Novel Cell Proliferation Marker for Identification of a Growth Center in the Developing Human Cricoid

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Background: Developmental histomorphology of the human cricoid cartilage has never been well described. Regional growth centers in the cricoid have been hypothesized, but have never been demonstrated in histological sections.

Objectives: To apply Mib-1 immunostaining, a monoclonal antibody directed at a nuclear proliferation marker, in human cricoids to identify a growth center and to study the changing histomorphology of the developing cricoid.

Design: Immunohistochemical Mib-1 studies were performed on postmortem cricoid sections of 2 fetuses (gestational age, 18.5 and 33 weeks), 1 newborn (full term, 41 weeks), and 3 children (aged 1, 4, and 13 years, respectively). Cell counts, surface areas, and organizational patterns of the chondrocytes were studied and described in hematoxylin-eosin–stained sections.

Results: Differential Mib-1 staining was found. The 18.5-week fetus showed diffuse cell proliferation throughout the cricoid. The cricoid sections of the 33-week fetus and 1-year-old child revealed a distinct ring of proliferation in the outer third of the cricoid ring. The 4- and 13-year-old exhibited no cell proliferation. Histomorphologically, with increasing age came chondrocyte hypertrophy, decreasing cell count per standard square, and increasing organization from a scattered to radial columnar pattern.

Conclusions: Growth of the cricoid involves a diffuse pattern of cell proliferation throughout the cricoid in fetal tissue. At term and until age 1 year, the region of proliferation is more restricted to the outer subperichondrial surface. By age 4 years, cell proliferation has stopped. Histomorphologic changes in the developing cricoid include decreasing cell counts per standard unit area, but increasing surface area with age. The aging chondrocytes develop an increasingly organized layout to form a radially arranged columnar pattern similar to that in the growth plate of the developing limb bud.


Cricoid Cartilage abnormalities, whether acquired or congenital, are a frequent source of airway compromise in both pediatric and adult populations. Historically, there was a conservative attitude toward surgical intervention owing to concerns of disrupting laryngeal development, but because of a mortality rate as high as 24% associated with long-term tracheotomy, treatment emphasis has shifted toward earlier surgical intervention. The anterior cricoid split procedure as an alternative to tracheotomy in the premature child with upper airway compromise was introduced by Cotton and Seid in 1980. Wright et al reported that tracheotomies involving the cricoid resulted in arrested development of the ring. Holinger et al criticized the anterior cricoid split as potentially disrupting a growth center presumably present in the anterior midline cricoid. However, Cotton and Seid claim that growth centers in the cricoid are located laterally rather than in the midline. There are no studies or conclusive evidence supporting the existence of a growth center. In fact, very little is known about the normal cricoid maturation processes. Furthermore, the cellular and molecular basis for a successful anterior cricoid split has not been well described. Studying developmental changes is important since it is generally accepted that the anterior cricoid split is successful in children up to age 2 years.

Studies of cricoid development have mainly focused on review of formalin-fixed archival tissue and have predominantly described gross anatomic dimensions. Very little is known about the normal developmental histomorphology. How does the cricoid ring grow? Do
MATERIALS, AND METHODS

STUDY POPULATION

Human fetopsy and autopsy larynges were obtained from the Department of Pathology at Children’s Hospital and Brigham and Women’s Hospital, Boston, Mass, over the past 3 years. Seven larynges, 1 each from an 18.5-week fetus, a 33-week fetus, a 41-week term infant, and 1-, 4-, and 13-year-old children, were included in our study. Each larynx was fresh with an iatrogenic posterior laryngeal split performed as part of a routine autopsy. The cricoid ring was harvested from each larynx, inked anteriorly, and embedded in cryostat mounting fixative and stored at -80°C.

SAMPLES

Cryostat sections of 6-µm thickness were used for hemotoxylin-eosin immunostaining. Two slides with 2 cricoid sections per slide were stained for each age.

IMMUNOHISTOCHEMICAL ANALYSIS

Mib-1 (catalog No. 0505; Immunotech-Coulter Company, France) monoclonal antibody to a 1002–base pair Ki-67 complementary DNA fragment was used for immunohistochemical analysis. The avidin-biotin complex method using a Vectastain Mouse Elite ABC Kit and AEC (3-amino-9-ethylcarbazole) Substrate Kit (Vector Laboratories, Burlingame, Calif) was used for staining.

Sections of 6-µm thickness were cut from each frozen block and placed on Fischer Gold Plus slides (Fischer Scientific, Pittsburgh, Pa). The slides were air dried for 20 minutes and then fixed in acetone for 8 minutes. After being air dried briefly following the acetone dip, the slides were blocked with normal mouse serum for 20 minutes. The excess serum was drained off, and the sections were incubated with a 1:50 dilution of Mib-1 primary antibody in a moisture chamber for 60 minutes. All incubations for this experiment were performed at room temperature. After several rinses in phosphate-buffered saline, the sections were treated with biotinylated antimouse IgG secondary antibody and avidin-biotin horseradish peroxidase complex. The antigens were visualized by means of the peroxidase reaction with 3-amino-9-ethylcarbazole as chromogen. Color development time ranged from 5 to 20 minutes. The sections were counterstained with diluted Gills 1X hematoxylin (Fischer Scientific) and blued in saturated lithium carbonate solution. Control sections were stained using mouse nonimmune serum for the negative control. Staining of the submucous glands within each section provided the internal positive control.

CELL COUNTING AND MEASURING

Slides were studied using NeuroLucida software (MicroBrightfield Inc, version 3, 1998). The hemotoxylin-eosin–stained cricoid sections were examined from low to high power to study and describe the changing histomorphology with increasing age. Each cricoid section was divided into anatomic regions: anteromedial, anterolateral, posterolateral, and posteriomedial. A standard square measuring 1 × 10^4 µm^2 at ×40 magnification was used as an outline within which we counted numbers of chondrocytes and measured cell and extracellular matrix surface areas. Measurements obtained from 10 squares for each region were averaged for each parameter investigated.

RESULTS

IMMUNOHISTOCHEMICAL ANALYSIS

Mib-1 immunostaining revealed differential positive staining of cricoid sections of various ages (Figure 1). The cricoid from the 18.5-week fetus showed diffuse chondrocyte proliferative activity with scattered positive staining throughout the entire ring. In contrast, the cricoid of the 41-week term infant exhibited few positive cells throughout the ring. The specimens from the 33-week fetus and the 1-year-old child revealed a discrete ring of positively stained cells along the outer third of the cricoid ring subperichondrially. The cricoids of the 4- and 13-year-olds showed no positive staining. Each section had positive brown staining of the sub-
mucous glands acting as our internal positive control. Immunostaining studies were reliable and consistent when repeated multiple times.

**CELL COUNTING AND HISTOMETRY**

Histometric calculations were gathered from anteromedial, anterolateral, posterolateral, and posteromedial sites. There were no significant differences in cell counts at any of these sites (Figure 2). The cell count per standard rectangle decreased with increasing age. The cell surface area, however, increased with age. Again, there were no significant regional differences (Figure 3). The matrix surface area remained constant for all ages. The specimen from the 13-year-old was not included in these measurements because of technical difficulties in maintaining an intact section on the slide.

**HISTOMORPHOLOGY**

Comparative studies of the cricoid sections of various ages revealed changes in chondrocyte size, surface area, and organizational pattern. The chondrocytes of the 18.5-week fetus were arranged in small clusters similar in ap-

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**Figure 1.** Mib-1 immunostaining of horizontal sections through the cricoid (Mib-1 immunostaining; original magnification × 20). Arrowheads point toward the inner lumen of the cricoid ring. A, Proliferating cells stain reddish-brown and are scattered throughout the entire ring of the specimen from the 18.5-week fetus. B, Proliferating cells are localized to the outer third of the cricoid in the 33-week fetus (demarcated by dashed lines). C, Positive cells are located in the outer third of the cricoid ring of the 1-year-old (demarcated by dashed lines). In B and C, Small arrows point to examples of positively stained proliferative cells. D, No staining occurred on the specimen from the 4-year-old.
Bean et al\textsuperscript{12} found that with increasing age, the hyaline cartilage of the cricoid ring loses its capacity to participate in wound healing. Following an anterior cricoid split in young rabbits, the cut ends showed a zone of “neocartilage” with reactive mitotic activity. In comparison, transection of the cricoid in adult rabbits caused necrosis with no evidence of reaction along the stump edges.

Age-related differences in chondrocyte mitotic activity in the developing human cricoid could account for the cartilage growth potential seen in young rabbits undergoing anterior cricoid split. There have also been studies on human septal cartilage supporting age-related factors in growth. Vetter et al\textsuperscript{13} demonstrated higher degrees of cell replication and proliferative capacities of septal chondrocytes in children than in adolescents, and higher replication rates and proliferation capacities in adolescents than in adults.

Unfortunately, the inability to study human croids of specific ages at our convenience or to perform in vivo procedures in patients preclude efficiently repeating the animal experiments on humans. Repeating the Mib-1 immunostaining experiment in an animal model could shed light on chondrocyte proliferation at the desired ages and reveal the effects of cricoid split on cell proliferation. The age at which chondrocyte proliferation ceases and whether the event concurs with the loss of regenerative capacities following croid splits could be investigated.

Our Mib-1 study does not support the presence of a regional growth center hypothesized by Holinger et al\textsuperscript{14} and by Cotton and Seid.\textsuperscript{1} In our study, there was no preferential staining of regions located anteriorly or laterally. Instead, we saw the diffuse cell proliferation throughout the entire cricoid of the 18.5-week fetus localize to an obvious ring of chondrocyte proliferation in the outer third of the croids of the 33-week fetus and the 1-year-old child. There was no cell proliferation in the croid of the 4-year-old. The reason for the lack of staining observed in the 41-week term infant was unclear. Given the trend of staining pattern observed, one would expect to see some proliferative activity. A possible explanation is a problem with the specimen itself or with its processing, especially because the internal positive control staining was also suboptimal in these sections. Again, an animal model could provide less variation in specimen handling.

Starting sometime between 18.5 weeks and 33 weeks in utero, there is a transition from diffuse proliferative activity to a more localized region of growth, suggesting appositional growth of the outer third of the croid ring followed by cessation of proliferative activity somewhere between ages 1 and 4 years. At this point, the croid may grow as a result of cell hypertrophy rather than cell division. This is further supported by concurrent histometric measurements. The cell counts per standard square decrease in number with advancing age, but cell volume increases.

The histomorphologic changes observed in our hematoxylin-eosin sections parallel those described in the developing limb growth plate.\textsuperscript{15,16} Chondrocytes of the limb start as mesodermal precursor cells that are close and crowded with indistinguishable boundaries. As cells...
Figure 4. Hematoxylin-eosin–stained horizontal sections through the cricoid (original magnification ×20). Arrowheads point toward the inner lumen of the cricoid. A, Cells are crowded into small clusters with a homogeneous appearance and no discrete organizational pattern in the specimen from the 18.5-week fetus. B, Chondrocytes are more distinct and larger in the specimen from the 41-week term infant; those chondrocytes near the luminal perichondrium are flattened. C, Chondrocytes in the 1-year-old show further hypertrophy and increased lacunar size. Columnar formation has begun radially around the cricoid. D, Further progression is evident in the specimen from the 4-year-old; the chondrocytes are clearly organized into radial columns and cell surface area has increased with increased age.
enlarge and differentiate, they secrete interstitial amorphous matrix and collagen. They progress through a chondrocyte differentiation program, starting as proliferating chondrocytes near the perichondrium.\textsuperscript{17} As the interstitium builds, cells become isolated in individual compartments or lacunae and gradually take on the cytological characteristics of mature chondrocytes, also called hypertrophic chondrocytes.\textsuperscript{17} In epiphyseal cartilage of growing long bones, cell division occurs in a consistent orientation resulting in lacunae arranged in long columns parallel to the axis of the long bone. The hypertrophic chondrocytes are then eventually removed and replaced by bone.

The specimen from the 18.5-week fetus exhibits the characteristics of mesodermal precursor cells; the cells are crowded with no identifiable organization. From 18.5 weeks' gestation to age 4 years, we see chondrocyte borders grow more distinct and increase in lacunar surface area. Histometry demonstrates that chondrocytes decrease in number per standard square, but they hypertrophy so that matrix surface area remains constant. We also see increasing radial columnar organization of the chondrocytes.

There is a striking resemblance in the columnar organization in developing limb growth plates and the older cricoid. Perhaps the cricoid ring represents a circular growth plate that undergoes radial growth. The Mib-1 studies suggest a proliferative “front” in the outer third of the ring. At some point between ages 1 and 4 years, proliferation subsides. Perhaps it is at this point that cricoid growth occurs via cell hypertrophy and cartilage matrix deposition.

These experimental data support the dynamic growth during the perinatal and early postnatal period in the development of cricoid. Fetal cartilage exhibits a period of rapid cell division. Older specimens demonstrate less cell division. It seems plausible that time-related differences in growth dynamics can account for differences in the cricoid’s response to injury as well as changes in form and size of the cricoid ring during early development. Growth patterns in the nondiseased state are described in this article and will lend insight into investigations concerning the diseased or traumatized cricoids in the future.

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