glycosaminoglycans in normal and arthritic cartilage. Cartilage sections were stained with alcian blue, pH 1.8. The reactivity of the intercellular matrix to alcian blue is maximum in the transitional zone of normal tissue **(A)**, but, in contrast, is more uniform throughout the arthritic tissue **(B)**. ×125.

same order of magnitude, but the figures relative to chondrocytes from pathological tissue are slightly larger than those of normal cartilage (1.3 vs. 0.9 nmoles·min^{-1}·μg DNA^{-1}).

DISCUSSION AND CONCLUSIONS

The presence of zinc and the different distribution of sulfur and zinc in the normal and arthritic cartilage are the new elements derived from our studies on the elemental composition of articular cartilage. The increase of calcium concentration in the transitional zone of arthritic cartilage with respect to that relative to the normal tissue gives an analytical support about the presence of calcified nodules seen by Ali et al. ('92) in the deeper zones of articular cartilage where calcified cartilage appears to advance into the noncalcified tissue. The type and amount of proteoglycans present in the different zones of this tissue have been thoroughly studied in the past by traditional methods (Maroudas and Kuettner, '90). It is therefore of relevance that the sulfur distribution profiles obtained by means of SRIXE are strictly related both to those obtained with classical methods for proteoglycan analysis in normal cartilages and to the histological evidence shown in Figure 3. It is known that, in arthritis, proteoglycans are removed by proteolytic degradation from the tissue which appear eroded in the zones interested by lesions (Thonar and Kuettner, '87). In our experiments, pathological cartilage sections were scanned by the X-ray probe across the noneroded zones of the tissue, and the results indicate that proteoglycan concentration, as measured by sulfur distribution profile, is dramatically lower with respect to normal tissue. In addition to this, proteoglycans appear to be rather homogeneously distributed throughout the tissue width.

Another interesting piece of information is the presence and the distribution of zinc. In a previous paper (Vittur et al., '92), we have put forward the idea that, in pig pre-osseous cartilage, the zinc atoms detected by SRIXE might be those linked to the metalloenzyme alkaline phosphatase. The same inference can be made in the present study. Despite the fact that zinc distribution in normal and pathological tissues is different, the order of magnitude of zinc concentration is the same, with a significantly higher level of this element in arthritic cartilage (superficial and transitional zones). The activity of the zinc-containing alkaline phosphatase is slightly higher in the chondrocytes isolated from pathological cartilage than in those isolated from the normal one. In addition to this, the activity of the enzyme present in the homogenate from arthritic cartilage of either rabbit (Mokondijmobe et al., '91) or human cartilage (Ali et al., '92), is three times higher than that relative to the alkaline phosphatase from normal tissue. Ali et al. ('92) suggested that this higher alkaline phosphatase activity could be correlated to the presence of an extended matrix vesicle-containing calcified area. In fact, matrix vesicles are the locus of the initial mineralization in cartilage (Anderson, '67; Bonucci, '67) and are characterized by a very high alkaline phosphatase-specific activity (Pollesello et al., '90). The matrix vesicle presence in the intermediate zone of arthritic tissue supports the idea of a mineralization extended to this zone and justifies the high calcium content (Table 1) of the tissue. The above-reported evidence strengthens the hypothesis that the zinc detected by means of SRIXE is connected to the presence of alkaline phosphatase.

ACKNOWLEDGMENTS

The fundamental support of Dr. S. Sutton (Brookhaven National Laboratory, Upton, NY) and the technical assistance of Mr. B. Gazzin and Mr. Bortoletto are greatly acknowledged. This work was supported by the M.U.R.S.T., by the C.N.R. (Italy), by Fidia S.p.a. (Abano Terme, Italy), and by the U.S. Department of Energy (contract DE-AC02-76CH00016).

LITERATURE CITED

Ali, S.Y., J.A. Rees, and C.A. Scotchford (1992) Microcrystal deposition in cartilage and in osteoarthritis. Bone Mineral, *17*:115–118.

Anderson, H.C. (1967) Electron microscopic studies of induced cartilage development and calcification. J. Cell Biol., *35*:81–101.

Bockman, R.S., M.A. Repo, R.P. Worrel, Jr, J.G. Pounds, G. Schidlowsky, B.M. Gordon, and K.W. Jones (1990) Distribution of trace levels of therapeutic gallium in bone as mapped by synchrotron x-ray microscopy. Proc. Natl. Acad. Sci. U.S.A. *87*:4140–4153.

Bonucci, E. (1967) Fine structure of early cartilage calcification. J. Ultrastruct. Res. *20*:33–50.

Ghadially, F.N. (1978) The fine structure of joints. In: The Joints and Synovial Fluid, Vol. 1. L. Sokoloff, ed. Academic Press, New York, pp 105–176.

Labarca, C., and K. Paigen (1980) A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem., *102*:344–352.

Mannik, M., and B.C. Gilliland (1983) Degenerative joint disease. In: Harrison's Principles of Internal Medicine. R.G. Petersdorf, R.D. Adams, E. Braunwald, K.J. Isselbacher, J.B. Martin, and J.D. Wilson, eds. McGraw-Hill, London, pp. 1999–2002.

Maroudas, A., and K. Kuettner, eds. (1990) Academic Press Inc., San Diego, Methods in Cartilage Research.

Mokondijimobe, E., E. Vignon, C. Hecquet, R. Bourbouze, and

M. Adolphe (1991) Hexosaminidase et phosphatase alcaline du cartilage et des chondrocytes en culture issus de lapins normaux et arthrosiques. Pathol. Biol., *39:*759–762.

Pollesello, P., P. D'Andrea, M. Martina, B. de Bernard, and F. Vittur (1990) Modification of plasma membrane of differentiating preosseous chondrocytes: evidence for a degradative process in the mechanism of matrix vesicle formation. Exp. Cell Res. *188:*214–218.

Stagni, N., G. Furlan, F. Vittur, M. Zanetti, and B. de Bernard (1979) Enzymatic properties of the Ca^{2+}-binding glycoprotein isolated from preosseous cartilage. Calcif. Tissue Int., *29:*27–32.

Thonar, E.J.-M.A., and K.E. Kuettner (1987) Biochemical basis of age-related changes in proteoglycans. In: Biology of Proteoglycans. J.N. Wight and R.P. Mecham, eds. Academic Press Inc., San Diego, pp. 211–246.

Vittur, F., C. Tuniz, S. Paoletti, R. Rizzo, and K.W. Jones (1992) Elemental analysis of growth plate cartilage by synchrotron-radiation-induced X-ray emission (SRIXE). Biochem. Biophys. Res. Commun., *188:*1010–1017.

Vittur, F., M. Grandolfo, E. Fragonas, C. Godeas, S. Paoletti, P. Pollesello, B.J. Kvam, F. Ruzzier, T. Starc, J.W. Mozrzymas, M. Martina, and B. de Bernard (1994) Energy metabolism, replicative ability, intracellular calcium concentration, and ionic channels of horse articular chondrocytes. Exp. Cell Res., *210:*130–136.