

Sutures, Growth Plates and the Craniofacial Base-Experimental Studies in the Toothless (*tl*-Osteopetrotic) Rat

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Abstract

The craniofacial skeleton develops from a base in which coordinated growth at sutures and growth centres assures the development of normal form. In this report we describe features of retarded postnatal craniofacial development in the osteopetrotic mutation, toothless (tl), in the rat in which bone growth in both the nasal area and the cranial base is reduced, suggesting that the mutation affects bone formation in sutures and growth plates. We began a systematic search for potential mechanisms by analysing the expression in time and intensity of RNA coding for collagens type I (Col I) and type III (Col III) analysed by in situ hybridisation of cells in the premaxillary-maxillary suture (PMMS). In the centre of the PMMS of tl rats, cells expressing Col I and Col III appeared later than in normal littermates and exhibited lower signal. During osteoblast recruitment from the suture centre into the bone domains, Col III RNA expression is switched off. Osteoblasts expressing Col I in abundance, but no Col III, appeared in the flanking bone regions of tl rats later than in normal littermates. It is proposed that the tl mutation restricts the number of available osteoblast progenitor cells, and that the shortage of these cells affects bone growth in the PMMS and in the cranial base. Additional analyses are needed to test this hypothesis and to understand the developmental dynamics in the cranial base.

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Introduction

Craniofacial development is a complex multidimensional process that depends in large part on growth in the cranial base at several growth centres posteriorly and a suture anteriorly. Coordinated development of these sites produces the normal shape of the skull.^{1,2} The suture between the premaxilla and the maxilla (PMMS) anteriorly and the growth centres around the basisphenoid drive the development of the craniofacial base (Fig. 1).

Cranial development is retarded in osteopetrotic rats transmitting the mutation toothless (*tl*). This stunted development can be clearly identified in the cranium when viewed from below (Fig. 2) comparing normal (N) and mutant (*tl*) littermates. In this figure, the PMMS (1) and the growth regions anterior (2) and posterior (3) to the basisphenoid bone are clearly marked on the normal

rat skull (N). (These growth centres of the craniofacial base are identified by the same numbers in Figure 1).

Sutures provide a mechanism for bone growth by supplying cells to bone surfaces while maintaining an unmineralised domain between areas of appositional growth of mineralised tissues.³ Recently Wurtz and colleagues⁴ have demonstrated that cells in the PMMS express genes for different collagens as some of these cells move from their central positions in the suture toward adjacent bone surfaces to participate in bone growth.⁵ Centrosutural cells have fibroblastic morphologies and express RNA for collagens I, III and V at low to moderate levels. As these cells or their progeny migrate toward bone surfaces, RNA expression for collagens III and V disappears and expression for collagen I is highly upregulated. This fits in well with the generally accepted role of type III collagen during develop-

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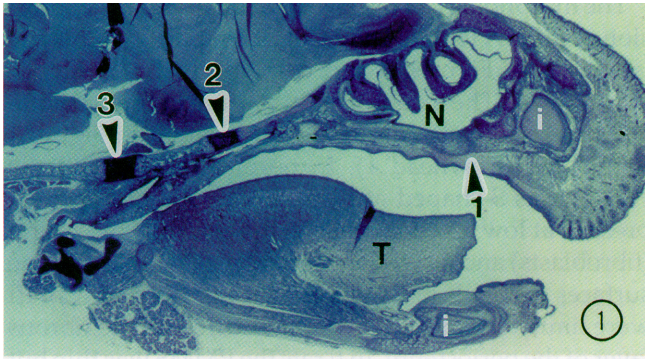


Fig. 1. Sagittal section of the anterior cranium of a 7-day-old normal rat illustrating the locations of the suture between the premaxilla and the maxilla (PMMS: arrowhead 1) and the growth plates anterior (arrowhead 2) and posterior (arrowhead 3) to the basisphenoid. These three sites are responsible for the majority of the growth in the craniofacial base. T = tongue; N = nasal cavity; i = incisor. Toluidine blue $\times 6$.

ment to regulate the diameter of type I collagen fibrils.⁶

Given this clear demarcation of sequential expression of fibrillar collagen mRNAs in the PMMS during facial growth, we began our analysis of potential mechanisms for the stunted perinatal craniofacial growth in the *tl* rat mutation (Fig. 2) by analysing the time of appearance and intensity of expression of mRNAs for collagens I and III in the PMMS.

Materials and Methods

All the experiments performed were approved by the Animal Care and Use Committee of the University of Massachusetts Medical Center. Animals were maintained there and used according to the recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985).

Source and Treatment of Animals

Rats were obtained from our breeding colonies and mutants (*tl/tl*) were identified radiographically at birth⁷ by the failure of development of marrow cavities in long bones. Normal homozygotes (+ / +) and heterozygotes (*tl* / +) are distinguishable only by breeding, and normal littermates included both these genotypes. Untreated rats of normal and mutant phenotypes were sacrificed by cervical dislocation at birth and 2, 4, 6 and 10 days. The skulls of 10-day-old normal and mutant rats were skinned, cleared and stained with alizarin⁸ to illustrate regional differences in development of the craniofacial base. Other heads were bisected and immersion fixed in freshly prepared 4% paraformaldehyde in RNase-free phosphate-buffered saline, pH 7.0, at 4°C for 24 hours. Hemi-sectioned heads were then demineralised in 0.3M EDTA, pH 7.0, at 4°C, dehydrated and embedded in paraffin under vacuum. Sections, cut at 7 μ m in a slightly oblique sagittal plane, included the maxillary incisor,

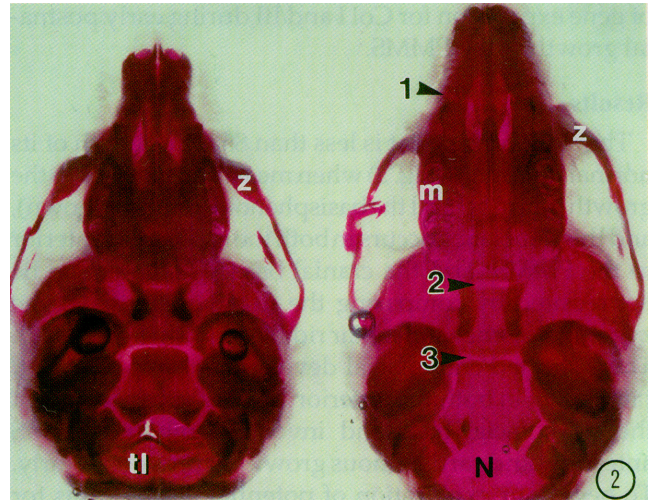


Fig. 2. Representative skulls, cleared and stained with alizarin, from a normal (N) rat and its 10-day-old toothless (*tl*) littermate, viewed from below. The locations of the premaxillo-maxillary suture (1) and the growth plates anterior (2) and posterior (2) to the basisphenoid are indicated by arrowheads. z = zygomatic process of the maxilla; m = molar ridge. Magnification $\times 3$.

molars and the PMMS. Serial sections were floated on RNase-free water and mounted on clean glass slides pretreated with aminopropyl triethoxysilane (Sigma, St. Louis, MO). Blocks and glass mounted sections were stored at 4°C until used.

Probes for Hybridisation

RNA probes were synthesised in the presence of digoxigenin (DIG)-modified UTP by an *in vitro* transcription kit (Boehringer Mannheim, Mannheim, Germany). Probes were generated from templates as previously described⁴ and the oligonucleotides transcribed for Collagen $\alpha 1$ (I) (Col I) and Collagen $\alpha 1$ (III) (Col III) were specific, with no cross-reactivity.⁴

In Situ Hybridisation

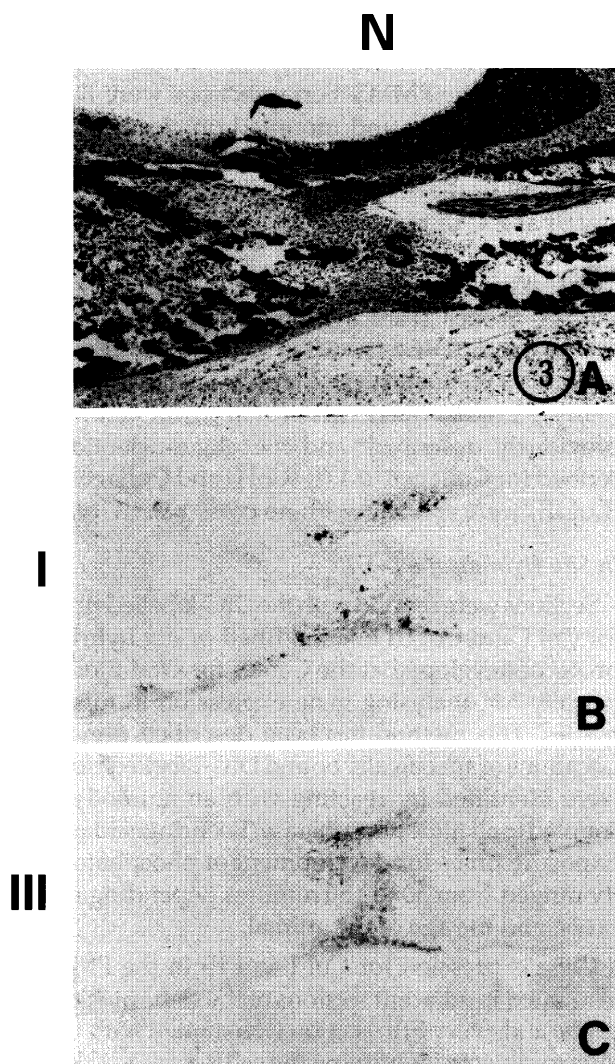
Sections were reacted with the DIG-labelled riboprobes for Col I and Col III in a modified *in situ* hybridisation protocol developed at the Center for Oral Biology specifically for analysing gene expression in mineralised tissues. This method has been described previously.^{4,9} Locations of specifically bound DIG-labelled riboprobes were identified by reacting with an antibody to DIG coupled to alkaline phosphatase (Boehringer Mannheim). Exposure times for development of phosphatase activity ranged from 50 to 150 minutes depending upon the probe and the age of the animal.

Gene expression for Col I and III in the PMMS was evaluated in adjacent sections of the same block for each probe and every fifth section was stained with toluidine blue to assist in identifying the cell populations expressing each gene. At least 5 sections for each probe from at least two animals of each age and genotype were analysed to determine the sites, times, intensity and patterns

of gene expression for Col I and III during early postnatal growth at the PMMS.

Results

The skull of the *tl* rat is less than 85% the length of its normal littermate (Fig. 2) when measured anterior to the growth centre behind the basisphenoid (data not shown), and this deficiency occurs in both the nasal area (anterior to the PMMS) and the cranial base. The latter can be appreciated by comparing the relative lengths of the zygomatic arches and molar ridges (z and m in Figure 2). Thus, stunted craniofacial development in perinatal *tl* rats occurs in both the anterior and posterior aspects of the craniofacial base and involves both the PMMS anteriorly and cartilaginous growth centres posteriorly. To begin our exploration of potential mechanisms for reduced facial growth in *tl* rats, we have focused on the perinatal expression of certain collagen genes by cells of the PMMS as recently described.⁴



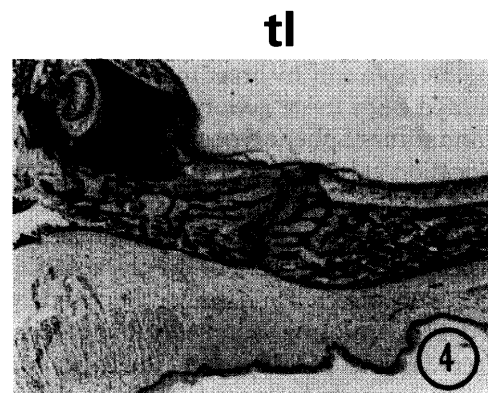
The morphology of the PMMS at birth and the expression of mRNA for Col I and Col III by its cells at this age is shown in Figures 3 and 4 for normal and *tl* rats, respectively. The location of this suture in the craniofacial base is shown at lower magnification in Figure 1 (noted by arrowhead 1). In normal rats (Fig. 3) the PMMS was S-shaped at birth. RNA for Col I was expressed at low levels by cells in the centre of the suture (fibroblasts) and at a much higher level by cells on bone surfaces (osteoblasts). Cells expressing RNA for Col III were limited to the centre of the suture and the fibrous layer of the periosteum. In *tl* rats the PMMS was thin but of normal morphology at birth (Fig. 4) and did not contain cells expressing RNA for either Col I or Col III (data not shown).

On the second and fourth postnatal days, RNA expression for Col I and III in the PMMS of normal rats was slightly higher but expressed by the same cell populations as at birth. In *tl* rats cells expressing Col I could be detected in the suture and on bone surfaces by day two but both the cell density and the intensity of gene expression were less than that in normal rats by day 4. However, cells expressing Col III in and around the PMMS in mutants were at or below our detection limit on the second and fourth postnatal day (data not shown).

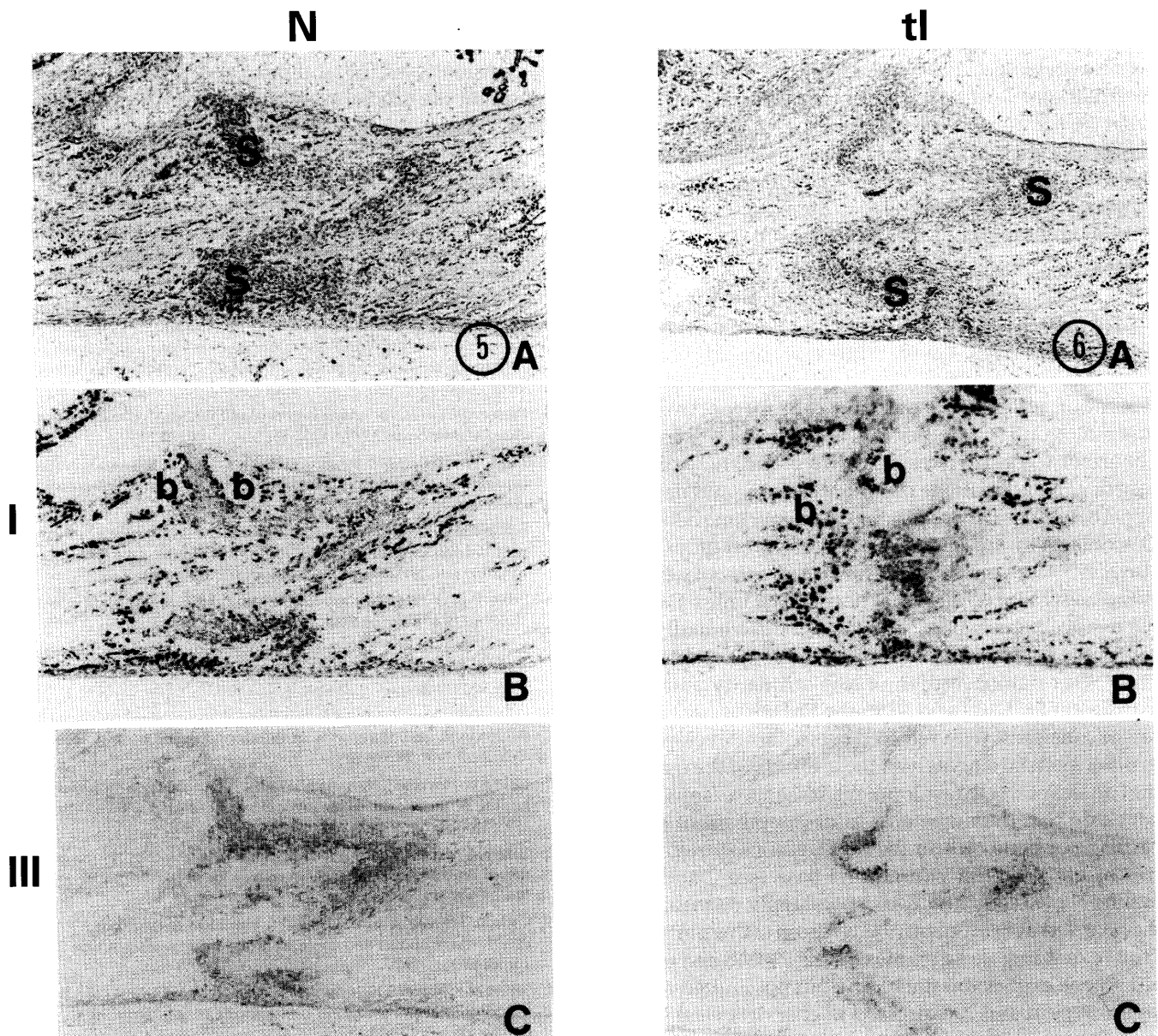
By the sixth postnatal day, cells expressing RNA for Col I and Col III in the PMMS of normal rats were greater in both number and intensity (Fig. 5) than at earlier ages. In mutants the PMMS continued to contain fewer cells than normal but gene expression for Col I by cells of the PMMS had increased over earlier ages (Fig. 6B). At this age cells expressing RNA for Col III were routinely present in the mutant PMMS (Fig. 6C) but were fewer in number than in normal littermates (Fig. 5C).

Discussion

These data confirm the differential expression of RNA for collagens I and III in the PMMS during normal postnatal development of the anterior facial skeleton



Figures 3 & 4 are representative photomicrographs of the PMMS (S) in newborn normal (N-Fig. 3) and toothless (*tl*-Fig. 4) rats. The suture area is well developed in normal rats but is thin with reduced cellularity in mutants. In normal rats sutural cells on bone surfaces express RNA for type I (I-Fig. 3B) collagen and cells in the centre of the suture and in the fibrous periosteum express RNA for type III collagen (III-Fig. 3C). Gene expression for these two collagens could not be detected in the PMMS of mutants (data not shown). Figs. 3A and 4: Toluidine blue x93.



Figs. 5 & 6 are representative photomicrographs of the PMMS (S) in 6-day-old normal (N-Fig. 5) and toothless (tl-Fig. 6) rats. The suture (S) is wide and highly cellular in normal rats (Fig. 5A) and thinner with fewer cells in mutants (Fig. 6A). In both normal rats (Fig. 5B) and mutants (Fig. 6B) osteoblasts on bone (b) surfaces flanking the suture express RNA for Col I at high levels while centrosutural cells (fibroblasts) express this gene (I) at lower levels. Gene expression ¹⁰ Col III (Figs. 5C & 6C) is by centrosutural cells only in both genotypes but expression is more widespread in the normal suture (Fig. 5C) than that of the mutant (Fig. 6C). Figs. 5A and 6A: Toluidine blue x 97.

first reported by Wurtz and colleagues⁴ and demonstrate that retarded development of the craniofacial base in *tl* rats is associated with the retarded appearance of cells expressing RNA for both collagens I and III.

Col III expression appears to be related to Col I expression during development." Mice in which the Col III gene has been knocked out in embryonic stem cells have a shortened life span, dying of ruptured aortae.⁶ Col I fibrils are of reduced number and variable diameter in all tissues but facial form does not appear to be hypoplastic.⁶ During bone formation, Col III fibres provide the initial filamentous framework for the deposition of type I collagen.¹⁰ A similar network of Col III fibres

provides the scaffolding for Col I deposition during bone formation in the maxilla¹¹ and for the deposition of collagen I during fracture repair.^{12,13} Thus, Col III and I exhibit an intimate sequential association during skeletal development that parallels gene expression for these proteins in osteoblasts and their progenitors in the PMMS. Initially, osteoblast precursors express Col III mRNA which produces the fibrous Col III core onto which is deposited the major protein of bone matrix, Col I, produced by the differentiated osteoblast.⁴ If bone matrix is thus deposited onto a network of Col III and associated cell attachment proteins, then the formation of this network could be a rate-limiting step in bone development.

Our data suggest that this may be the case for anterior facial development in *tl* rats where the appearance of cells expressing Col III mRNA in the PMMS is both retarded and lower than in normal littermates. Thus, what we have observed in *tl* rats appears to be distinctly different from that described following a failure to produce Col III.⁶ These observations indicate that a more thorough evaluation of collagen gene expression in the normal and mutant PMMS is warranted.

In the toothless mutation in the rat, there are reductions in the number of bone cells and this may be reflected in both the low cellularity and the hypoplasticity of the PMMS (Figs. 4 & 6). While it is generally considered that reduced osteoclast differentiation and bone resorption are the primary causes of osteopetrosis, in this mutation^{14,15} chondrocytes and osteoblasts are also abnormal. Chondrocytes in growth plates show premature degeneration in the proliferating zone and delayed hypertrophy and mineralisation in mutant long bones.^{16,17} Osteoblasts in mutants are not present on older bone surfaces.¹⁷ On younger bone surfaces, osteoblasts lack cytoplasmic stress fibres that normally stabilise them in the extracellular matrix¹⁸ and cannot differentially sort RNAs for actin, a major component of these stress fibres.¹⁹ Our demonstration of low cellularity and gene expression for Col I and III in the PMMS of neonatal *tl* rats is consistent with reduced osteoblast development in other skeletal sites. Given these effects of the *tl* mutation on skeletal cell development, it seems reasonable to conclude that derangements in development of either cartilage or bone cells in the cartilaginous growth centres of the posterior craniofacial base (see 2 and 3 in Figure 1) are likely and could potentially contribute to the retarded development of this region in this mutation. Thus, extending these studies on the PMMS and evaluating gene expression (including but not limited to collagens type II and X expressed in chondrocytes) in the growth centres of the posterior craniofacial base in mutants are likely to provide clues about normal development of this region.

These studies can potentially contribute to our understanding of normal craniofacial development and help identify factors contributing to both normal facial form and normal (class I) occlusion. In addition, together with similar studies of the brachymorphic mutation in the mouse,²⁰⁻²² they may provide strategies for the early diagnosis, treatment and even prevention of craniofacial abnormalities.

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