Advances in Anatomy, Embryology and Cell Biology

Frederic Shapiro

Disordered Vertebral and Rib Morphology in Pudgy Mice

Structural Relationships to Human Congenital Scoliosis

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Abstract

Disordered vertebral and rib morphology in the pudgy mouse, due to mutations in the *delta-like 3* (*Dll3*) gene of the Notch family, has been studied using whole mount preparations, specimen radiographs, histology, and computerized three-dimensional reconstructions of serial histologic sections. Studies were done in 68 mice, 37 pudgy (pu/pu) and 31 non-affected (pu/+), from late embryo to 3 months of age. *Vertebral bodies* showed malformations including: hemivertebrae; wedged vertebrae; bifid vertebrae (butterfly, double hemivertebrae at same level); and fused vertebrae (partial unilateral bony bar or complete bone block). *Intervertebral discs* adjacent to malformed vertebrae were always abnormal regarding: size (smaller or larger than normal); position (oblique or vertical); shape (round, hexagonal, curvilinear, and other patterns); disorganized peripheral fibers of annulus fibrosus; partial presence across width of vertebral column; and/or complete absence between adjacent vertebrae. *Rib* malformations included: decreased numbers; asymmetric shape and spacing; fused and widened ribs; branching from fused origins into two, three, or more separate ribs; and paravertebral continuous longitudinal cartilage accumulations from which fused ribs originated. The vertebral, intervertebral disc, and rib abnormalities were different in each pudgy mouse. This variability indicates that a single gene mutation does not account solely for the deformities making it necessary to explain by other concepts such as epigenesis. Mutations of genes involved in the oscillatory clock/segmentation mechanism (delta-ll3, Mesp1, lunatic fringe, and others), expressed as early as the presomitic mesoderm stage of embryogenesis, have been clearly implicated in congenital vertebral and rib malformation in mice and humans. Invariable intervertebral disc abnormalities adjacent to abnormal vertebrae are observed, leading to consideration of additional pathogenetic mechanisms. Normal and abnormal structures of vertebral bodies appear directly related to shape and position of the intervertebral discs. The center of the cartilage model of a developing vertebral body and its endochondral bone lie along a line extending (approximately) perpendicular to the central portion of the long axis of the closest section of the adjacent intervertebral disc. Hemivertebrae and bifid vertebrae form in relation

to vertically oriented generally midline discs; the wedged vertebrae, and some hemivertebrae, form in relation to obliquely oriented discs; and bony bars (unilateral or block) form in relation to cartilage vertebral body models not separated by discs. The more abnormal the intervertebral discs, the more abnormal are the adjacent vertebral bodies. The final deformity is not fully patterned in the early embryo; it is due to a combination of primary genetic/epigenetic factors, secondary "obligate" mechanisms, and tertiary biomechanical forces.

Findings in pudgy mice involving all vertebrae and ribs and a case of severe human congenital scoliosis are remarkably similar. Vertebral morphogenesis is reviewed, including developmental concepts described over several decades and stressing current molecular concepts of variations in the oscillatory clock which patterns segmentation. An overview of human congenital scoliosis is outlined especially the severe deformities (spondylocostal dysplasias). The notochord is a precursor of the nucleus pulposus of the intervertebral disc, and the disc is invariably abnormal in relation to malformed vertebral bodies. Whether a convoluted notochord has a role in primary causation or is solely a passive displaced structure, its abnormal positioning could further induce vertebral body variability. Renewal of embryonic studies linking notochord/neural tube removal and transplantation experiments inducing vertebral malformation combined with assessments of gene/molecular components of the oscillatory segmentation clock and notochord should further clarify abnormal morphogenesis.

Keywords: **P**udgy mouse; Histopathology; Vertebral, intervertebral disc, and rib abnormalities; Congenital scoliosis; Gene mutations; Segmentation clock; Notochord

Contents

Chapter 1
Introduction

Normal and abnormal vertebral development have been studied over the past 200 years at increasing levels of resolution as techniques for biological investigation have improved. Disordered development of the axial skeleton from the early embryonic period on leads to structurally malformed vertebrae and intervertebral discs and ribs causing the severe deformities of scoliosis, kyphosis, and kyphoscoliosis. Developmental malformation of the axial skeleton therefore has led to considerable biological and clinical interest. This work will detail our studies on the structural deformities of the vertebral column and adjacent ribs in the pudgy mouse [1] caused by mutations in the delta-like 3 (*Dll3*) gene of the Notch family [2]. While gene abnormalities in the pudgy mouse have been outlined, there has been no in-depth assessment of the histopathology of the pudgy vertebral and rib abnormalities that this study will provide. In addition, although congenital scoliosis has been recognized as a clinical problem since the mid-nineteenth century (1800s) [3] and accurately defined by radiography since the early twentieth century (1900s) [4–6], there have been few detailed histopathologic studies of human cases. We will also relate our histopathologic findings in the pudgy mouse to the histopathology of human vertebral and rib malformations in clinical cases of congenital scoliosis, one of which we defined in detail previously [7].

The pudgy mouse was recognized in the 1950s and described initially by Grünberg in 1961 [1]. The underlying recessive gene was induced by X-rays secondary to "specific locus" experiments carried out at the Oak Ridge National Laboratory in Tennessee (USA). The pudgy mouse was subsequently bred there and at Jackson Laboratories in Maine (USA). The entire pudgy mouse vertebral column is shortened with marked structural irregularities of the vertebrae, intervertebral discs, and adjacent ribs. The tail is shortened, twisted, and deformed due to the vertebral abnormalities. The bones of the appendicular skeleton are normal. A few affected newborns fail to thrive and die within two or three days of birth but the majority are systemically healthy, survive, and move actively about their cages.

In this study we concentrate on the structural changes of the vertebral column and ribs in pudgy mice compared with age-matched non-affected siblings. We

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outline the development of late embryonic and postnatal structural irregularities at varying ages. Multiple breeding pairs were used to accumulate the affected specimens. The pudgy mouse studies include whole mount preparations, high-resolution specimen radiographs, plastic-embedded toluidine blue-stained histologic sections, and computerized three-dimensional reconstructions of serial histologic sections.

The biological understanding of both normal and abnormal vertebral development over the past several decades has been furthered by investigational studies including (i) surgical interventions in the chick embryo (removal of specific regions/structures, grafts/transfers of tissue regions, and chick-quail chimeras), (ii) genetic mutants in the mouse both naturally occurring and transgenic (genetically modified), and (iii) clinical-radiographic studies in the human (with some histopathology and gene analysis). Normal and abnormal embryologic and early postnatal development in the chick, mouse, and human will be discussed in relation to pudgy mouse findings.

Chapter 2
Materials and Methods

2.1 Source, Distribution, and Ages of Pudgy and Non-affected Mice

Mice obtained for this study were products of pudgy breeding pairs from Jackson Laboratories, Bar Harbor, Maine. For the affected pudgy mice, heterozygous unaffected littermates served as controls. An affected pudgy mouse (pu/pu) can be identified at birth since it is approximately three-quarters the length of its non-affected littermates (pu/+) and has a markedly shortened, twisted tail. The mice were sacrificed by intraperitoneal injections of sodium pentobarbital. Vertebral and rib assessments were performed in 68 mice, 37 affected (pu/pu) and 31 non-affected (pu/+) age-matched siblings from the late embryo to 3 months of age. There were eight sets of births (litters) in which two or more of the sibling littermates were affected, allowing for a comparison of rib and vertebral anomalies in pudgy mice from the same mother and same pregnancy as well as with all other pudgy mice.

2.2 Whole Mount Preparations

Twelve mice underwent whole mount preparation, seven pu/pu and five pu/+. Following sacrifice the skin, heart, lungs, and abdominal contents were removed. Extremity muscles were not excised. The specimen was exposed to running cool tap water for 1 h, dried gently with paper towels, and placed in a fixative-stain solution composed of absolute alcohol (80 ml), acetic acid (20 ml), and alcian blue 8GX (Sigma Chemical Co., St. Louis, MO) (15 mg). The specimen was left in this solution for 24–48 h, the length of time being dependent on the intensity of alcian blue staining of cartilage portions of the skeleton. Following this the specimen was dehydrated in absolute alcohol for 5 days. Further staining was performed with

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alizarin red (Sigma) prepared using 10 mg alizarin red in 1 % potassium hydroxide. The staining continued until a deep red color indicated those skeletal parts that had converted to bone tissue. The specimen was then transferred for clearing to Mall solution composed of 79 ml water, 20 ml glycerin, and 1 g of potassium hydroxide. Once clearing of the adjacent soft tissues had been completed, the specimen was transferred to glycerin in increasing amounts from 70 to 80 to 90 %. The specimen was stored eventually in 100 % pure glycerol at room temperature in a closed drawer to minimize exposure to light that could lead to fading of the stain. Examination was performed to assess vertebral and rib patterns from dorsal, volar, and right and left lateral planes using a dissecting microscope (Carl Zeiss, Germany) equipped with a Contax RT5 electronic SLR system camera (Yashica, Japan). This method was adapted from those described previously [8–10].

2.3 Radiographic Studies

The vertebral column and ribs (primarily adjacent posterior and lateral ribs) were assessed using specimen radiographs. Radiographs were taken in 32 mice, 19 pu/pu and 13 pu/+. In those specimens that were to undergo whole mount preparation, the sternum and ventral portions of the rib cage were removed prior to radiography to allow clear visualization of the vertebrae and posterior ribs. In all other specimens, the vertebral column with the adjacent rib cage was excised in one piece from the base of the skull to the pelvis. Anteroposterior (ventral-dorsal) specimen radiographs were made using a General Electric Faxitron machine. Varying times and exposures were used for groups of specimens to obtain the sharpest images. The most common exposure used was for 48 s at 30 kV but times varied between 30 to 48 s and kilovolts used between 25 and 40.

2.4 Histologic Studies

Histologic studies were performed on 56 mice, 30 pu/pu and 26 pu/+. Following sacrifice and dissection, the spines and rib segments were fixed immediately in 10 % neutral buffered formalin. Fixation continued for 2 weeks following which the tissue segments were transferred to 25 % formic acid for decalcification. Once the tissues were soft, they were cut into smaller segments and infiltrated in JB4 medium (Polysciences, Warrington, PA) for several weeks. Tissues were embedded in JB4 plastic for sectioning. Sectioning was performed primarily in the coronal plane but additional sections were cut in the sagittal and transverse planes. Sections were cut at 5 μm and stained with 1 % toluidine blue.