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

Jennifer C. Kim, MD; Leila A. Mankarious, MD

**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 hemotoxylin-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.

the chondrocytes follow the sequence of chondrogenesis that has been well established in the developing limb bud? Until these questions are answered, we are less able to predict, prevent, modify, or treat disease processes that affect cricoid growth.

The goal of our study was to identify a growth center, characterize the pattern of Mib-1 staining in the developing human cricoid, and describe histomorphologic changes in the cricoid that may be analogous to those seen in the developing limb bud. Immunohistochemical staining with Mib-1 monoclonal antibody is accepted as one of the best methods to measure cell proliferation. It provides direct and rapid identification of proliferating cells by recognizing a proliferating nuclear antigen that proliferating nuclear cell antigen is expressed and seen in the developing limb bud. Immunohistochemical changes in the cricoid that may be analogous to those occurring in the developing human cricoid, and describe histomorphologic changes that occurred with increasing age. Histometric analyses have been performed in animal models studying growth dynamics of the cricoid. No studies or descriptions could be found on human cricoid specimens. Cell counts and histometric measurements may give information on how the ring grows; ie, does it do so by increasing cell number, size, and/or extracellular matrix?

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.

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-
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 cricoids 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 cricoid splits could be investigated.

Our Mib-1 study does not support the presence of a regional growth center hypothesized by Holinger et al\textsuperscript{4} and by Cotton and Seid.\textsuperscript{5} 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 cricoids of the 33-week fetus and the 1-year-old child. There was no cell proliferation in the cricoid 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 cricoid ring followed by cessation of proliferative activity somewhere between ages 1 and 4 years. At this point, the cricoid 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 lumenal 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. As the interstitial builds, cells become isolated in individual compartments or lacunae and gradually take on the cytological characteristics of mature chondrocytes, also called hypertrophic chondrocytes. 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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Reprints: Leila A. Mankarious, MD, Department of Otolaryngology, Massachusetts Eye and Ear Infirmary, 243 Charles St, Boston, MA 02114-3096.

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