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Role of uppermost superficial surface layer of articular cartilage in the lubrication mechanism of joints

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ABSTRACT

The uppermost superficial surface layer of articular cartilage, the 'lamina splendens' which provides a very low friction lubrication surface in articular joints, was investigated using atomic force microscopy (AFM). Complementary specimens were also observed under SEM at −10 °C without dehydration or sputter ion coating. Fresh adult pig osteochondral specimens were prepared from the patellas of pig knee joints and digested with the enzymes, hyaluronidase, chondroitinase ABC and alkaline protease. Friction coefficients between a pyrex glass plate and the osteochondral specimens digested by enzymes as well as natural (undigested) specimens were measured, using a thrust collar apparatus. Normal saline, hyaluronic acid (HA) and a mixture of albumin, globulin, HA (AGH) were used as lubrication media. The surface irregularities usually observed in SEM studies were not apparent under AFM. The articular cartilage surface was resistant to hyaluronidase and also to chondroitinase ABC, but a fibrous structure was exhibited in alkaline protease enzymes-digested specimens. AFM analysis revealed that the thickness of the uppermost superficial surface layer of articular cartilage was between 800 nm and 2 μm in adult pig articular cartilage. The coefficient of friction (c.f.) was significantly higher in chondroitinase ABC and alkaline protease enzymes digested specimens. Generally, in normal saline lubrication medium, c.f. was higher in comparison to HA and AGH lubrication media. The role of the uppermost, superficial surface layer of articular cartilage in the lubrication mechanism of joints is discussed.

Key words: Uppermost superficial surface layer; articular cartilage; lubrication; atomic force microscopy; protein.

INTRODUCTION

Adult articular cartilage is a remarkably resilient tissue, characterised by a highly differentiated structural anisotropy. Articular cartilage is composed of about 70% water, 25% extracellular matrix protein and 5% cells. Histologically, adult articular cartilage consists of 4 zones, i.e. superficial or tangential, transitional, radial and calcified zone. The superficial zone, an acellular nonfibrous region is called the uppermost superficial surface layer (Fig. 1). Zonal organisation in the deeper layers, which is largely responsible for the special mechanical properties of the tissue, especially its compressive and tensile properties (Torzilli, 1984; Mow et al. 1992; Guilak et al. 1994), has been investigated by Wong & Hunziker (1998).

The normal structure and properties of the superficial zone are of great importance for the weight bearing function of cartilage (Setton et al. 1993) and its morphology influences the wear and lubrication mechanism operating between the joint surfaces. (Swanson, 1979) The intact, superficial surface provides a very highly efficient lubrication mechanism with an extremely low coefficient of friction. Hence, any damage to the superficial cartilage zone may leads to cartilage breakdown and osteoarthrosis.

Superficial articular cartilage is covered with a highly viscous fine granular electron dense material. The structure and composition of the articular surface

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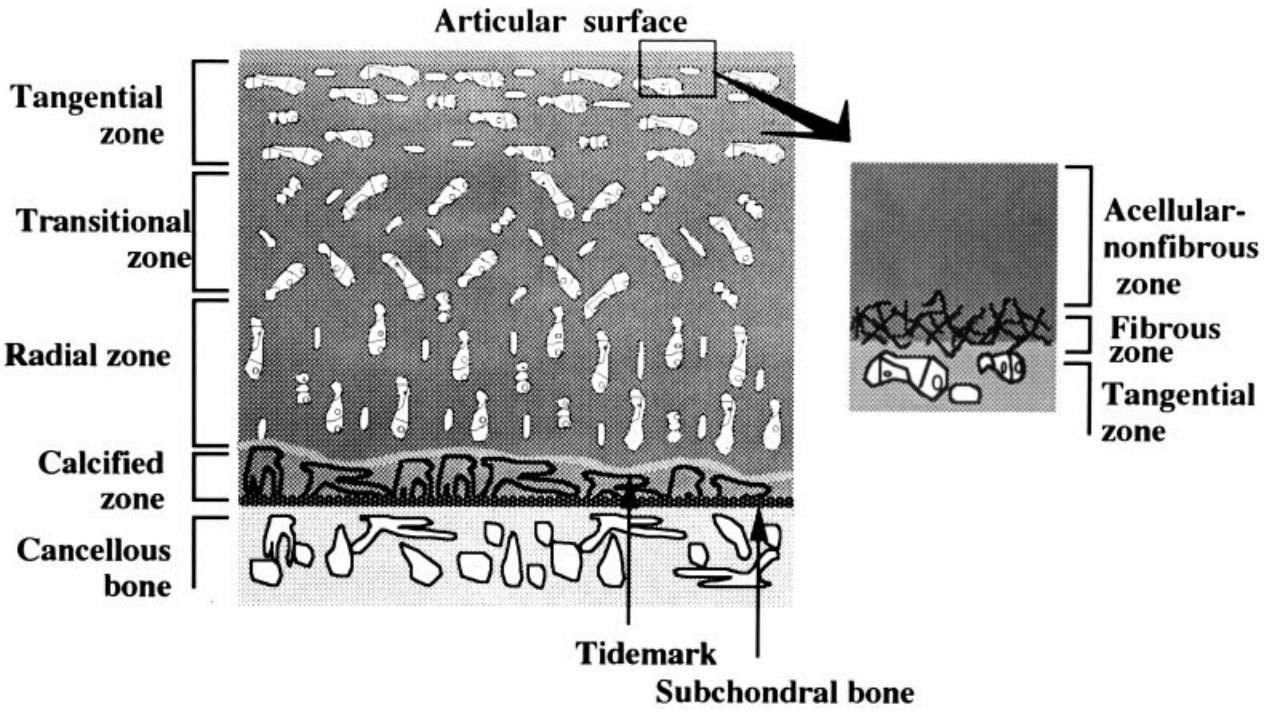

Fig. 1. Schematic representation of adult articular cartilage showing zonal structure. The uppermost superficial surface layer of articular cartilage represents an acellular nonfibrous zone, a fibrous zone and an underlying tangential zone (inset).

has been the subject of considerable debate in the recent past (Weiss et al. 1968; Clarke, 1971; Gardner, 1972; Ghadially et al. 1982; Stanescu et al. 1985; Jeffery et al. 1991; Kobayashi et al. 1995; Jurvelin et al. 1996). There is a growing body of evidence indicating that the surface irregularities such as humps, pits, ridges, and undulations observed under conventional SEM are shrinkage artifacts (Bloebaum & Wilson, 1980; Kirk et al. 1993). Jurvelin et al. (1996) reported that the most superficial layer, typically 200–500 nm thick is acellular and nonfibrous and cannot be eliminataed or digested completely with chondroitinase AC. Stanescu et al. (1985) reported that the articular surface is covered by a fine granular electron dense material, proteinic in nature. According to him, this proteinaceous material can be digested with trypsin and chymopapain and is resistant to testicular and microbial hyaluronidase, keratanase and chondroitinase ABC.

Atomic (or scanning) force microscope (AFM/SFM), and cryoscanning electron microscopy (Cryo SEM) are advanced research instruments and methods to observe the compliant articular cartilage surface in its hydrated state. Briefly, in AFM, a sharp tip stylus, attached to a small cantilever scans the surface of the sample within a subnanometer range of resolution (Binnig et al. 1986). In a constant force mode of operation, the cantilever signal, i.e. contact force, remains constant owing to a sensitive servo system which adjusts the sample height during scanning. Hence the servo signal is a direct function of surface height (z) at any point (x, y) on the scan plane and thereby serves to map the surface topography of the object. In addition, measurement of the tip indentation and contact force renders possible an estimation of local stiffness, elasticity and viscosity (Pethica & Oliver, 1987; Radmacher et al. 1993; Hansma & Hoh, 1994).

In this study, we used AFM to characterise the surface topography of fresh pig patellar articular cartilage maintained in a physiological solution. Using various enzymes, we tried to determine the composition of the uppermost superficial surface layer of articular cartilage and its effects on the coefficient of friction (c.f.). In addition to AFM, matched samples were also examined by low temperature SEM (LTSEM) in their natural physiological conditions, i.e. without critical point dehydration and sputter ion coating.

MATERIAL AND METHODS

Intact knee joints of adult pigs were obtained from a local slaughter house immediately after death and stored at -20 °C, but not for more than 48 h. Two

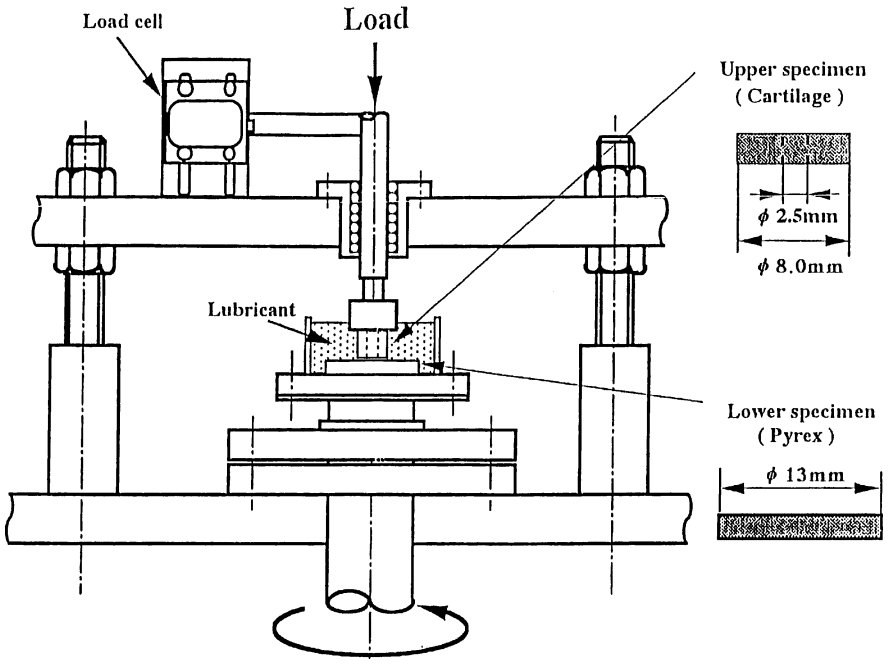

Fig. 2. Thrust collar apparatus for friction tests. The lower shaft supported by ball bearings was driven by a variable speed motor. The upper shaft supported by a slide roll bearing, can move vertically and rotate with negligible friction. Load was applied with dead weights, which were attached on the upper end of the shaft. Friction force was measured by a bar attached to the upper shaft which was connected to the load cell and to the recorder.

8 mm specimens were taken from the central portion of each patella, a total of 36 specimens in all. Full thickness osteochondral specimen cylinders, 8 mm in diameter, were drilled from the patella after opening the joints immediately before enzymatic digestion. About 2–4 mm of underlying subchondral bone was retained in order to produce a full thickness osteochondral specimens. The specimens were irrigated with PBS during preparation. Enzymes used in this experiment were hyaluronidase, chondroitinase ABC (protease free) and alkaline protease. In each part of study, at least 3 osteochondral specimens were used for friction tests and at least 2 specimens were scanned under AFM and LTSEM.

Enzymatic digestion prior to friction testing

Hyaluronidase digestion. Hyaluronate lyase from *Streptomyces hyalurolyticus* (Seikagaku corporation, Tokyo, Japan) at a strength of 200 TRU/ml was constituted and digestion was carried out for 12 h in each specimen.

Chondroitinase ABC '*protease free*' (*chondroitinase ABC*) *digestion*. Chondroitinase ABC lyase from Proteus vulgaris (Seikagaku corporation, Tokyo, Japan) at a strength of 1 unit/ml and 5 units/ml was prepared and the digestion was carried out for 12 h in each specimen.

Alkaline protease digestion. Microbial serine proteinase from *Streptomyces griseus* (Seikagaku corporation, Tokyo, Japan) at a strength of 1 unit/ml and 50 units/ml were constituted and digestion was carried out for 2 h in each specimen.

Each enzyme was prepared according to the supplier's specifications. The duration of digestion and concentration of various enzymes were followed, either according to the supplier's specification or according to available literature.

Friction test

Friction tests were performed in a thrust collar apparatus (Fig. 2). In each test 3 osteochondral specimens were used. In each test, load applied was 27 N and the revolving speed was 1 mm/s. All friction tests were performed between the osteochondral specimens and a pyrex glass plate. After each test, the glass plate was either changed or cleaned with alcohol.

After the initial load for 3 min, each test was run for 10 min. Normal saline, hyaluronic acid 'molecular weight 190' (HA190), and a combination of albumin 3 wt%, globulin 0.5 wt%, and hyaluronic acid

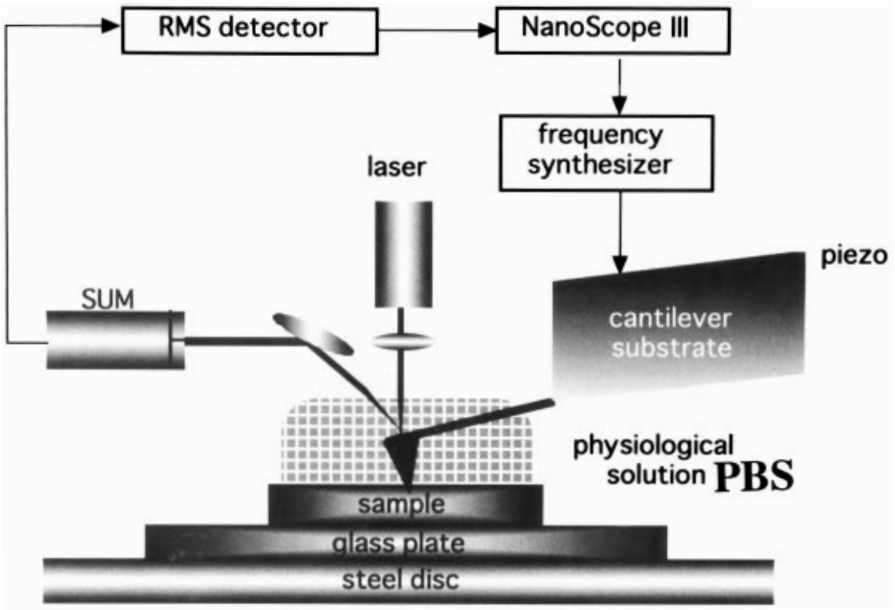

Fig. 3. Arrangement for AFM imaging of the articular cartilage surface. A cylinder of full thickness osteochondral specimens either digested with enzymes or undigested was mounted on the piezoscanner and covered with PBS solution. Oxide-sharpened Si_3N_4 tips were used for imaging. The site of analysis could be accurately determined, with a stereomicroscope.

0.375% (AGH) were chosen as lubrication media. Undigested (natural) specimens, i.e. without enzymatic treatment, were tested as controls. The results of measurements of coefficient of friction were analysed by Fisher's PLSD. c.f. was calculated by the simple equations.

c.f. = frictional force/load

Specimens after friction tests were observed under AFM and SEM.

Atomic force microscopy (*AFM*)

For AFM observations the bony surface of each specimen disc was dried with a paper towel and immediately glued to a glass slide using fast drying aronalfa glue. During glueing, the articular surface was moistened with drops of phosphate buffered saline (PBS) solution and the cartilage surface was not touched at any time before the scanning. Using double-sided adhesive tape, the glass slide was fixed to a steel disc which was magnetically mounted on the piezoscanner of the AFM. The sample was then submerged in PBS solution (Fig. 3). All observations were conducted in an aqueous environment using the liquid cell of the AFM (Nanoscope III digital instrument, Santa Barbara, CA). Cantilevers with a nominal force constants of $k = 0.094$ and $k = 0.022$ N/m and oxide-sharpened Si_3N_4 tips (Olympus Ltd. Tokyo, Japan) were used for imaging. At least 2 specimens in each study were observed under AFM and about 60% of total surface was scanned in each specimens.

In AFM, as the tip encounter elevation changes on the surface, the cantilever deflects. An optical lever is typically used to detect and amplify this deflection. Thus the height changes and raster scanning in the x and y directions were all accomplished with a piezoelectric crystal. In order to image the sample gently, the deflection signal was minimised by optimising gains and the scan speed. The scan speed was 1–3 lines per second and scan size was ranging from 50 μm to 1 μm. Images were obtained in the constant force mode or height mode. Images taken in this mode (height mode) were generally displayed in shades of grey, such that the highest areas were lightest and the lowest darkest.

Low temperature scanning electron microscopy (*LTSEM*)

For the SEM examination specimens were the same as in AFM examination. Surface observations were carried out after each friction test using SEM (Hitachi S-2380N) with a cool state control unit. For the SEM examinations, the specimens were frozen at −20 °C and loaded on the specimens holder without dehydration or sputter ion coating. Temperature at the

specimen holder was then maintained at -10 °C. Specimens were examined at accelerating voltages of 10 kv.

RESULTS

Surface morphology of natural articular cartilage under AFM using scanning probe microscopy system showed a consistently smooth surface. The surface was featureless and nonfibrous and a high surface viscosity was manifested (Fig. 4). The surface appearances were almost unchanged in the specimens digested by hyaluronidase and by chondroitinase ABC, which showed similarly amorphous features.

A fibrous surface structure appeared when the surface of articular cartilage was digested by alkaline protease (1 unit/ml) for 2 h and became more prominent in a increased concentration of 50 unit/ml and digested for the same period of 2 h (Fig. 5*a*). The observed fibrous structure in constant force mode (height mode) was confirmed in phase mode (Fig. 5*b*). AFM analysis revealed the thickness of uppermost superficial surface layer was 800 nm ~ 2 μm in pig articular cartilage.

Table 1 summarises the results for the coefficient of friction between the undigested (natural) articular cartilage and the pyrex glass plate in comparison with specimens digested by hyaluronidase, chondroitinase ABC and alkaline protease in 3 lubrication media, i.e. normal saline, HA190 and AGH. The friction coefficient was almost the same in cases of undigested specimens and the specimens digested by hyaluronidase enzyme. However, the coefficient of friction between the pyrex glass plate and the specimens digested by chondroitinase ABC was significantly higher when compared with the undigested specimens or those digested by hyaluronidase in the AGH lubrication medium or in saline.

In cases of alkaline protease enzyme treated specimens, a significantly high coefficient of friction was recorded in comparison with the undigested specimens in each of the 3 lubrication media (Table 1). Moreover, in alkaline protease enzyme digested specimens, in the first 3–5 min of running a high coefficient of friction was noted and thereafter a stable or irregular phenomenon was observed (Fig. 6).

Generally the coefficient of friction was slightly greater in normal saline and lower in hyaluronic acid and AGH lubricant media in each specimens (Table 1 and Fig. 6).

The surfaces of the treated or untreated articular cartilage specimens under AFM after friction tests did

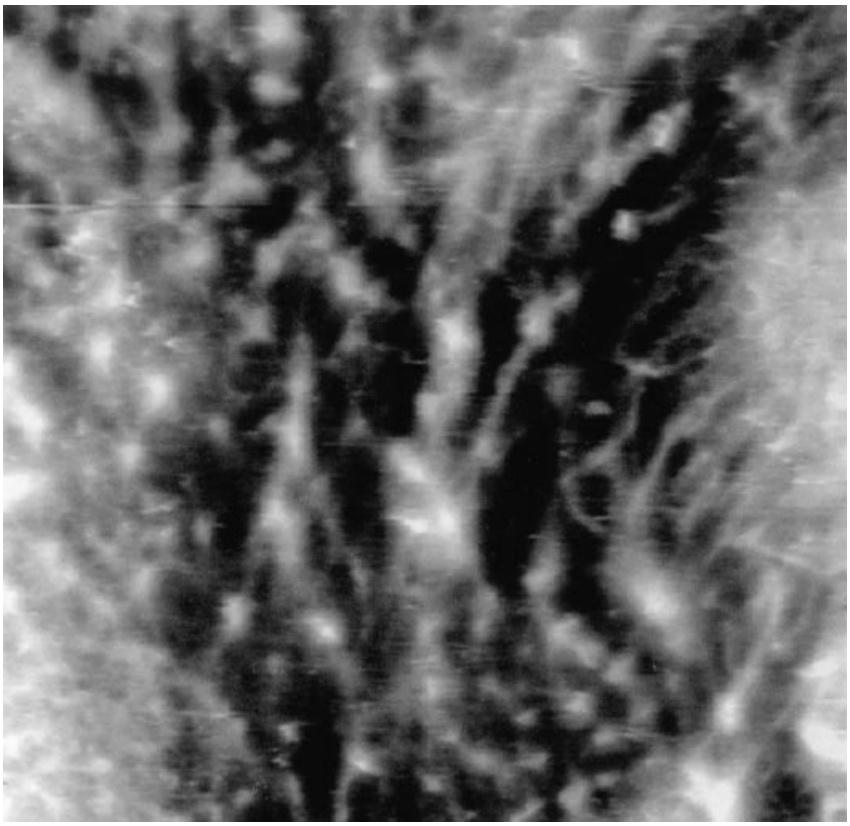

Fig. 4. AFM images of adult pig articular cartilage surfaces. Undigested (natural) articular cartilage reveals a relatively featureless and nonfibrous surface, covered with a high viscocity material. Full scan size 30 μm; full grey ranges 1700 nm.

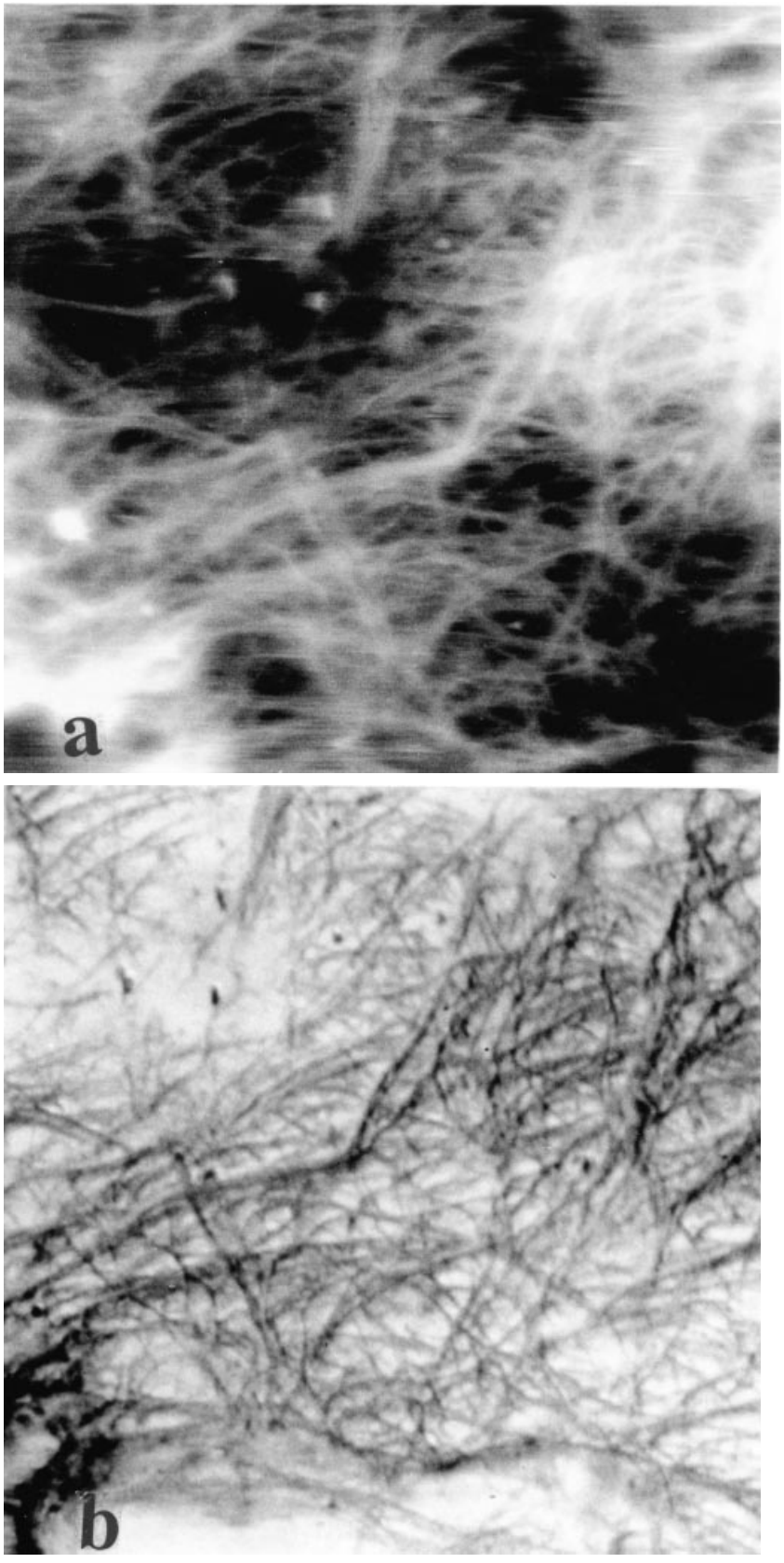

Fig. 5. AFM images of adult articular cartilage surface after digestion with alkaline protease enzyme (50 unit/ml) for 2 h. In constant force mode (height mode); network of fibrils (tangential zone) aligned predominantly parallel to the surface is seen. Phase mode at $0 \sim 30°$ confirms the existence of a fibrous network.

Table 1. *Friction co-efficient between articular cartilage* (*undigested and digested by hyaluoronidase*, *chondroitinase ABC*, *alkaline protease enzymes*) *and Pyrex glass plate in various lubrication medium*

Lubricant	Undigested (natural) (n = 3)	Hyaluronidase 200 TRU/ml 12 h (n = 3)	Chondroitinase ABC (potease free) 1 unit/ml 12 h (n = 3)	Alkaline protease 1 unit/ml 2 h (n = 3)
Normal saline	0.0028 ± 0.0007	0.0025 ± 0.0012	0.0043 ± 0.0013*	0.0070 ± 0.0003**
HA (m.w. 190)	0.0025 ± 0.0005	0.0025 ± 0.0012	0.0038 ± 0.0005	0.0067 ± 0.0004**
AGH (albumin-3.5wt% globulin-0.5wt% HA-0.375%)	0.0024 ± 0.0005	0.0017 ± 0.0010	0.0048 ± 0.0009*	0.0053 ± 0.0003**

Statistical significance was evaluated between undigested and digested samples. *, $P < 0.05$; **, $P < 0.01$

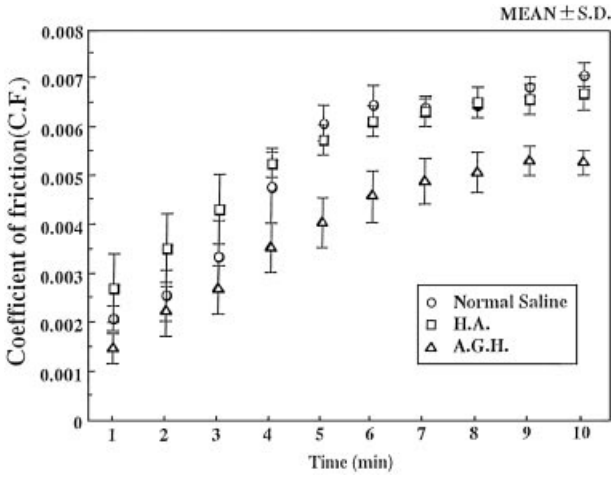

Fig. 6. Coefficient of friction between articular cartilage digested with alkaline protease enzyme (1 units/ml) for 2 h and the pyrex glass plate. Tests were performed in 3 lubricants media, i.e. normal saline, HA (M.W.190), AGH (3.5wt% albumin, 0.5wt% globulin and 0.375% HA). A load of 27 N and a speed of 1 mm/second were kept constant in each friction test. Each test was performed for 10 min.

not show any particular features. Streaks of viscous material were visible as wear tracks on the surface, but the surface was nonfibrous (Fig. 7).

Although examination under SEM at −10 °C and without dehydration and sputter ion coating was possible, the resultant images were of low resolution. When examined under LTSEM, the specimens that were observed under AFM showed short ridges and undulations (Fig. 8).

DISCUSSION

Until now the characterisation of the surface and the subsurface has been performed either by techniques such as SEM and TEM involving dehydration, (Meachim & Roy, 1969; Ghadially, 1983; Jeffery et al. 1991) or by cryotechniques such as cryoSEM (Gardner et al. 1981; Kobayashi et al. 1995). However, fixation and subsequent dehydration produces distortions and shrinkage artifacts in conventional SEM (Bloebaum et al. 1980; Ghadially, 1983; Kirk et al. 1993). Crystal formation in cryospecimens is probably the source of artifacts in cryoSEM. Furthermore, LTSEM without sputter coating reduces conductivity and thereby diminishes resolution. In the present study, the images under LTSEM were also of low resolution. Gardner et al. (1981) reported that low temperature microscopy of cartilage revealed a pattern of secondary surface irregularities and of tertiary elevations closely resembling those seen by conventional scanning electron microscopy of fixed dehydrated hyaline cartilage. According to their observations the free surface of the cartilage was obscured by a layer of vitrified fluid. It was considered to be frozen aggregates of synovial fluid, of synovial fluid debris or of synovial fluid cells. In joint lubrication, fluid on and in the articular cartilage plays a very important role both in fluid film and boundary lubrication. To investigate the latter lubrication mode, it is necessary to observe the surface in wet conditions.

The atomic (or scanning) force microscope (AFM/SFM) in this aspect, furnishes a new technique for imaging the surface topography of a number of biological structures maintained in physiological solution. (Binnig et al. 1986) It has been a useful technique to examine the compliant articular cartilage in its natural physiological conditions (Jurvelin et al. 1996). The present study has also shown its applicability in investigating the structure of normal and digested articular cartilage before and after friction tests.

Ghadially (1983), in a study on rabbit articular cartilage, reported that the surface of articular cartilage is covered with a surface coat, 30 nm ~ 1 μm thick, woolly in texture and transient in nature. He suggested that the composition of this electron dense

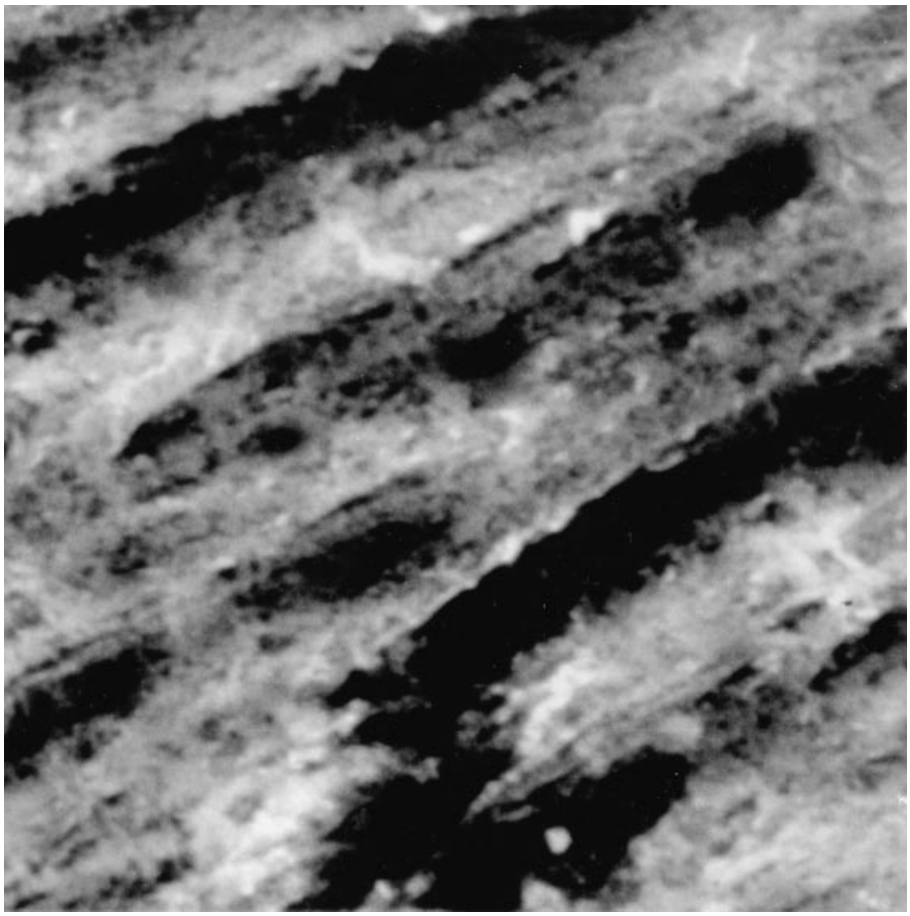

Fig. 7. AFM images of articular cartilage surface after friction tests in normal saline as lubrication medium. Articular cartilage surface, digested with hyaluronidase enzyme (200 TRU/ml) for 12 h. Streaks of viscous material are visible as wear tracks on the surface, but the surface is nonfibrous. Full scan size 10 μm; full grey ranges 700 nm.

particulate layer may be a mixture of extraneous material (e.g. precipitated synovial fluid) and/or material extruded from the cartilage (e.g. lipid debris and degraded metabolites discharged into the joint space). Orford & Gardner (1985) reported that in canine articular cartilage, 2 collagen-poor ultramicroscopic layers were present which were distinguished by staining with the electron-dense cationic dye, 'Cupromeronic blue', with a critical electrolyte concentration technique and by digestion with testicular hyaluronidse. The superficial layer, 50 nm thick, was stained at low electrolyte concentration, but failed to stain in conditions specific for sulphated glycosaminoglycans. It was hyaluronidase-resistant and could either have been glycoprotein or protein in nature. The deeper layer, 100 nm $\sim$ 400 nm thick, stained positively at an electrolyte concentration specific for sulphated glycosaminoglycans. This layer, they suggested, probably represented a chondroitin sulphate rich proteoglycan. These layers are important in the lubrication of the articular surface. Balaz et al. (1966) suggested that the uppermost superficial surface layer may represent a synovial protein-hyaluronate complex. Our AFM analysis confirmed the existence of a nonfibrous surface which is typically 800 nm $\sim$ 2 μm thick in adult pig articular cartilage. It is resistant to hyaluronidase, partially digested with chondroitinase ABC (protease free) and is completely digested by alkaline protease, which suggests that the superficial layer is either glycoprotein and/or protein. However, the specific nature of this proteinous material needs to be investigated.

Oike et al. (1980) reported that commercial preparations of chondroitin ABC lyase and chondroitin AC lyase have significant amounts of proteinase activity and suggested that studies which employed these commercial enzyme preparations for their presumed ability to degrade only glycosaminoglycans may require re-evalution. In present study, protease free chondroitinase ABC was used which suggested that the superficial layer not only contains glycosaminoglycans but also proteinaceus material.

The excellent lubrication in natural joints can be attributed to fluid film lubrication in which joint surfaces rarely come into direct contact (Oka et al. 1990; Ikeuchi et al. 1994). However, at a constant

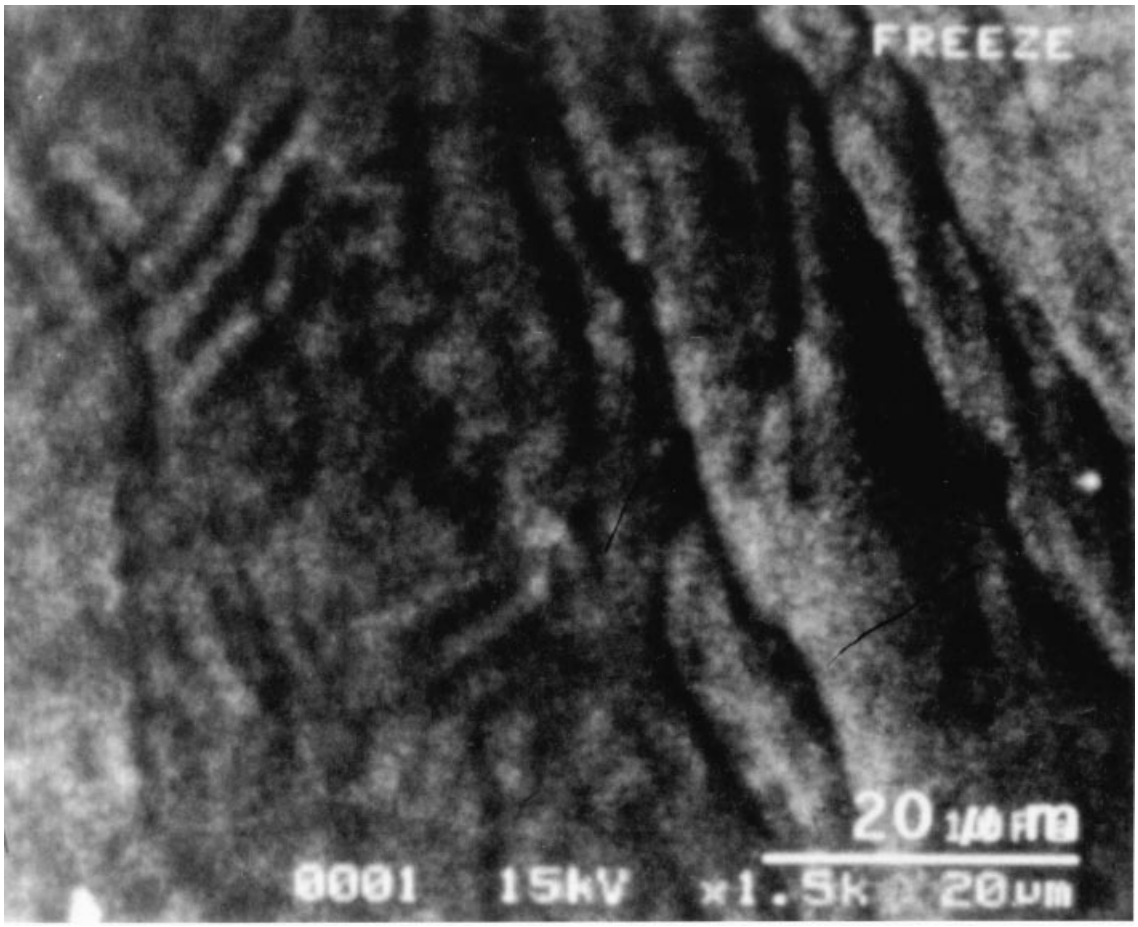

Fig. 8. The articular cartilage surface of same specimens after friction tests analysed in TSEM. Short ridges and undulations are seen on untreated specimens.

level of weight bearing, boundary and weeping lubrications also operates. In boundary lubrication, some high molecular weight substances, for example, HA and protein, are important. In joint lubrication, a role for protein and hyaluronic acid present in synovial fluid has been investigated by Chikama (1985). David et al. (1981) reported that glycoprotein-1 (LGP-1) is the molecule responsible for the lubricating properties of synovial fluid.

The results of the present study show that at a low speed of 1 mm/s, the coefficient of friction was low between pyrex glass plate and natural (undigested) articular cartilage, as well as between pyrex glass plate and hyaluronidase digested specimens in normal saline lubrication medium. This suggests that the surface layer of articular cartilage is probably composed of absorbed molecules which forms a gel film on the rubbing surface. Hyaluronic acid and AGH lubricant media further facilitate the lubrication mechanism, leading to a decrease in the friction coefficient. However, articular cartilage digestion with chondroitinase ABC leads to a high coefficient of friction which may be due to loss of some lubrication activity at the cartilage surface, most probably, the glycosaminoglycan activity which is digested selectively by chondroitinase ABC enzyme.

Finally, the enzymatic digestion with alkaline protease denuded the articular cartilage surface of its amorphous layer, thereby exposing the underline fibrillar structure. It also digested the cartilage protein as a whole, which leads to softening of the cartilage and a increase in creep deformation and wear.

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