In Vitro Enzymatic Treatment and Carbon Dioxide Laser Beam Irradiation of Morphologic Cartilage Specimens

Jiannis K. Hajiioannou, MD; Antonios Nikolidakis, MD; Irene Naumidi; Emmanuel Helidonis, MD; Giannis Tzanakakis, MD; George A. Velegrakis, MD

Objectives: To determine the role of the main cartilage components in the internal system of interlocked stresses and to clarify the effect of laser beam irradiation on cartilage.

Design: Control and experimental series.

Subjects: Rabbit ear cartilage.

Intervention: Rabbit ear cartilage strips incubated in collagenase and hyaluronidase enzyme solutions for specific periods were examined, and the observed changes in shape, strength, and elasticity were recorded, as well as the effect of carbon dioxide laser irradiation. Laser-pretreated cartilage strips were also incubated in the enzyme solutions to determine whether the laser-provoked changes were susceptible to enzymatic action. All cartilage pieces were examined by light and electron microscopy.

Results: Collagenase-treated cartilage strips gradually lost their interlocked stresses, while hyaluronidase-treated strips mostly maintained their shape and their physical characteristics. Hyaluronidase-incubated cartilage strips altered their shape when they were laser treated. Collagenase-treated cartilages did not modify their shape when they were laser treated. Laser-pretreated cartilage pieces lost their new form in collagenase solutions but kept their laser-evoked shape when put in hyaluronidase solutions.

Conclusion: The macroscopic observations combined with light and electron microscopy findings argue for the distinct role of the collagen network in morphologic cartilage shape and tensile strength preservation and provide a probable mechanism of cartilage transformation owing to carbon dioxide laser irradiation.


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tilage specimens) maintain their shape indefinitely because of their molecular structure and because of a system of interlocked stresses inherent in their molecular structure and configuration.10

Results of investigations by Fry11 suggest that the internal stress alignment is a function of the protein polysaccharide complex. Therefore, the collagen network only acts as a skeleton for binding the mucopolysaccharides of the protein polysaccharide complex and without any specific role in the stress system. Subsequent investigations showed a relationship between tensile stiffness and the proteoglycan component of the cartilage.12 No such relationship between tensile stiffness and the proteoglycan component was established.

The collagen network resists tensile and shear stresses and strains, while the fluid component resists the high hydrostatic compressive stresses that are generated. The cartilage cells are directly attached to the extracellular matrix and are part of the fluid continuum of the tissue. The cells do not provide any special structural resistance, but their cellular shapes, pressures, and chemical environments are directly altered by deformations created in the extracellular matrix and by local fluid pressure and flow.13

In our study, we determined the role of the cartilage components in the internal system of interlocked stresses that maintain its shape and elasticity. We also clarified the effects of laser beam irradiation that modify the natural characteristics of cartilage.

## METHODS

In 1967, Fry11 used enzymatic incubation of cartilage pieces to determine whether collagen or proteoglycans were responsible for the cartilage internal stresses. We modified his experimental method by adding laser irradiation and microscopic examination. Institutional guidelines regarding animal experimentation were followed. Rabbit ear cartilage was used because of its relative uniformity and its sizeable surface. Numerous pieces of equally sized ear cartilage obtained from recently humanely killed rabbits of the same age were used as experimental material. Previous investigation showed that any 2 adjacent ear cartilage strips of the same size have almost identical histological and physical characteristics; furthermore, the deformation produced by partial release of the interlocked stresses is virtually identical as long as both pieces are treated in the same manner.11

Ear cartilage was peeled off the skin and cut in pairs of pieces of approximately the same width, length, and thickness (1 cm × 2 cm × 1 mm). One of the pair served as a control specimen, with the other comprising the experimental series. To determine whether chondrocytes play some role in the internal stress system, 8 matched pairs of ear cartilage were frozen in liquid nitrogen to cause cellular death. Cartilage pieces were then thawed. The observed deformation in control and experimental pieces was equal, suggesting that the internal stress system did not depend on the participation of metabolizing chondrocytes but was rather a property of the components already produced by the cells.

A control series and an experimental series of matched cartilage strips were incubated at 37°C in small plastic vials. Each vial was filled with 8 mL of Krebs-Ringer phosphate buffer solution containing 1.2 g of amoxacillin sodium plus clavulanic acid per liter to avoid bacterial growth in the solution. Collagenase (from Clostridium histolyticum type II; Sigma Chemical Co, St Louis, Mo) and hyaluronidase (bovine testes sterile fil-

terized lyophilized powder, Sigma Chemical Co) were added to the vials of the experimental group. The amount of enzyme added to the experimental incubating vials was weighed. The concentrations of the hyaluronidase solutions were 0.5 mg/mL, 0.75 mg/mL, and 1 mg/mL; the collagenase solution concentration was 0.25 mg/mL. These concentrations were based on previous experiments of enzymatic actions on cartilage.11

Cartilage strips were removed from the vials at different time points (at 2, 8, 16, 24, 48, and 72 hours) after their incubation. They were photographed, macroscopically studied, and prepared for microscopic examination. In addition, a series of enzymatically treated strips and their controls at the same time point were irradiated with carbon dioxide laser, and the alterations in their shape were recorded and digitally photographed. Strips that had already been treated by laser irradiation and had obtained a curved shape were also incubated in the enzymatic solutions to detect whether the laser-provoked shape modifications were susceptible to enzymatic action.

The laser beam was delivered in the central area of a single surface in the group of irradiated cartilages by means of a carbon dioxide laser (model 1040; Sharplan Laser Industries, Ltd, Tel Aviv, Israel) at a wavelength of 10.6 µm. The laser beam was focused to a spot size of 2 mm with a barium fluoride lens, transparent at 10.6 µm, and with a focal length of 400 mm mounted on a surgical microscope. Intermittent exposures were used; the exposure time was 0.5 seconds, and the output power was 3 W. In each exposure, the energy level reached 48 J/cm². To remodel each cartilage, 20 to 30 pulses of 0.5 seconds each, equivalent to 60 to 90 J, were required. These laser variables were based on the results of previous experiments.3

The specimens were fixed in cold 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). After overnight fixation in glutaraldehyde, samples were postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) for 1 hour at 4°C, dehydrated in a series of alcohols and in propylene oxide, and then embedded in epoxy resin.

For light microscopic examination, 1-µm sections were prepared. They were stained with modified trichrome stain for epoxy resin-embedded specimens.

For electron microscopic examination, the selected areas were thin sectioned and stained with uranyl acetate and lead citrate. Imaging was carried out with an electron microscope (JEM 100CX; Hitachi, Tokyo, Japan).

## RESULTS

### ENZYMATIC AND LASER-INDUCED ALTERATIONS

Hyaluronidase has a selective enzymatic effect on the acid polysaccharide chains of proteoglycans. This enzyme depolymerizes chondroitin 4 sulfate, chondroitin 6 sulfate, and hyaluronan without affecting the protein core of the complex.14-16

The incubation experiments were carried out in Krebs-Ringer phosphate buffer solution using 30 matched pairs of cartilage strips. Cartilage pieces incubated in hyaluronidase in the higher concentration (1 mg/mL) did not show perceptible alterations after 72 hours. The strips seemed to be slightly softened and somewhat augmented after 48 hours and 72 hours of incubation, a finding also noted in the control group that might be attributed to cartilage water intake (Figure 1A).

Up to 40% of the dry weight of cartilage matrix is collagen in the form of an interlacing fabric of fine fibrils
100 Å to 200 Å in diameter. The enzyme collagenase is specific for collagen fibers and was used in a concentration of 0.25 mg/mL in Krebs-Ringer phosphate buffer solution to act on the experimental cartilage pieces. Twenty-four matched pairs of rabbit ear cartilage were used. Cartilage incubated in collagenase gradually loses its integrity as the collagen dissolves in the solution, progressively abolishes its interlocked stresses, and loses its elasticity, while keeping its shape (Figure 1B and C). With further degradation, the cartilage strip finally loses its form and turns into a gel mass (Figure 1D).

When laser irradiated, cartilage strips of the control group curved predictably toward the laser beam, obtaining a new permanent shape. Hyaluronidase-incubated strips responded in a similar way to laser irradiation, bending toward the beam direction (Figure 2). In contrast, cartilage strips incubated in collagenase for 8 hours curved randomly when laser was applied to a surface (Figure 3). Sixteen hours after incubation in collagenase solution, no response of cartilage to laser was noted. Strips pretreated by laser that had obtained a curved shape before their incubation in enzymatic solutions mostly kept their curved shape when they were incubated in nonenzymatic and hyaluronidase-containing solutions. Laser-pretreated cartilage strips seemed to gradually alter their shape after 8 hours of incubation in collagenase solution and returned to their original form after 16 hours. The enzymatic and laser-induced macroscopic alterations on cartilage are summarized in Table 1 and Table 2.
Table 1. Hyaluronidase and Laser Irradiation Effect on Cartilage

<table>
<thead>
<tr>
<th>Cartilage Strip</th>
<th>Hyaluronidase Concentration, mg/mL</th>
<th>Time, h</th>
<th>Internal Stresses</th>
<th>Laser Irradiation Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>2</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.75</td>
<td>2</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>0.75</td>
<td>2</td>
<td>Present</td>
<td>Not irradiated</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>48</td>
<td>Present, slightly softened</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>1.00</td>
<td>48</td>
<td>Present, slightly softened</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>1.00</td>
<td>48</td>
<td>Present, slightly softened</td>
<td>Not irradiated</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>72</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>1.00</td>
<td>72</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>1.00</td>
<td>72</td>
<td>Present</td>
<td>Not irradiated</td>
</tr>
</tbody>
</table>

Table 2. Collagenase and Laser Irradiation Effect on Cartilage

<table>
<thead>
<tr>
<th>Cartilage Strip</th>
<th>Collagenase Concentration, mg/mL</th>
<th>Time, h</th>
<th>Internal Stresses, Elasticity, Shape</th>
<th>Laser Irradiation Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>8</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.25</td>
<td>8</td>
<td>Partially abolished, softened, kept shape</td>
<td>Bent unpredictably</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>0.25</td>
<td>8</td>
<td>Partially abolished, softened, shape slightly changed</td>
<td>Not irradiated</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>16</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.25</td>
<td>16</td>
<td>Abolished, lost elasticity, kept shape</td>
<td>No effect</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>0.25</td>
<td>16</td>
<td>Abolished, lost elasticity, returned to pretreatment shape</td>
<td>Not irradiated</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>24</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.25</td>
<td>24</td>
<td>Abolished, lost elasticity, kept shape</td>
<td>No effect</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>0.25</td>
<td>24</td>
<td>Abolished, lost elasticity, kept shape</td>
<td>Not irradiated</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>48</td>
<td>Present</td>
<td>Bent toward beam direction</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.25</td>
<td>48</td>
<td>Abolished, lost elasticity, absent</td>
<td>No effect</td>
</tr>
<tr>
<td>Laser pretreated</td>
<td>0.25</td>
<td>48</td>
<td>Abolished, lost elasticity, absent</td>
<td>Not irradiated</td>
</tr>
</tbody>
</table>

Figure 4. Cartilage sample treated with collagenase (incubation time, 2 hours). No visible difference from intact samples can be seen. The structures of the cartilage are distinguishable and are well stained (light microscopy, original magnification ×40).

MICROSCOPIC RESULTS

Light Microscopy

Light microscopy of the specimens processed with hyaluronidase revealed no change in cartilaginous morphologic characteristics based on the hyaluronidase concentration or on the hyaluronidase incubation time (range, 2-72 hours). In addition, staining with modified trichrome stain and methylene blue showed no difference among the specimens in the hyaluronidase-treated group.

The trichrome stain is known to stain pink the collagen structures of extracellular matrix. Therefore, the depth of discoloration characterizes the thickness of the tissue layer where the collagenase destroyed the collagen structures. In the group of specimens processed with collagenase, a significant difference in staining intensity was revealed relative to the incubation time. Incubation for 2 hours did not change the metachromatic appearance of the sample compared with intact rabbit ear cartilage (Figure 4). In contrast, collagenase incubation of trichrome-stained samples for 8 hours resulted in the disappearance of pink coloring of the collagen structures (Figure 5).

The depth of the tissue layer that is nonstainable for collagen increases with the duration of collagenase processing (Figure 6). It was not our goal to determine the association of the time characteristics of collagen destruction with the penetration depth of the enzyme. However, the preliminary conclusion that may be drawn from our observations is that this dependence is nonlinear. The specimens that were processed with enzyme for 16 hours vs 24 hours demonstrated slight if any difference in stain-
ing for collagen. In contrast, in the specimens processed for 48 hours (the maximum incubation time), the depth of the zone of destroyed collagen was only twice as deep compared with the specimens processed for 8 hours. The arrangement of chondrocytes and their morphologic characteristics in the specimens of this group were unchanged compared with intact tissues.

Transmission Electron Microscopy

Transmission electron microscopy of the samples incubated in collagenase revealed destruction of filament structures of the extracellular matrix. In the specimens incubated for 2 hours, the penetration depth of the enzyme was shallow or indistinguishable, even on the electron microscopic level. Enzyme incubation of the specimens for 8 to 48 hours resulted in the creation of a layer of discoloration on the light microscopy specimens. The electron microscopic analysis of these zones confirmed that total destruction of collagen structures occurred within these discoloration zones formed after processing with collagenase (Figure 7). The border of this layer had similar appearances for any incubation time within the range of 8 to 48 hours and moved toward the inner layers with increased duration of incubation.

In the zone of total collagen destruction, the electron microscopic analysis revealed only fine-grained and microfibrillar structures, with few fibrillar fragments of extracellular matrix. The diameter of such fibrils was 2 to 3 times smaller than that of collagen fibrils in the intact tissue, while the cross striation, which is characteristic of collagen fibers, was indistinguishable.

Beneath the zone of total collagen destruction, the extracellular matrix responded slightly to staining for collagen. In addition to granular and microfibrillar components, the electron microscopic analysis of this zone demonstrated the presence of a small amount of fibrils with typical morphologic characteristics of collagen fibrils, which include primarily the cross striation of the fibers and their orientation and diameter. Figure 8 shows the network of widely spaced collagen fibers, which is typical for the zone of partial destruction of collagen. The extracellular matrix of the deeper layers was indistinguishable from that of intact cartilage. The orientation...
of cellular fibrils and their cross striation and diameter were typical for rabbit ear cartilage (Figure 9).

**COMMENT**

Cartilage is an avascular tissue that consists of chondrocytes and an extensive extracellular matrix produced and maintained by chondrocytes. Cartilage matrix is composed predominantly of aggrecan and type II collagen. It is believed that type II collagen is primarily responsible for tensile strength, while aggrecan molecules, entrapped within the type II collagen lattice, provide compressive strength. The highly sulfated glycosaminoglycan side chains of chondroitin sulfate and keratan sulfate enable the matrix to structure large amounts of water and thereby create a large osmotic pressure.17 Long chains of hyaluronan are present throughout the matrix and serve to specifically bind aggrecan and to link protein to retain the aggrecan molecules within the cartilage extracellular matrix.18

Mixture models have been successfully used to describe the response of articular cartilage to various loading conditions. In their classic study, Mow et al19 formulated a mixture model of articular cartilage in which the collagen-proteoglycan matrix is modeled as an intrinsically incompressible porous permeable solid matrix, and the interstitial fluid is modeled as an incompressible fluid. This biphasic model has been used to describe the response of articular cartilage in confined compression creep, stress relaxation, and dynamic loading.19-22 By incorporating the tension compression nonlinearity of the solid matrix into this biphasic framework, the model has been used to describe the response of cartilage in unconfined compression as well.23-25 However, the negatively charged proteoglycans in articular cartilage produce an osmotic pressure, which swells the tissue and contributes to its compressive stiffness.26-30 Proteoglycans are also responsible for various electromechanical effects. These include streaming potentials and currents, reduced tissue permeability.26,39

Lai et al28 proposed a triphasic model of articular cartilage as an extension of their biphasic theory that models cartilage as a mixture of the following 3 phases: (1) an intrinsically incompressible porous permeable charged solid phase, (2) an intrinsically incompressible interstitial fluid phase, and (3) an ion phase with 2 monovalent ions (anion and cation). In this theory, the motive forces for water and ions are described by the gradient of chemical or electrochemical potentials. These driving forces are balanced by the frictional forces between the phases as one phase flows through the other. Stress and strain in cartilage is determined by a balance of tissue elastic properties, fluid flow or shift, and electrostatic charge. This model was later extended to incorporate multiple polyvalent ions by Gu et al.40

In our experiments, inhibition of cellular metabolism by means of the freezing of chondrocytes did not affect the interlocked stresses in the cartilage. This suggests that the maintenance and release of the interlocked stresses are not cellular functions.

The physical outcomes of enzymatic incubating of cartilage strips are notable. The enzymatic effect of hyaluronidase on the protein or polysaccharide portion of the extracellular matrix does not seem to affect the interlocked stresses in the cartilage. This suggests that the maintenance and release of the interlocked stresses are not cellular functions.

The physical outcomes of enzymatic incubating of cartilage strips are notable. The enzymatic effect of hyaluronidase on the protein or polysaccharide portion of the extracellular matrix does not seem to affect the interlocked stresses even in high concentrations of the enzyme. In addition, the laser irradiation response, which
reshapes cartilage by remodeling tensile stresses,7 seemed to be the same for the hyaluronidase-treated cartilage and for the control group of cartilages.

Light microscopy of the hyaluronidase-processed specimens revealed no association of morphologic characteristics of the cartilage with the hyaluronidase concentration or with the hyaluronidase incubation time. No difference among the specimens in the hyaluronidase-treated group was noted.

Surface collagen decomposition (which most likely represents collagen types I, II, and V41) seems to abolish cartilage tensile stresses without altering its shape until total degradation occurs. Internal stress alignment is dependent to a significant degree on the collagen component of the cartilage and on the fixed-charge density attributed to proteoglycans and water flow or shift. This observation is further supported by the effect of laser irradiation on cartilage 8 hours after collagenase digestion. Cartilage strips reshaped in an unexpected fashion, and no response to laser treatment was noted with increased incubation time. Collagenase-treated cartilage strips showed no tendency to curve, suggesting that this was due to a lack of the collagen component.

In the group of specimens processed with collagenase, a significant difference in staining intensity was microscopically seen, appearing as discoloration zones, depending on the incubation time. The electron microscopic analysis of these discoloration zones confirmed the total destruction of collagen structures within them. The border of this layer had a similar appearance for any incubation time within the range of 8 to 48 hours and moved toward the inner layers with increased duration of incubation. In the zone of total collagen destruction, the electron microscopic analysis revealed only fine-grain structures, with few fibrillar fragments of extracellular matrix.

The nonsynchronous-appearing destruction of collagen fibrils could be evidence of the presence of collagen structures with different resistance to collagenase or could be attributed to a nonsynchronous effect of the collagenase type used in different subtypes of collagen.

Cartilage remodeling has been the subject of extensive study in the past. The permanent remodeling of a cartilage implant that is destined to serve as a framework for the reconstruction of traumatized or deformed areas of tissue continues to be a challenge. Thermochondroplasty is a promising new technique in which living cartilage is altered by laser beam application and remains viable afterward. Sobol et al5 suggest that cartilage stress relaxation is due to a phase transformation of water molecules from a bound state (binding to proteoglycans or collagen) to a free or mobile state.

Microscopic findings showed the distortion of the proteoglycans and alterations in the collagen skeleton that were unsatisfactorily elucidated and underestimated. In a previous study9 in which cartilage was irradiated, electron microscopy revealed collagen skeleton alterations of the irradiated specimens compared with control specimens. More specifically, (1) in the plane of histological section, many collagen fibers seemed shorter than normal, (2) the number of ring-shaped collagen components was significantly increased, and (3) the collagen fibers, which normally have a symmetric orientation, especially around the borders of the chondrocyte nests and in the vicinity of the perichondrium, lost their spatial orientation in the irradiated areas. Orientation became more random, resulting in a homogeneous appearance of the matrix.

If cartilage internal stresses and laser reshaping effect were mainly proteoglycan dependent, then the hyaluronidase-invoked disruption should at least partially release them, and the laser effect should be somewhat modified. Also, it should be expected that the laser-pretreated cartilage strips after hyaluronidase incubation would return to approximately their original size. Because the structural integrity in these strips seems to be intact, it is reasonable to suggest that the proteoglycans are at least not directly involved in laser reshaping.

In contrast, as observed in our experimental study and as supported by the microscopic results, collagen degradation was a substantial factor that leads to the release of the cartilage tensile stresses. Laser irradiation did not reshape cartilage strips treated with collagenase after a specific period, and laser-pretreated cartilage strips that already had been reshaped lost their form and returned to their original shape after collagen degradation occurred.

The proteoglycan role is not negligible, and collagen-proteoglycan interaction is vital for mechanical stress and laser-induced stress relaxation in cartilaginous tissue.42 Based on modern triphasic mechanoelectrochemical theory, the internal stress in cartilage is due to the interplay of the tensile collagen II framework, the electrostatic repulsion from charge or deproteinized carboxyl or sulfoxyl groups, and the movement of water. Stress and strain in cartilage are determined by a balance of tissue elastic properties, fluid flow or shift, and electrostatic charge.28

Our observations combined with electron microscopic findings suggest that the contribution of collagen to cartilage internal stresses is more important than is generally believed. In addition, collagen plays a key role in the cartilage laser-reshaping effect, and it may be the primary cartilage constituent responsible for cartilage transformation. Laser shaping is a promising cartilage reconstructive technique that can overcome the problems of the traditional methods such as tissue paucity and shape memory effect and can achieve precise shaping of the newly formed graft, resulting in the desired aesthetic outcome. This study presents the laser-shaping concept from a new perspective and adds to ongoing research in the area of cartilage-shaping techniques.

Submitted for Publication: October 1, 2005; final revision received December 29, 2005; accepted March 15, 2006.

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Drafting of the manuscript: Hajiioannou and Nikolidakis. Critical revision of the manuscript for important intellectual content: Hajiioannou and Velegrakis. Administrative, technical, and material support: Hajiioannou, Nikolidakis, and Velegrakis. Study supervision: Hajiioannou and Velegrakis. Microscopic and histologic analysis: Naumidi and Tzanakakis.

Financial Disclosure: None reported.

Funding/Support: This work was funded and supported by the Department of Otolaryngology, University Hospital of Crete, and by the authors' personal funds.

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