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Collagen Fibrils Are Differently Organized in Weight-Bearing and Not-Weight-Bearing Regions of Pig Articular Cartilage

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ABSTRACT The magnetic resonance (MR) appearance of the weight-bearing ("loaded") and not-weight-bearing ("unloaded") regions in T_2-weighted images of pig articular cartilage is different. On the hypothesis that this difference may be ascribed, at least in part, to a different collagen fibre organization in the two regions, this organization was studied using biochemical, histological, and X-ray diffraction methods. While the mean concentrations of collagen and of its cross-links were the same in the two regions, a regular small angle X-ray diffraction pattern was observed only for the habitually "loaded" tissue. It was also seen by light microscopy that the four typical functional zones were well displayed in the "loaded" cartilage whereas they were not clearly depicted in the "unloaded" tissue. Collagen presented a high concentration of fibrils forming an intricate and dense meshwork at the surface of both "loaded" and "unloaded" cartilage. A second zone of high collagen concentration was present at the upper layer of the deep zone of "loaded" cartilage. By contrast, this lamina of highly concentrated fibrils was lacking in "unloaded" cartilage and collagen fibrils appear thinner. Our study proves that the organization of collagen fibres is different for the "loaded" and "unloaded" regions of articular cartilage. It also suggests that this different organization may influence the MR appearance of the tissue. *J. Exp. Zool. 287:346–352, 2000.*

Articular cartilage is a dense connective tissue with the functions of load-bearing and reducing friction in articulating joints. This tissue consists of cells dispersed in an extracellular matrix composed primarily of proteoglycan aggregates, collagen type II, and water. Collagen fibrils appear differently oriented in the different zones of the tissue habitually weight-bearing in vivo: they appear tangentially oriented in the superficial layer (about 100 μm thick) and radially oriented in the deepest zone (Goodwin and Dunn, '98). Magnetic resonance imaging (MRI) represents the most powerful modality for the clinical investigation of articular cartilage (Mc Cauley and Disler, '98). It is well known that normal articular cartilage appears laminated when placed at certain orientations with respect to the external magnetic field (Rubenstein et al., '93; Xia et al., '94; Mlynárik et al., '96). In a recent study on pig articular cartilage of the proximal humeral end (Fragonas et al., '98) it was observed that the MRI laminar appearance of the in vivo habitually weight-bearing cartilage ("loaded") was different from that of the normally not-weight-bearing ("unloaded") tissue of the same joint. This behaviour was ascribed to the protons of water molecules interacting with collagen and it was proved that the laminar appearance of the tissue is influenced by the sample

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orientation in the magnetic field and also by the concentration of collagen in the different cartilage zones. However, the correlation between collagen concentration and laminar appearance was not perfect and the influence of additional factors on the MR images was suspected. Therefore, it was hypothesized that collagen fibril orientation and/or packing of collagen macromolecules in the fibrils may also have some relevance to the MRI appearance of cartilage. The aim of this investigation was to ascertain if in "unloaded" articular cartilage collagen fibres are organized differently from those of "loaded" cartilage. The collagen fibril organization was investigated by biochemical methods, histology, and small angle X-ray scattering.

MATERIALS AND METHODS

Cartilage samples were obtained from 150–200-kg pigs within three hours of the slaughter of the animals. Cylindrical plugs of the tissue, 5 mm in diameter, were excised from the "loaded" and "unloaded" regions of the articular cartilage of the proximal head of pig humera (Fragonas et al., '98). Samples were stored in DMEM (Dulbecco's modified minimum essential medium, Seromed, Berlin) at 4°C until they were imaged by magnetic resonance microscopy (MRM). A portion of subchondral bone was left attached to the cartilage samples after excision and was taken as reference for sample orientation in the MRM experiments.

Magnetic resonance microscopy

The cartilage plugs were set into 5-mm NMR tubes with the articular surface placed parallel to the axis of the tube. The angular orientation of the specimen was adjusted so that the cartilage surface was perpendicular to the frequency encoding direction along the x-axis.

The MRM experiments were performed at 24°C with a Bruker AM300 console equipped with a standard microimaging unit and coupled to a Spectrospin vertical wide bore (WB) superconducting magnet, operating a 7.05 T (300 MHz for ^{1}H).

High-resolution images were obtained using a modified spin-echo sequence (Hsu et al., '96). The repetition time between phase encoding steps was 1.5 sec and an echo time of 34 msec led to the predominance of T_2 contrast. The matrix size of 128 × 128 and the field of view of 6 mm resulted in an in-plane resolution of 47 μm.

Biochemical techniques

Samples were also characterized for their mean collagen content by determination of hydroxyproline content according to Jamall et al. ('81). The pyridinolinic groups were quantitatively estimated by fluorimetry after HPLC fractionation of the hydrolysis products of collagen (16 hr at 108°C in 6 M HCl) (Eyre et al., '88). Collagen preparations for the determination of pyridinolinic groups were obtained from samples of "loaded" and "unloaded" cartilage depleted of proteoglycans by extraction with 4 M guanidinium chloride according to Sajdera and Hascall ('69) (48 hr of extraction at ambient temperature in 4 M guanidinium chloride, 50 mM TRIS, pH 7.4).

Histology

After fixation in formol-phosphate, samples of "loaded" and "unloaded" cartilage were paraffin embedded. Thin slices (10 μm thick) were then cut and stained for collagen by the procedure of van Gieson (AFIP, '57). Some samples were embedded in glycol-methacrylate and serial sections were cut both vertically and tangentially to the articular surface. Sections were digested by hyaluronidase type IV and stained for collagen by the osmium-silver methenamine procedure (Hwang et al., '90).

Small angle X-ray scattering (SAXS) experiments

Immediately after MRM experiments, 1-mm thick slices were cut from the imaged cartilage plugs perpendicularly to the cartilage surface and fixed in 3% phosphate buffered formaldehyde (pH 7.4) for one week before SAXS experiments (Yarker and Hukins, '84).

Measurements were performed at the 5.2L SAXS wiggler beam line (Amenitsch et al., '95) of ELETTRA (Science Park, Trieste, Italy). Fixed samples were rinsed in physiological saline, mounted in a home-made thermostable cell provided with two polypropylene windows (SPEX Industries, Edison, NJ, 5 μm) to maintain samples in a wet state during diffraction experiments. Samples were impinged with an X-ray beam of 200 × 500 μm (horizontal × vertical). The sample-to-detector distance was 2850 mm and the photon energy was 8 keV (0.15 nm). A monodimensional gas-filled detector (Gabriel type) was used.

RESULTS AND DISCUSSION

Both "loaded" and "unloaded" cartilage types exhibit a mean collagen content of 39 ± 2% of the dry tissue weight (n = 12) as already reported (Fragonas et al., '98).

The lateral and longitudinal packing of collagen molecules in fibril formation is favored and stabi-

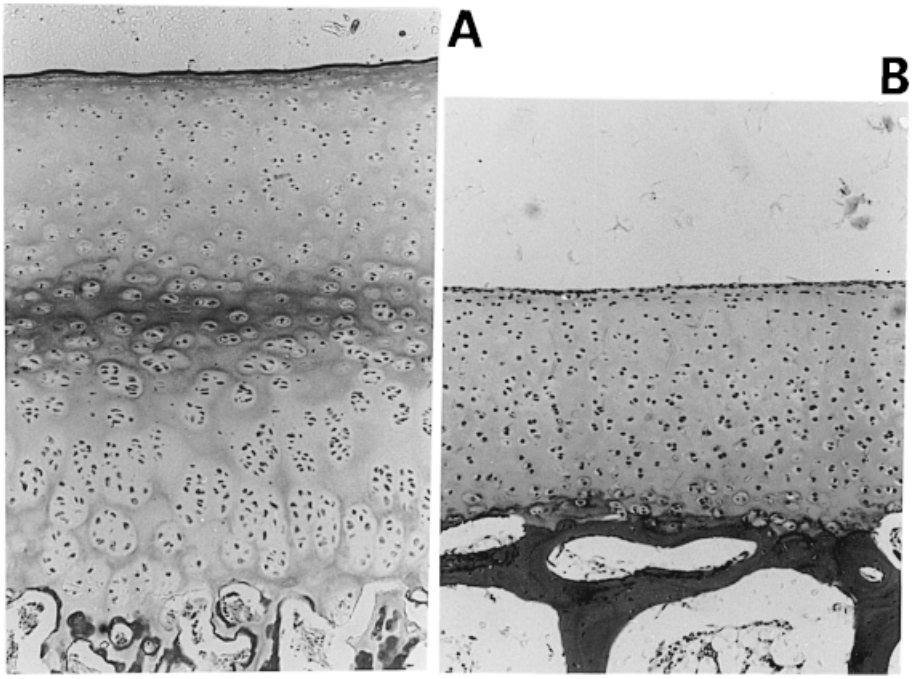

Fig. 1. Collagen distribution in "loaded" (**A**) and "unloaded" (**B**) articular cartilage as shown by the trichromic staining procedure of van Gieson. Original magnification × 100.

lized by the formation of pyridinolinium cross-links (Knott et al., '97). For this reason, an evaluation of the number of pyridinolinium cross-links per unitary weight of collagen can potentially help in a gross estimation of the relative dimensions of collagen fibrils in the two zones of articular cartilage. Unfortunately, the number of pyridinolinium cross-links for a unitary amount of collagen was the same for both cartilage types (4.5 μmoles/mmole of hydroxiproline) giving no indication about a difference in the mean dimension of fibrils.

Histology (Fig. 1A,B) shows that "loaded" tissue collagen is mainly localized at the articular surface for a depth of about 100 μm with a second zone of intense staining at about half of the cartilage thickness. A quantitative distribution of col-

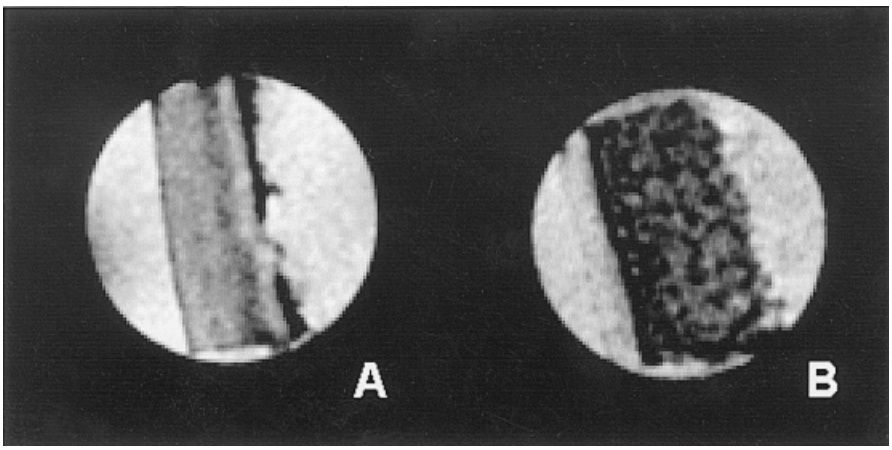

Fig. 2. T_2-weighted magnetic resonance microimages of porcine cartilage plugs derived from the habitually loaded (**A**) and habitually unloaded (**B**) tissue. The in-plane resolution of the images is 47 × 47μm. In each image the surface of the cartilage is on the left and exhibit a reduced signal intensity. In (A) an additional intermediate lamina of reduced signal intensity is evident. A residual bone portion appears as a thin dark lamina on the right side of (A). The thick spongy-like structure visible on the right side of (B) is the trabecular subchondral bone.

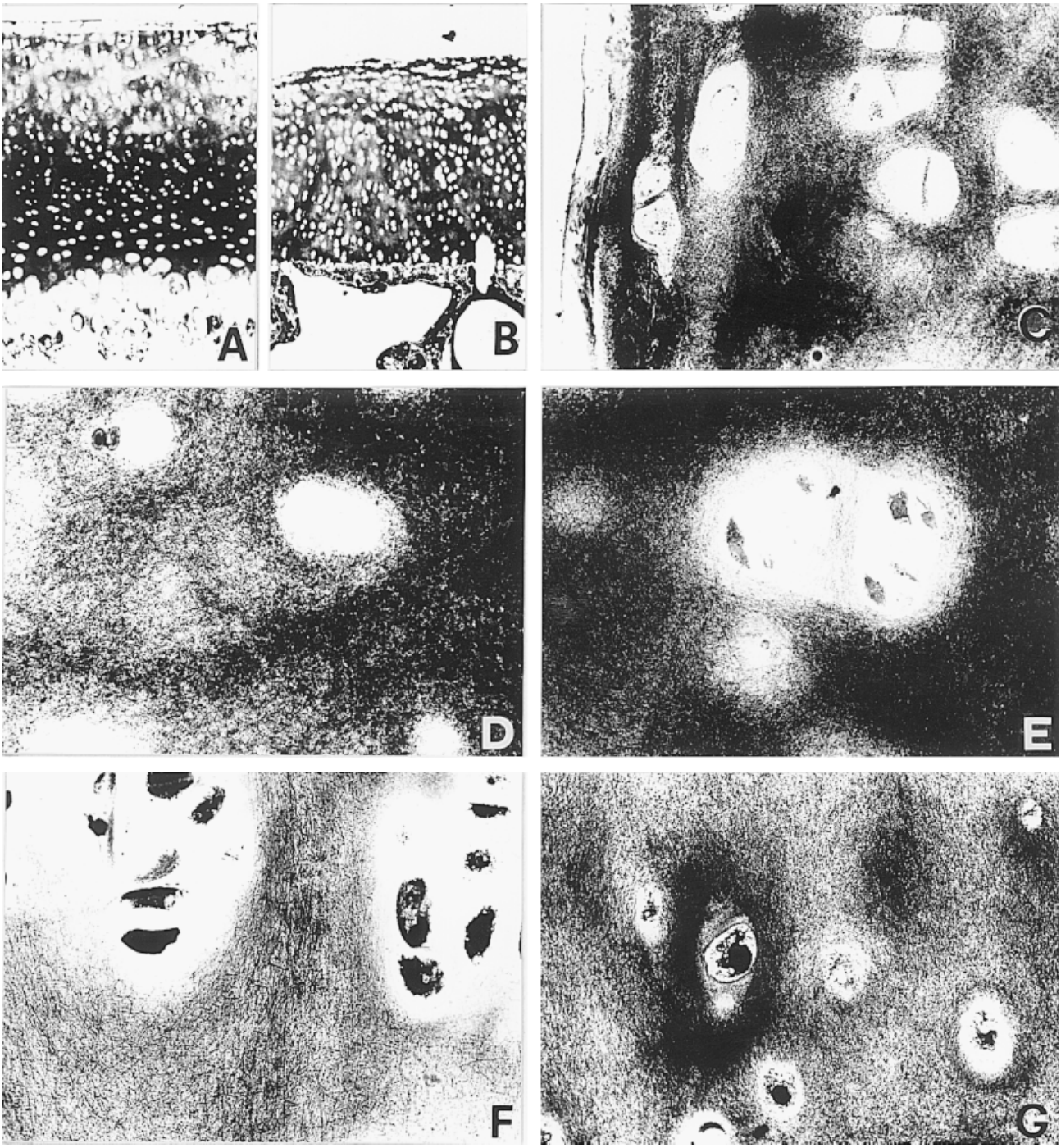

Fig. 3. Collagen distribution in "loaded" and "unloaded" cartilage as determined by staining of the tissues by osmium-silver methenamine method (Hwang et al., '90). The field is 900 μm wide in A–B and 110 μm in C–G. **A:** "Loaded" cartilage. **B:** "Unloaded" cartilage. **C:** "Loaded" cartilage superficial zone. **D:** "Loaded" cartilage transitional zone at the limit with the deep lamina. Bundles of collagen fibrils are seen running towards the surface (to the left). **E:** Deep layer zone. The collagen meshwork is very dense in the interterritorial matrix. **F:** Deepest zone over calcified cartilage. Collagen fibrils run in vertical or transversal directions. **G:** "Unloaded" cartilage bulk zone. The collagen meshwork is fine. Dense collagen bundles are also seen to surround the chondrocytes.

lagen was previously assessed by biochemical data (Fragonas et al., '98). More uniform appears the staining of the "unloaded" tissue where the collagen content is highest at the articular surface.

Figure 2A,B shows the MRI appearance of the same "loaded" and "unloaded" samples. The "loaded" tissue presents a laminar structure with two zones of low signal intensity, at the surface and in an intermediate zone, while only the dark superficial lamina is present in the image of the "unloaded" tissue.

When applied to vertical sections of articular cartilage, the staining procedure of Hwang revealed that the "loaded" cartilage displays the four typical functional zones (superficial, transitional, deep, and calcified). Two zones are heavily stained for collagen: the superficial (about 70 μm thick) and a layer (or lamina) about 350 μm thick within

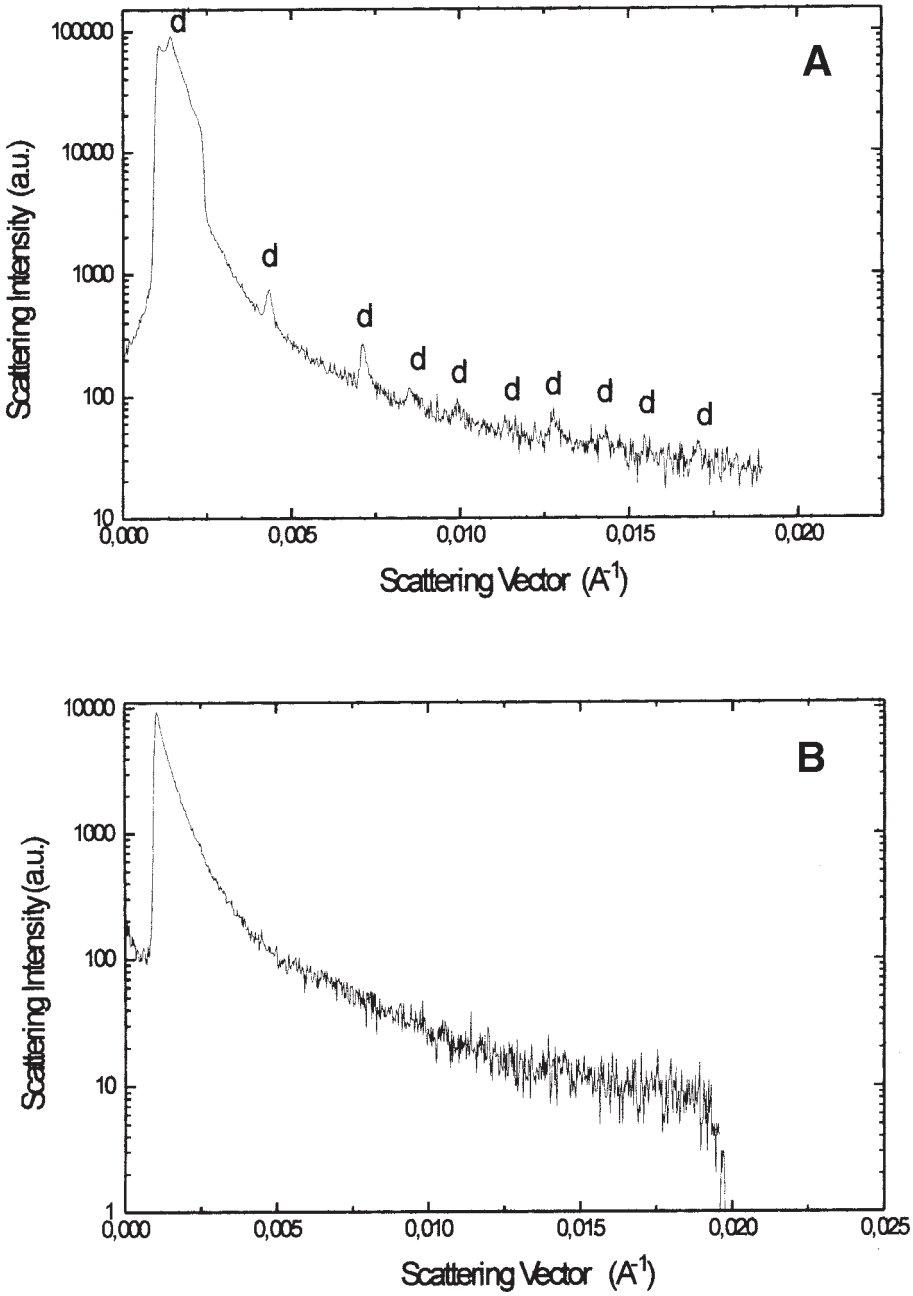

Fig. 4. Small angle X-ray diffraction patterns derived from the habitually loaded (**A**) and habitually unloaded (**B**) cartilage. A repetition periodicity of 670 Å (d) is evident only for samples of the "loaded" tissue.

the deep zone (Fig. 3A). In "unloaded" cartilage the collagen meshwork appears differently organized; while the superficial zone is heavily stained as in "loaded" cartilage, the bulk of cartilage shows a patched stain with no definite layers (Fig. 3B). High-resolution light micrographs show dense bundles of collagen fibrils at the superficial zone (Fig. 3C), classic Benninghof arcades at the stem from the deep layer (Fig. 3D), as well a dense interterritorial meshwork within the deep layer (Fig. 3E). In the deepest zone (over the calcified cartilage) where isogenic groups of chondrocytes are identified, the collagen fibrils form a less dense meshwork and individual fibrils can be traced running transversally or vertically (Fig. 3F). In "unloaded" cartilage collagen bundles alternate to form a fine or a dense meshwork (Fig. 3G). The examination of serial tangential sections, collected at different depth of the tissue and providing right angle views respect to the vertical ones, shows in the superficial zone of "loaded" cartilage bands of heavy staining and in transitional zone a layer (600–900 μm in depth) where a complete staining of all the interterritorial matrix occurs. On the contrary, the staining was patched in the transitional zone (100–400 μm in depth) and in the bulk of "unloaded" cartilage. A comparison of high-resolution light micrographs from vertical and tangential 1 μm thick sections at the level of the deep layer shows no distinguishable differences. In this case, collagen fibrils form a dense-packed three dimensional network with no predominant direction of fibril bundles. The only zone that had a preponderant (but not unique) fibril orientation was the deepest one in "loaded" cartilage where collagen bundles with a predominant fibril orientation at right angles to the articular surface were observed.

When studied by SAXS, "loaded" and "unloaded" cartilage display two different, specific diffraction patterns (Fig. 4A,B). Regularly spaced peaks appear in the diffraction pattern of loaded cartilage revealing a periodicity attributable to the typical organization of collagen molecule in fibrils (d spacing = 670-Å period). On the contrary, no diffraction peak is evident in the patterns from "unloaded" cartilage. From X-ray diffraction experiments on the "loaded" regions of tibial plateau cartilage of pigs and dogs, Yarker and colleagues ('84) obtained similar results. The relatively large dimensions of the X-ray beam employed in this study allowed us to obtain a signal of the average arrangement of the collagen fibrils in the two regions of articular cartilage and it was not possible to determine the X-ray diffraction patterns at the level of the single laminae, evident in the MR images. The lack of regular repetition peaks in the SAXS patterns from "unloaded" cartilage may potentially be explained by the lack of thick collagen fibres at the deep zone of the tissue and of the corresponding ordered structure of collagen fibres, and/or by a random distribution of collagen fibres of small diameter generating a powder diffraction pattern of low intensity with repetition signals which cannot be collected by the 1D detector used for the experiments.

These results pointed out that, despite the fact that the average collagen concentration in the two zones of the articular cartilage is the same, about 39% of the dry weight, the structure, and distribution of collagen fibrils appear to be different. This may reasonably be the result of the different metabolic activity of the chondrocytes of the two regions under influence of the mechanical loading (Martina et al., '97). In the region of cartilage where loading is normally high, collagen fibres should be highly organized in order to realize a three-dimensional network able to resist to the compressive forces. In the zones normally not subjected to load, the cartilage depth is reduced, the lamina of concentrated collagen at the upper deep zone is lacking, the collagen fibres can be more disorganized, and the fibres may be randomly dispersed in the amorphous proteoglycan gel. The difference in proteoglycan concentration between the two tissue zones is well known (Fragonas et al., '98) and it is also known that proteoglycan concentration influences collagen fibre packing (Katz et al., '86).

Finally, it is of interest to note that both human and porcine "loaded" articular cartilages show four histological zones but they differ in the quantitative distribution of collagen (Venn, '78, '79; Fragonas et al., '98; Cova et al., '98). The fact that human and porcine "loaded" cartilages give different MR images (Cova et al., '98; Fragonas et al., '98) confirms the importance of collagen concentration in the MRI appearance of the tissue. In addition "loaded" and "unloaded" pig cartilages differ in collagen organization and also produce different MR images. As a result, we suggest that the spatial organization (i.e., the orientation, the local concentration, the physical dimensions) of collagen fibres might also influence the MRI appearance of the tissue.

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