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Short Report

Palatal shelf adhesion is mediated by TGF-β_3 induced chondroitin sulphate proteoglycan

ANGEL GATO[1], CONSUELO TUDELA[2], ISABEL ALONSO[1], JOSÉ A. MORO[1], Mª JOSÉ RIVAS[2], MIGUEL A. FORMOSO[2], CARMEN MAESTRO[2], AURORA DEL RÍO[2], JORGE MURILLO[2], CARMEN BARRIO[2] and CONCEPCIÓN MARTÍNEZ-ÁLVAREZ [2]*

[1] Departamento de Anatomía Humana. Facultad de Medicina, Universidad de Valladolid, Spain and
[2] Departamento de Ciencias Morfológicas I. Facultad de Medicina, Universidad Complutense de Madrid, Spain

During palate fusion in mammals, the adhesion of opposing medial edge epithelium (MEE), the epithelium covering the tips of palatal shelves, is an essential event whose alteration causes cleft palate (Greene and Pratt, 1977). The initial adhesion of these epithelia seems to be related to the appearance of certain changes in their most superficial cells occuring just prior to the contact between palatal shelves. The most striking change observed is the presence of a coat formed by glycoconjugates, whose experimental removal causes the *in vitro* inhibition of palatal shelf adhesion (Greene and Pratt, 1977). Amongst the different proteoglycans that could be forming part of these glycoconjugates and have a role during palatal shelf adhesion, our recent demonstration of the presence of chondroitin sulphate proteoglycan (CSPG) covering the superficial MEE cells (Martínez-Álvarez *et al.,* 2000a), strongly points to its direct participation in this mechanism.

A distorted palatal shelf adhesion has been suggested to be the origin of the cleft palate produced in the absence of transforming growth factor beta 3 (TGF-β_3) (Proetzel *et al.*, 1995; Martínez-Álvarez *et al.,* 2000b). The gene of *TGF*-β_3 is strongly expressed in palatal MEE cells of the still vertical palatal shelves and this expression persists until the midline epithelial seam disappears (Pelton *et al.*, 1990). It has not been investigated so far whether the glycoconjugates that cover the MEE surface and facilitate palatal shelf adhesion are altered in these mice. However, this could be the case, as TGF-β regulates the expression of glycoconjugates and proteoglycans in several tissues (Rapraeger, 89).

The aim of this work was to determine whether the presence of CSPG on the mouse MEE surface (Martínez-Álvarez *et al.*, 2000a) is directly involved in palatal shelf adhesion and is induced by TGF-β_3. Immunolabelling with the anti-chondroitin sulphate CS-56 (Sigma) monoclonal antibody was performed on paraffin sections of Carnoy fixative fixed specimens. Embryonic day 14 (E14) and E14.5 Swiss (CD1) or C57BL/6J TGF-β_3 wild type (TGF-β_3 +/+) and null mutant (TGF-β_3 -/-) mouse palates were utilized for the *in vivo* studies, whilst E13.5 similar mice and stage 35 Hamburger and Hamilton chick embryos were used to perform palate cultures. Either paired or isolated mouse palatal shelves and chick isolated palatal shelves were cultured in Trowell's culture (Brunet *et al.,* 1995), whereas chick paired palatal shelves were cultured in 0.5 % agar gels as described in Sun *et al.*, 1998, using 1 % ascorbic acid supplemented DMEM/F12 as culture medium. In all cultures, medium was replaced each 24 hours. Cultures were then fixed in 4 % paraformaldehyde in PBS or Carnoy fixative and processed respectively for histological (haematoxylin and eosin staining) or immunohistochemical procedures. In order to alter CSPG expression, 10.8 mg/ml β-D-Xyloside (Sigma) or 9 U.I./450 μl chondroitinase AC (Sigma) were added to the medium of CD1 palate cultures. Some cultures were added 10 U.I./100 μl heparitinase type II (Sigma) or were not supplemented to be used as controls. 10 ng/ml TGF-β_3 (Sigma) was added to the culture medium in those experiments requiring this condition.

Immunolabelling of E14 and E14.5 mouse palates with the anti-CSPG CS-56 monoclonal antibody showed no anti-CSPG positive material amongst MEE cells or on the MEE surface until palatal shelves had approached each other at E14.5. At this time point, an anti-CSPG positive material covered most of the MEE surface, greatly increasing when the contact between the palatal shelves became imminent (Fig. 1). Positive staining was never seen amongst MEE cells, but was observed surrounding the bulging superficial MEE cells, in consonance with our previous observations (Martínez-Álvarez *et al.*, 2000a). Significantly, in the zones where palatal shelf contact had occurred, the midline epithelial seam showed totally negative anti-CSPG staining, thus indicating that after the initial contact, adhesion amongst MEE cells is not mediated by CSPG. As the epithelial seam disrupted, anti-CSPG positive mesenchyme occupied the midline, leaving only small islands of totally negative anti-CSPG labelling.

When palatal shelves are cultured for 36 hours, always fuse. However, the addition of the CSPG synthesis inhibitor β-D-Xiloside or the CSPG degrading enzyme chondroitinase AC to E13.5 palate cultures resulted in a complete absence of fusion of palatal shelves, which remained either separated or minimally adhered after 36 hour culture (Fig. 2). Palatal shelf adhesion was not impeded when adding the heparan sulphate degrading enzyme heparitinase. These results demonstrate the specificity of the expression pattern of CSPG on the MEE surface and its direct involvement in the initial adhesion of palatal shelves, thus pointing to its participation in the mechanism of cell recognition between opposing MEE cells required for palatal adhesion and fusion. CSPG could be forming part of extracellular matrix molecules present on the MEE surface, as certain cadherins and N-CAM, that are expressed during the early development of the palate and contain CSPG (Kerrigan *et al.,* 1998).

We then investigated whether the presence of this superficially placed CSPG is induced by TGF-β_3. The immunolabelling of E14.5

***Address correspondence to:** Concepción Martínez-Álvarez. Departamento de Ciencias Morfológicas I. Facultad de Medicina, Universidad Complutense de Madrid, Spain. e-mail:calvarez@eucmax.sim.ucm.es

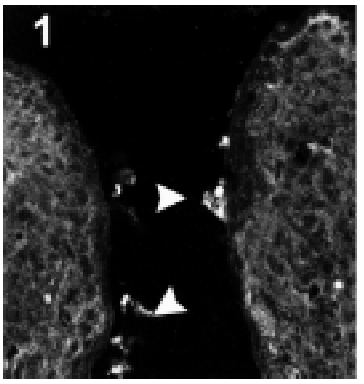

Fig. 1. E 14.5 mouse palate showing CSPG positive material on the apical surface of the MEE.

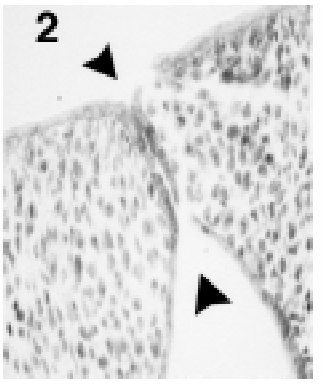

Fig. 2. Palatal shelf adhesion in culture is prevented by chondroitinase AC.

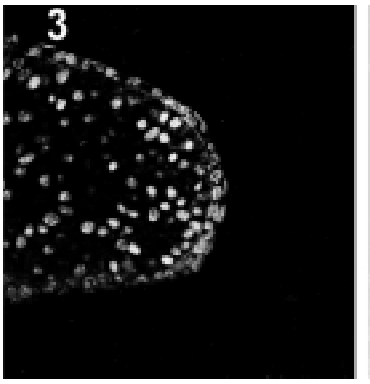

Fig. 3. TGF β3 -/- palatal shelves do not express CSPG at the MEE apical surface.

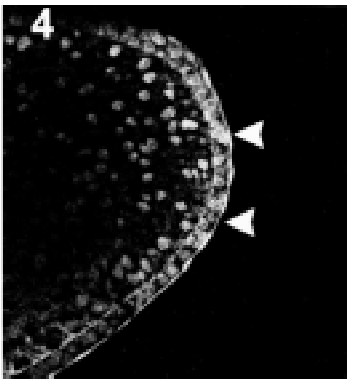

Fig. 4. The expresion of CSPG at the MEE apical surface is induced by TGFβ3 addition to the culture medium.

TGF-β_3 homozygous negative mouse palates with the anti-CSPG CS-56 monoclonal antibody showed a striking reduction of CSPG on the MEE surface when compared to controls and similar results were obtained when isolated E13.5 TGF-β_3 +/+ and -/- palatal shelves were cultured for 12 hours and compared. In the TGF-β_3 +/+ cultures, expression of CSPG was observed on the MEE surface, whilst this expression was never seen in the TGF-β_3 -/- cultures (Fig. 3). Moreover, when cultured in pairs for 24 hours, TGF-β_3 -/- palatal shelves failed to adhere adecuately and had not anti-CSPG positive staining on the MEE surface. However, the addition of TGF-β_3 to both 12 hour TGF-β_3 -/- isolated palatal shelf cultures and 24 hour TGF-β_3 -/- palate cultures resulted in an increase in both the expression of CSPG on the MEE surface (Fig. 4) and in palatal shelf adhesion.

Chick palatal MEE does not express TGF-β_3 (Sun *et al.,* 1998). When cultured in agar gels for 45 hours, paired chick palatal shelves adhered only partially and were frequently totally separated. The addition of TGF-β_3 to the culture medium strikingly increased palatal shelf adhesion, leading sometimes to palatal fusion. To determine whether this effect of TGF-β_3 on chick palatal shelf adhesion correlates with the induction of the expression of CSPG by MEE cells, we cultured isolated chick palatal shelves for 12 hours with or without adding TGF-β_3 to the culture medium and immunolabelled the sections with the anti-CSPG CS-56 monoclonal antibody. Confocal visualization of the control cultures showed little or no anti-CSPG staining in the MEE. However, a great expression of CSPG both amongst MEE cells and on the MEE surface was noticed in the TGF-β_3 treated cultures.

These results evidence that CSPG expression by superficial MEE cells in both mouse and chick embryos is induced by TGF-β_3 and suggest that the role of TGF-β_3 on palatal shelf adhesion is due, at least in part, to the presence of a CSPG containing material on the MEE surface. The ability of TGF-β_3 to regulate the synthesis and secretion of extracellular matrix molecules, including proteoglycans, in different tissues is well known (Bassols and Massagué, 1988; Rapraeger, 1989). However, to date, the regulation of proteoglycans expression in MEE cells by TGF-β_3 had never been reported. TGF-β_3 could influence this expression either by regulating the transcription of the gene codifying for the proteglycan core protein or by controlling the process of addition of glycidic chains to proteoglycans (Inman and Colowick., 1985; Bassols and Massagué, 1988). An influence of TGF-β_3 on the activity of metalloproteinases, that regulate proteoglycan synthesis, should neither be rouled out.

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