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Immunohistochemical identification of TGF-β1 at the maxillaries in growing Sprague–Dawley rats and after muscle section

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ABSTRACT

Growth factors are currently being extensively studied in the literature to ascertain their role during maxillofacial development. Taking into account that few investigations refer to the functions of growth in the maxillaries, our aim was to identify the TGF-β1 immunohistochemical expression pattern in the maxillaries of growing rats. A secondary aim was to identify this pattern after orofacial function inhibition by muscle section. In the palate and the mandibular symphysis and body, we found that bone was formed through an endomembranous pathway with intense TGF-β1 staining inside chondroid cells during the maximum development stages. At the midpalatal suture and the mandibular symphysis and condyle, endochondral ossification was detected with an intense expression of TGF-β1 inside the chondrocytes when major growth occurred. After the muscle had been sectioned, at the mandible the maturation process was accelerated, this change being transitory until muscular function was recovered. However, at the palate, the intervention caused a greater disturbance of the growing pattern, which did not recover normality.

1. Introduction

Maxillary development has been widely studied as far as anatomy and histology are concerned. Although different hypotheses have been established regarding the linkage between macroscopic craniofacial growth and its regulatory factors, such as genes, growth factors, and environmental factors, this process is still not fully understood.[1,2]

In the past two decades, many investigations have increased knowledge about how growth factors take part in general development. However, the role of growth in orofacial development, especially during the postnatal period, remains unclear. The majority of the studies done to date have only analysed the *in utero* period.[3–8]

The transforming growth factor-β (TGF-β) is mainly stored in the mineralized extracellular bone matrix.[9] TGF-β controls important biological activities over the following tissues: (1) it takes part in the differentiation of the ectomesenchymal cells of the first branchial arch into orofacial cell types[10]; (2) it is the most potent mitogen for osteoprogenitor cells[11]; (3) it

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stimulates bone formation[12]; (4) it is a potent chemotactic over osteoblast cells[13]; (5) it increases bone protein synthesis[14] and inhibits its proteolysis[15]; and, (6) it inhibits bone resorption and osteoclast activation.[13] Consequently, it would appear that TGF-β, including its isoform TGF-β1, has a primordial role in the development of the calcified tissues in the maxillaries.

The aim of the present study was to identify the TGF-β1 immunohistochemical expression pattern in the upper maxillary and in the mandible during normal postnatal growth in rats, and to compare such expression with the pattern obtained when orofacial growth is modified by the unilateral section of the superficial masseter muscle.

2. Materials and methods

2.1. Animals

38 Sprague–Dawley rats were divided in two groups, both of which included males and females and both of which were kept in standardized conditions.

The expression of TGF-β1 was analysed at the following postnatal stages in the first group ($n = 20$): newborn (10 days old), lactation (12 and 19 days old), end of lactation (21 days old), raising growth peak (35 days old), prepuberty growth peak (45 days old), puberty (60 days old), and adulthood (75, 90 and 135 days old). Two subjects were observed for each developmental stage.

The second group of animals ($n = 18$) allowed us to examine the changes that occurred in the expression of TGF-β1 after muscle section. According to the first part of our study, the maximum postnatal growth occurred from 30 to 60 days after birth, and was chosen in order to observe a greater alteration of development. Therefore, the muscle section was done when the animals were 30 days old, and the effects were analysed 35, 45 and 60 days after the operation. Each sub-group was constituted by six subjects.

2.2. Unilateral section of the superficial masseter muscle

The method used to alter muscle function consisted in fibre contraction inhibition.[16,17] The animals were sedated using ketamine 20% 50 mg and xylazine 2% 50 μL per kg of body weight. The superficial masseter muscle was sectioned unilaterally with a scalpel, cancelling its function of mandibular protrusion. Then, the incision was sutured at a cutaneous level in a way that allowed the fibres to be reinserted after approximately 15–30 days, leading to the reestablishment of a normal masticatory function. The rats tolerated well the intervention, whereas other methods we had used previously in pilot studies, such as orthopaedic appliances which produced mandible protrusion, had not led us to any concluding results because of animal adaptative problems.

2.3. Preparation of sections

This part of the experiment had to be adapted empirically in order to preserve the antigenicity of bone proteins. The specimens were dissected from the midpalatal suture, and from the mandibular symphysis, body and condyle. Afterwards, they went through the following sequence:

1. Fixation in formol 40% for 48 h.
2. Decalcification with ethylenediaminetetraacetic acid (EDTA) 12.5%. The time depended on the age of the animal because the tissues calcified with age and needed longer to decalcify. For instance, 10-day-old tissue decalcified in 6 days, and 45-day-old tissue in 21 days.
3. Washing the excess EDTA with formol 40% for 24 h to avoid its precipitation in the tissue when it makes contacts with ethanol in the next step.
4. Dehydratation in an increasing graded series of ethanol: ethanol 30 °C for 1 h, ethanol 70 °C, 90 °C and absolute for 8 h each, and another absolute ethanol for 16 h.
5. Cleaning in three toluene solutions for 5 h and, finally, paraffin inclusion in two steps for 16 h each, applying the last one with a vacuum bomb.
6. Using an electronic micrometer (Leica) and specific blades for bone tissue (R35, Feather), the 5 μm-thick sections were obtained.

2.4. Immunohistochemical staining

At the time our investigation started, there were no publications concerning the immunohistochemical staining method to be used in bone tissue. Although other authors afterwards performed similar studies on bone, they did not detail the times or concentrations employed.[13] In our study, the immunohistochemical detection of TGF-β1 at the decalcified samples of the maxillaries was done following this method:

1. Deparaffin sections in 3 steps of xylol for 10 min each.
2. Rehydratation in a decreasing graded series of ethanol lasting 5 min per stage.
3. Endogen peroxidase blockage throughout immersion of sections for 5 min in a solution of 20 mL of hydrogen peroxide 30%, 20 mL of absolute ethanol and 160 mL of phosphate-buffered saline (PBS).
4. Washing in PBS in 3 steps for 5 min.
5. Background blockage with normal goat serum (Sigma) 1:30 diluted in PBS for 60 min.
6. Primary antibody incubation in a humid chamber at 4 °C overnight. The antibody applied was a monoclonal anti-TGF-β1 synthesised in rabbits (Santa Cruz Biotechnology Inc.) used at 1:50 dilution in PBS.
7. Application of a secondary antibody for 60 min. This was a polyclonal peroxidase goat to rabbit (Sigma) diluted 1:100 in PBS. As this antibody was labelled with peroxidase, the sections were immersed in a solution of 200 mL PBS, 200 μL hydrogen peroxide, and 2 mL diaminebenzidine (DAB) for 5 min, which resulted in the formation of brown benzidine precipitates and finally revealed the location of the TGF-β1.
8. Haematoxylin contrasting for 10 s, dehydratation in ethanol 70 °C and absolute ethanol for 3 min each. This was followed by cleaning with xylol 3 times for 5 min.

Negative control staining with normal serum was not immunoreactive under the method used (data not shown). Sections were analysed by means of a Nikon E800 microscope