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Patterns of Cartilage Structural Protein Loss in Human Tracheal Stenosis

Leila A. Mankarious, MD; Allison B. Adams, BA, MA; Valerie L. Pires, BS

Objectives: **The study sought to identify which of the major structural proteins in tracheal cartilage are lost in the inflammatory process, and to determine whether damaged cartilage shows signs of regeneration and whether this is an age-dependent phenomenon.** ***Study Design:*** **Immunohistochemical analysis.** ***Methods:*** **Archival human tracheal and subglottic stenosis segments removed for the treatment of airway compromise were investigated by means of immunohistochemical analysis for differential loss of collagen type I or type II or aggrecan.** ***Results:*** **Specimens were found to have preferentially lost collagen I and aggrecan in areas of severe disruption of the cartilage ring. Collagen II was preserved. In addition, areas of apparent cartilage regeneration were identified based on increased collagen II and aggrecan relative to baseline levels in uninjured sections of the rings. Regenerative capacity was present in most of the specimens investigated and was not age specific.** ***Conclusions:*** **Collagen I and aggrecan are lost in areas of severe ring compromise, indicating that at least one of these two molecules is responsible for structural integrity. The remaining cartilage has some regenerative capacity, but it is small relative to the degree of cartilage damage. No new collagen I was identified in the cartilage ring, indicating that, although an intense inflammatory reaction occurred, fibroblasts did not deposit new collagen I as seen in other scar tissues.** ***Key Words:*** **Tracheal stenosis, subglottic stenosis, collagen I, collagen II, aggrecan, cartilage regeneration.**

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INTRODUCTION

Acquired tracheal and subglottic stenosis most commonly results from inflammation and infection within the mucosa, submucosa, and cartilage of the subglottis and trachea. Often, the epithelium and submucosa are thickened and scarred.[1] It is likely that cartilaginous involvement depends on depth of injury and degree of inflammation. Histopathological assessment of resected, stenotic specimens often reveals a loss of cartilage integrity and bulk. Frequently, the cartilage rings collapse, further narrowing the airway. In the present investigation, we sought to identify which cartilaginous, structural proteins are most susceptible to the inflammatory process and thereby degraded. Also, we sought to determine whether the cartilage maintains a regenerative capacity and whether that capacity is lost with advancing age.

Hyaline cartilage is named for its glass-like, gross appearance (from the Greek, *hyalos*, for "glass"). It is found predominantly in articular, weight-bearing surfaces and in the trachea and larynx where flexibility and strength are necessary. Investigations of hyaline cartilage have focused mainly on articular cartilage, because of the large numbers of individuals with arthritic disabilities. Although mature hyaline cartilage in the joint and cartilage within the trachea are similar in appearance, they differ in their collagen and proteoglycan ratios.[2] Cohen et al.[3] have demonstrated a proportionately larger amount of collagen type I in tracheobronchial cartilage than in articular cartilage. In fact, the main structural components of cricoid and tracheal hyaline cartilage appear to be collagen I and aggrecan. Although collagen type II is also present in tracheal cartilage, it has a relatively low concentration, representing approximately 10% to 20% of collagen content based on cyanogen bromide digestion. In articular hyaline cartilage, collagen I plays a minor role, whereas collagen II and aggrecan are the main structural proteins. The significance of collagen type I versus collagen II as a structural protein is not clear because the two types have a similar structure.

Aggrecan is the principal proteoglycan secreted by the chondrocyte that provides the unique compressible characteristics of cartilage. Its structure consists of a long protein core to which many chondroitin sulfate and keratan sulfate side chains are attached. The end result is a "bottle-brush" structure. Because the side chains contain many negatively charged sulfate groups, inorganic counter ions are attracted, creating a markedly hydrophilic environment. Therefore, the entire bottle-brush structure is well hydrated and provides load-bearing and compressive properties. Many aggrecan molecules are

From the Department of Otology and Laryngology, Massachusetts Eye and Ear Infirmary, Harvard School of Medicine, Boston, Massachusetts.

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Send Correspondence to Leila A. Mankarious, MD, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114, U.S.A. E-mail: leila_mankarious@meei.harvard.edu

fixed in the cartilage matrix by their ability to bind to hyaluronan, a long-chain, unbranched protein.[4]

Collagen II is the principal collagen secreted by chondrocytes and is specific to cartilage. It is a marker for proliferating, differentiated chondrocytes and serves to distinguish the chondrocytes from their mesenchymal precursors, which secrete collagen type I. Collagen II structure comprises three identical polypeptide chains (alpha chains) twisted into a right-handed helix. Each helix is further bonded both internally and to other collagen II helices. The end result is a tightly bound lattice of collagen II fibrils. Collagen II provides the tensile strength of the cartilage structure; hence, a balance exists between the intrinsic tendency of the cartilage to swell, because of its hydrophilic aggrecan molecules, and the rigidity attributable to the collagen II lattice.[5,6]

Collagen I is the fibrillar collagen of bone, tendon, dentin, and dermis. The distribution pattern of collagen I suggests that it plays a major supporting role in tissues that normally exhibit little distensibility under mechanical stress, similar to collagen II in articular cartilage. Collagen I is thought to be produced by the mesenchymal precursors of chondrocytes; however, it is known to remain in the extracellular matrix after conversion of the precursor cells to mature chondrocytes.[2] It has a structure similar to that of collagen II in that it is composed of three polypeptide chains; however, not all three are identical (two alpha-1[I] and one alpha-2[I]).[5] Like collagen II, collagen I fibrils possess the ability to cross-link, thereby forming a rigid, lattice-like structure.[5,6]

Because collagen I is the predominant component of scar tissue, an area of investigation has centered around whether articular cartilage is able to heal with predominantly type I or type II collagen. The collagen II lattice has a specific tensile strength for load bearing, and this cartilage characteristic may be altered by replacement with a collagen I lattice. To date, the results have been conflicting; however, in general, articular hyaline cartilage was able to regenerate with collagen II if the defect was in proximity to the marrow or if the cartilage was relatively immature.[7] The present study investigates levels of collagen types I and II in the areas of tracheal cartilage fracture or attenuation to provide insight into which protein provides structural support to the cartilage ring, which proteins are most susceptible to the inflammatory process, and which may be regenerated. These findings will also provide information regarding the state of differentiation of the chondrocyte because cell function can be correlated with the production of distinct collagen types. In addition, we seek to identify a potential developmental difference in patterns of protein loss.

MATERIALS AND METHODS

Study Population

Archival, paraffin-embedded tissue blocks were obtained in accordance with the Internal Review Board from the Pathology Department of Massachusetts General Hospital (Boston, MA). Specimens consisted of segments of human trachea excised for repair of subglottic and tracheal stenosis because of intubation injury. Gross specimens were required to have gross cartilage integrity or fracture, or both, to be included in the study. The study population consisted of six human subjects (ages, 2, 4, 11, 15, 39, and 57 y). Data on sex of the patients and duration from time of injury to excision of stenosis were not controlled.

The authors recognize that a large selection bias is inherent in the present study because only specimens severe enough to warrant surgical intervention were used as the basis for the data. No determination can be made regarding each age group's individual tolerance to injury or biochemical response to injury as a function of duration of injury. Furthermore, appropriate age-matched control sections do not exist because normal, unaffected trachea is not usually resected and archived.

Samples

Gross specimens were examined to ensure that the area to be studied included the tracheal injury. Specimens were sectioned in the axial plane at a thickness of 6 μm, baked, and deparaffinized. One section from each age group was stained with hematoxylin and eosin (H&E); the remainder of the sections underwent immunohistochemical staining as described in "Immunohistochemistry." Areas of experimental interest consisted of the sites of cartilage ring fracture relative to the remaining, grossly normal ring. It is likely that the entire ring was exposed to the inflammatory process; however, on H&E staining, the areas of fracture represented a relatively greater loss of cartilage matrix and were adjacent to other, better-preserved sections of the ring.

Immunohistochemistry

Antibodies. The antibody II-II6B3 (Developmental Studies Hybridoma Bank, Iowa City, IA) is a monoclonal antibody that recognizes the triple helix of collagen II. It is specific for collagen II without cross-reactivity to any of the other known collagens. Antibody was used at a dilution of 25 μg/mL in 2% goat serum in phosphate-buffered saline (PBS).

The antibody 5-D-4 (lot 99401, Seikagaku Corp., Falmouth, MA) is a monoclonal antibody that recognizes the keratan sulfate region of the aggrecan molecule. This antibody was selected because it recognizes an epitope in and adjacent to the main site of proteolytic cleavage by many of the matrix metalloproteinases.[4] Antibody was used at a dilution of 5 μg/mL in 2% goat serum in PBS.

Rabbit anti-human collagen I (lot 4129, Caltag Laboratories, Burlingame, CA) is a polyclonal rabbit antibody that is specific for collagen I antigenic determinants with no cross-reactivity to collagen types II, III, IV, and V. Antibody was used at a dilution of 20 μg/mL in 2% goat serum in PBS.

Secondary antibodies consisted of goat anti-mouse and goat anti-rabbit antibodies linked to fluorescein (Jackson ImmunoResearch, Westgrove, PA). Antibodies were used at a dilution of 15 μg/mL in 2% goat serum in PBS.

Staining. For specimens stained for aggrecan and collagen II, bovine testicular hyaluronidase at a concentration of 1,600 units/mL acetate buffer was incubated with the tissue sections for 30 minutes at room temperature. For specimens stained for collagen I, a pepsin digestion was performed using 0.4% pepsin in 0.01 N HCl for 30 minutes. Slides were washed three times in Tris-buffered saline and blocked in 2% goat serum for 1 hour at room temperature. Incubations for the primary antibodies were performed overnight at room temperature. Secondary antibodies were incubated for 45 minutes at room temperature. Negative controls for all antibodies consisted of tissue sections treated as described earlier in the present study, without the use of the primary antibody.

Microscopy

All tissue sections were analyzed initially with an immunofluorescence microscope. Photographs were taken with a Leica (Wetzlar, Germany) confocal immunofluorescence microscope.

RESULTS

Aggrecan Staining in Damaged Tracheal and Cricoid Cartilage

Regions of the cartilage ring that had lost cartilage integrity or were fractured, or both, were noted to have a relative loss of aggrecan staining at the fracture site (Fig. 1) over the remainder of the ring. This finding was consistent throughout all age groups. For uninjured regions of the cartilage rings, aggrecan staining levels and patterns were noted to be similar to those already established[8] for normally developing cricoid cartilage rings that did not have intubation trauma.

Some of the specimens of stenosis (from the 4-, 11-, 39-, and 52-y-old subjects) demonstrated areas of apparent cartilage regeneration immediately adjacent to cartilage ring fracture or attenuation. The stain in these areas was intense relative to the baseline aggrecan seen in the uninjured regions of the ring. The apparent cartilage regeneration was inferred by identification of protrusions of the cartilage outside the normal morphological appearance of the ring. Furthermore, within these regions, chondrocytes were noted to be small and were contained within the extracellular matrix in a highly disorganized fashion, and lacunae were absent (Fig. 2). These histological characteristics are similar to those seen in fetal and neonatal cricoid cartilage (i.e., cartilage that is known to be in a stage of rapid growth, mainly because of cell division).

Collagen II Staining in Damaged Tracheal and Cricoid Cartilage

Areas in the cartilage rings that had lost cartilage integrity or were fractured, or both, did not show a loss of collagen II relative to the uninjured portion of the ring (Fig. 3). In areas of preserved cartilage that was not fractured, collagen II staining was identified throughout all age groups, which was similar to the staining seen in our previously published report on developmentally normal cricoid cartilages.[8] As with the aggrecan stain, a few of the specimens (from the 4-, 11-, 39-, and 52-y-old subjects) showed a relative increase in collagen II staining suggestive of regeneration of the extracellular matrix. The increased collagen II stain and aggrecan stain coincided with the same regions (Fig. 4).

Collagen I Staining in Damaged Tracheal and Cricoid Cartilage

In the areas of lost cartilage bulk or fracture, or both, relatively less collagen I staining was seen compared with that in the normal, undamaged cartilage ring (Fig. 5). Preserved cartilage that was not fractured demonstrated collagen I staining within the lacunae and extracellular matrix and at the perichondrium. This staining pattern was seen in all ages tested. Little to no collagen I staining was seen in areas of apparent cartilage regeneration.

DISCUSSION

In the present investigation, fractured cricoid and tracheal cartilage rings resected for the treatment of subglottic or tracheal stenosis were found to have lost aggrecan and collagen I in regions of cartilage ring attenuation

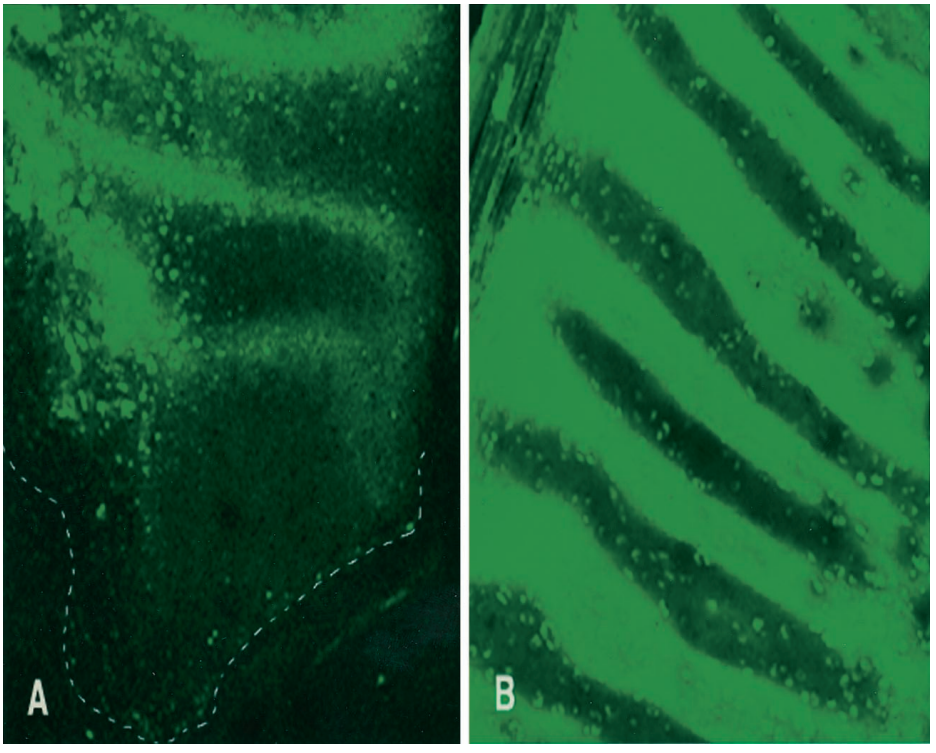

Fig. 1. (**A**) Immunohistochemical image taken using a confocal microscope demonstrating loss of aggrecan at the fracture site. Bottom: Fracture site is outlined in the white dotted line. Top: An area of cartilage attenuation; the ring remains intact. (**B**) Confocal microscopic image of cartilage ring of grossly normal caliber from the same specimen (from 4-y-old subject) (original magnification ×10).

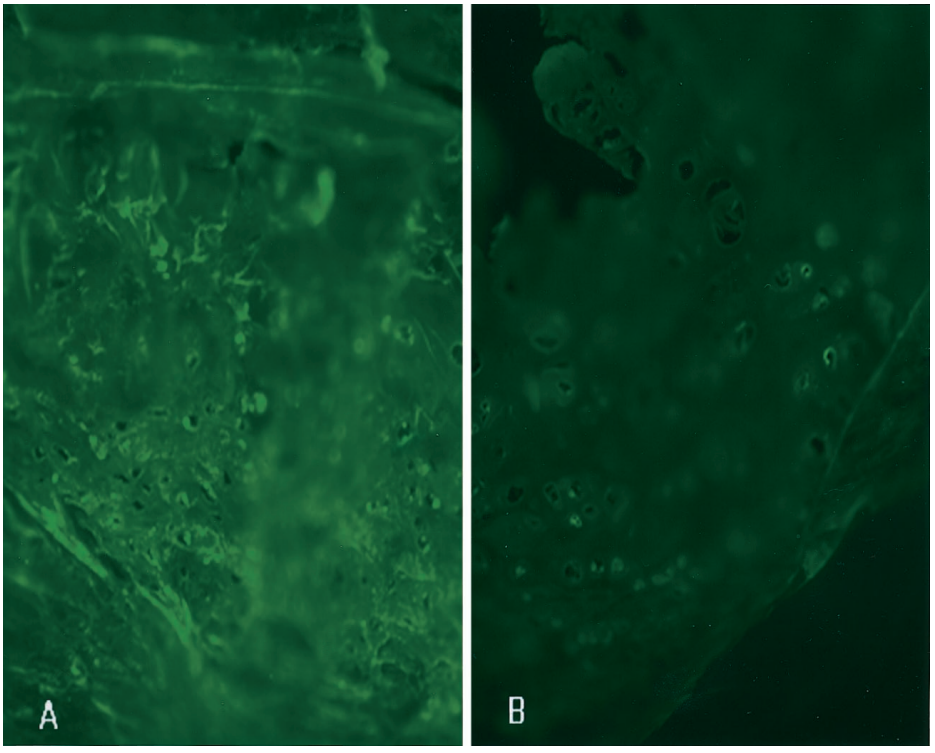

Fig. 2. (**A**) Immunohistochemical image demonstrating aggrecan regeneration near a cartilage ring fracture site in a specimen from a 52-year-old subject. The small size and disorganization of the chondrocytes are evident. (**B**) Area of aggrecan attenuation at the site of a cartilage ring fracture from the same specimen (original magnification × 20).

or fracture, or both. Only collagen II was preserved in areas of gross cartilage loss. Differential loss of aggrecan and collagen I suggests that these two moieties are more susceptible to the inflammatory process than is collagen II. Furthermore, at least one of these two molecules appears to be preferentially responsible for maintaining the shape of the cartilage ring because both were noted to be absent or attenuated (or both) specifically in regions of fracture or collapse. It is likely that loss of structural integrity is preferentially attributable to loss of collagen I because it provides rigidity and structural support to cartilage in general,[2] whereas aggrecan, in its highly hy-

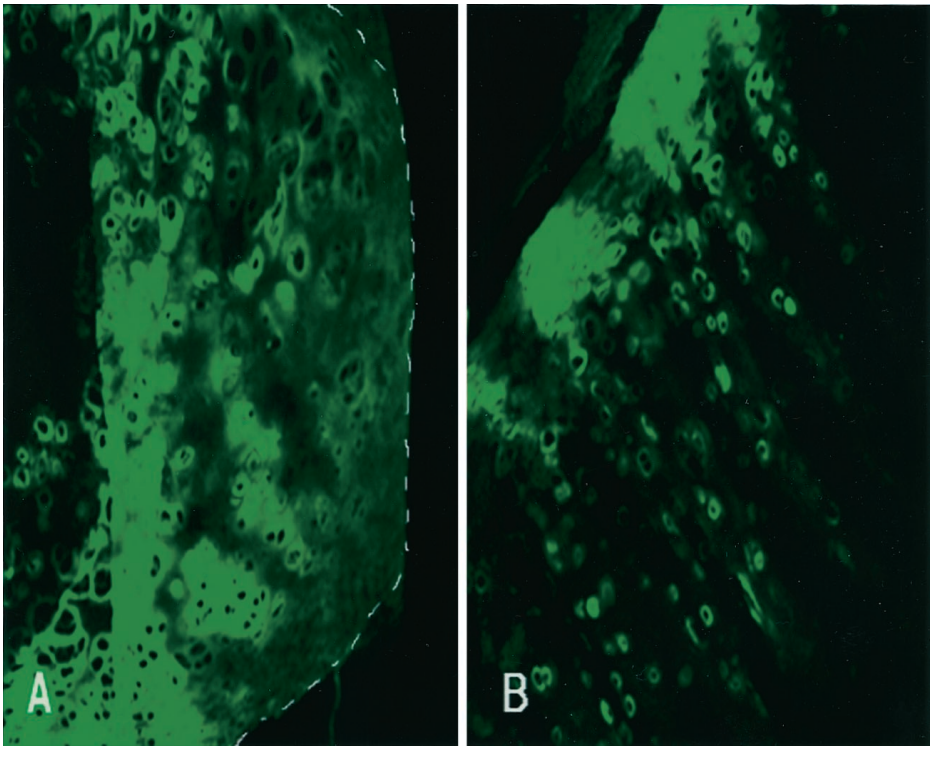

Fig. 3. (**A**) Immunohistochemical image taken using a confocal microscope demonstrating preservation of collagen II at the fracture site. Fracture site is indicated by the white dotted line. (**B**) Confocal microscopic image of cartilage ring of grossly normal caliber from the same specimen as in **A** (from 39-y-old subject) (original magnification × 20).

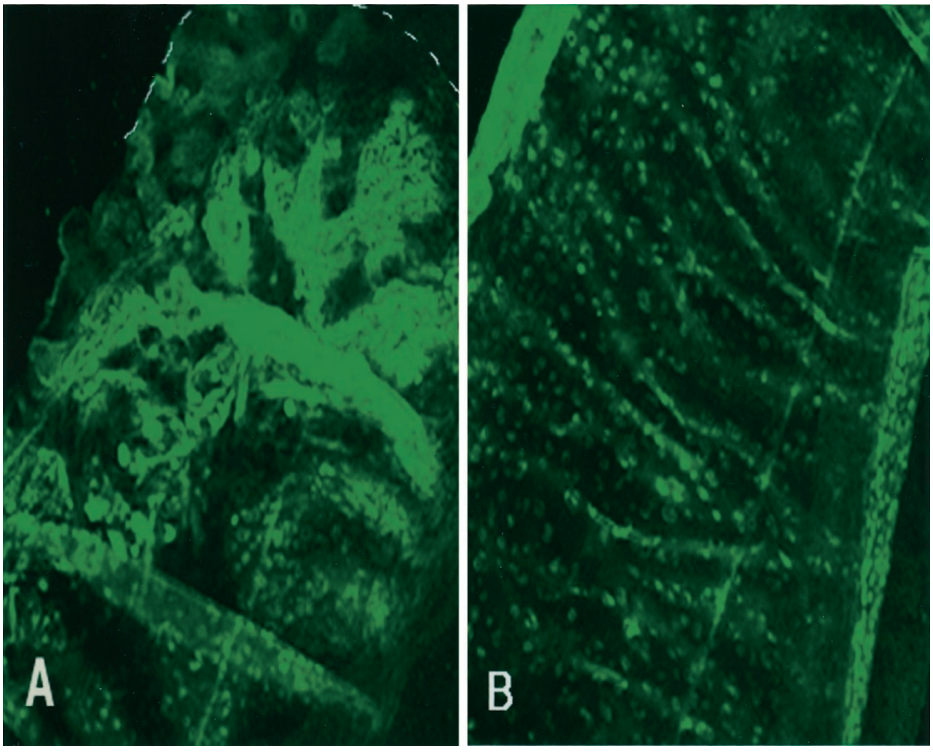

Fig. 4. (**A**) Confocal image of cartilage stump demonstrating an area of increased collagen II adjacent to a fracture site. The disorganized cell within that region is evident. (**B**) Confocal image demonstrating collagen II in cartilage ring of normal caliber from the same specimen as in **A** (from 4-y-old subject) (original magnification × 10).

drated state, provides more of the compressive qualities of cartilages.[4]

Possibly, regions of increased collagen II and aggrecan relative to the rest of the cartilage ring resulted from loss of extracellular matrix and a subsequent concentration of the proteins (proteoglycan). The end result would be a brighter stain when using immunofluorescence. However, this seems unlikely; because aggrecan represents a major component of the extracellular matrix, one would not expect to see increased aggrecan levels as a result of loss of the extracellular matrix. In addition, one might have expected to see the same phenomenon with collagen

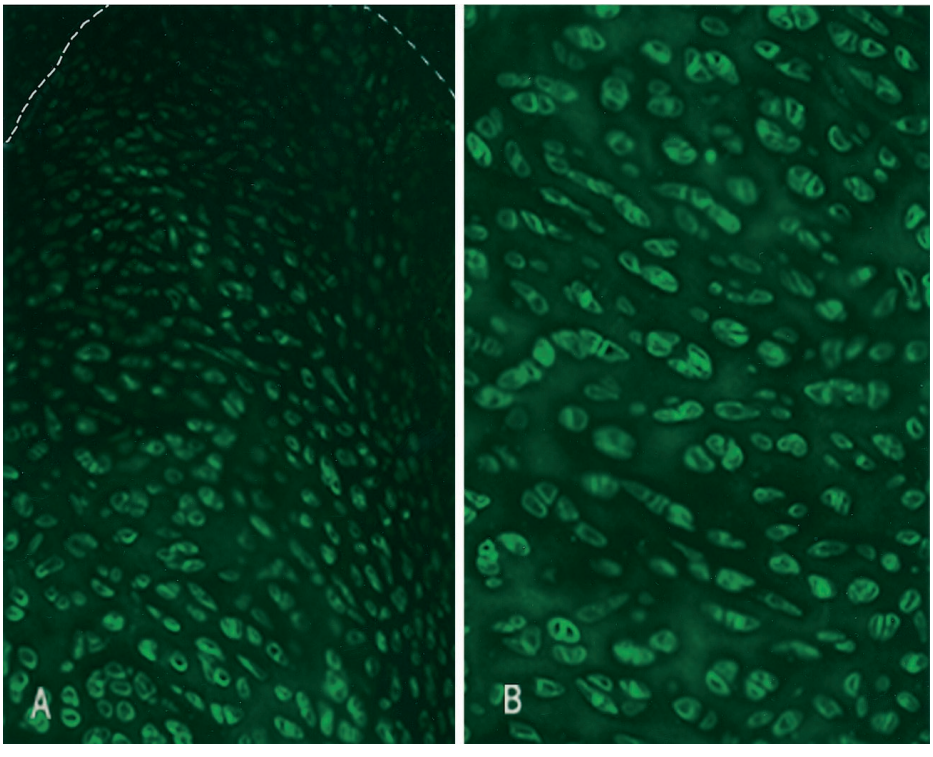

Fig. 5. (**A**) Immunohistochemical image taken using a confocal microscope demonstrating loss of collagen I at the fracture site. Fracture site is indicated by white dotted line. (**B**) Confocal microscopic image of cartilage ring of grossly normal caliber from the same specimen as in **A** (2-year-old child) (original magnification × 20).

I because both type I and type II collagen are similar in structure and stability. As mentioned earlier, an increase in collagen I was not seen in areas of apparent cartilage regeneration. Furthermore, regions of increased collagen II and aggrecan were limited to locations where the chondrocytes had an immature histomorphological appearance. The cells were reminiscent of fetal cricoid cartilage or the proliferative phase in the growth plate. Both of these areas are known to have rapidly proliferating cells.[9] Chondrocyte proliferation is known to dramatically decrease in the human cricoid cartilage between the ages of 1 and 4 years.[9] The chondrocytes become arranged in a radial, columnar fashion, and are located within lacunae. Therefore, it is more likely that regions of increased collagen II and aggrecan were present because these molecules were secreted from the cells after a proliferative phase.

Regions of increased collagen II and aggrecan were apparent in "outgrowths" of the rings. Taken together, the lack of collagen I concentration, the apparent immaturity of the adjacent chondrocytes, the fact that collagen II and aggrecan are postproliferative markers, and the location of the concentrated proteins within outgrowths of the ring suggest that these regions of relative increased collagen II and aggrecan represent the remaining cartilage's attempt to regenerate. This phenomenon was not found to be age dependent.

It has been widely accepted that within cartilage, only the mesenchymal precursors of chondrocytes are capable of producing collagen I.[2] Otherwise, other inflammatory cells such as fibroblasts can be responsible for the creation of collagen I during wound healing. No increase in collagen I was seen over that which is normally present in the cartilage ring. That is, although there was a large inflammatory process in the cartilage ring (enough to cause loss of structural integrity), no additional collagen I was deposited. Rather, as mentioned earlier, repair appeared to be limited to small areas of increased collagen II and aggrecan deposition located near regions of severe structural ring compromise. These findings suggest that only the remaining chondrocytes were responsible for repair of the ring because these two molecules were specific to chondrocytes. Although inflammation was present within the ring, the inflammatory cells did not deposit collagen I and hence did not contribute to the repair of the cartilage ring. Clearly, if an increase in collagen I were identified, the present study would not be able to identify the source of collagen I without the use of in situ hybridization to correlate cell type with protein production. The regenerative capacity of the chondrocytes was maintained throughout the majority of the ages tested. However, the regions of regenerated cartilage were haphazard, and some further compromised the airway by growing intraluminally. Future directions from the present study include identification of which inflammatory cytokine(s) and cell(s) are responsible for the majority of cartilage disintegration, and their potential action modification. In addition, the regenerative capacity of the chondrocytes can be enhanced after identification of the responsible growth factors.

CONCLUSION

Collagen I and aggrecan are lost in areas of severe ring compromise, indicating that at least one of these two molecules is responsible for structural integrity. The remaining cartilage has some regenerative capacity, but this is small relative to the degree of cartilage damage. No new collagen I was identified in the cartilage ring, indicating that, although an intense inflammatory reaction occurred, fibroblasts did not deposit new collagen I as seen in other scar tissues.

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