Cleft Palate Cells Can Regenerate a Palatal Mucosa in vitro

INTRODUCTION

Orofacial clefts are common congenital malformations in the head and neck region (Bellis and Wohlgemuth, 1999). A cleft palate is surgically closed to restore the integrity of the oral and nasal cavity, and to allow for normal feeding and speech. The primary repair of a cleft palate generally leaves lateral open wounds, causing wound contraction and scar formation (Kremenak et al., 1970; Wijdeveld et al., 1991; Pigott et al., 2002), although in a supraperiosteal flap technique, the bone remains covered (Ito et al., 2006). In either event, surgery impairs the growth and development of the dento-maxillary complex (Wijdeveld et al., 1991; Ross, 1995; Mølsted, 1999), which may require additional surgical and orthodontic intervention.

For implantation in full-thickness skin wounds, several types of tissue substitutes have been developed with keratinocytes and fibroblasts, and a dermal substrate. They seem to reduce wound contraction and scar formation (Kremer et al., 2000; Sheridan et al., 2001). Similar techniques have been used to construct oral mucosal substitutes (Cho et al., 2000; Izumi et al., 2003) that might reduce scar formation and subsequent growth disturbances after cleft palate repair.

Skin or mucosal substitutes are generally prepared from normal cells, but cells from children with cleft palate are genetically and phenotypically different. Variations in genes for transforming growth factor-alpha (TGF-α) and -beta 3 (TGF-β3) have been associated with cleft palate (Blavier et al., 2003; Jugessur et al., 2003; Krapels et al., 2006). These factors belong to the EGF and TGF-β superfamilies of growth factors, which are crucial in keratinocyte proliferation and differentiation (Hashimoto, 2000). Therefore, they may influence epithelial differentiation in culture. Moreover, palatal fibroblasts from children with cleft palate differ in the expression of TGF-β isoforms from control individuals (Bodo et al., 1999). They also show an increase in decorin, glycosaminoglycan, and collagen synthesis, and a decrease in hyaluronan synthesis (Bosi et al., 1998; Bodo et al., 1999). The capacity of cells from children with cleft palate to regenerate a mucosa in vitro has never been investigated. Therefore, we compared the regenerative capacity of keratinocytes and fibroblasts from children with cleft palate and age-matched individuals without cleft palate. In addition, mucosal substitutes were histologically and immunohistochemically compared with native palatal mucosa.

MATERIALS & METHODS

Participants and Sample Collection

Samples from eight children with cleft palate (age 18 ± 1 mos) and eight control individuals (age 22 ± 6 mos) were used. All children were treated in the Cleft Palate Craniofacial Unit of the Radboud University Nijmegen Medical Centre. They all had a non-syndromic cleft palate with or without cleft lip and alveolus, and were routinely screened for contra-indications for surgery (see online...
surgical protocol). The children and their parents were also included in the Eurocran Gene-Environment Interaction study. Control individuals were age-matched children without congenital malformations and scheduled for tonsillectomy at the Ear-Nose-Throat department. The Central Ethical Committee of The Netherlands approved the study protocol, which included written informed consent from the parents. During the primary surgical closure of the cleft palate, a small 3-mm biopsy of the hard palate mucosa was taken in the center of the hard palate, about 1 cm from the medial edge of the cleft. In control individuals, it was taken during tonsillectomy, at about 1 cm from the median. Five randomly chosen samples from each group were used for cell culture; the remaining three were used for histology.

## Cell Culture
The biopsy samples were cut into two parts, an upper part containing mainly epithelium, and a lower part containing mostly of the connective tissue. The epithelium was incubated in 0.25% trypsin solution (Invitrogen, Breda, the Netherlands) for 30 min at 37°C, and the connective tissue for 10 min. The epithelium was cut into small pieces and incubated in a T75 bottle on a 3T3 feeder layer in keratinocyte medium containing 10% fetal calf serum (FCS) (Invitrogen), adenine, cholera toxin, hydrocortisone, insulin (all from Sigma, St. Louis, MO, USA), epidermal growth factor (EGF) (AMS Biotechnology, GmbH, Frankfurt, Germany), and triiodo-L-thyronine (Sigma) (Rakhorst et al., 2006). A T75 bottle was chosen to allow a large number of colonies to form. After about 2 wks, colonies had formed, and the cells were passaged to a new feeder layer and cultured until confluent. Small pieces of the connective tissue were incubated in 1 well of a 24-well plate in DMEM supplemented with 10% FCS, 100 IU/mL penicillin, and 100 µg/mL streptomycin (all from Invitrogen). The fibroblasts were cultured until confluent in the 24-well plate, and passaged subsequently to a T25, a T75, and 3 T75 bottles. All cells were frozen in liquid nitrogen (passage 3 for fibroblasts, passage 1 for keratinocytes). Typically, 1 3-mm biopsy yielded from 8 to 10 million keratinocytes and from 6 to 9 million fibroblasts.

## Fibroblast Seeding
The fibroblast seeding was essentially performed according to previously published methods (El-Ghalbzouri et al., 2002a). De-epidermized connective tissue was prepared as previously described (Ponec et al., 1988). Briefly, a piece of connective tissue was placed on 1% agarose (basal membrane side down) in a 15-mL tube, and 1 mL medium containing 200,000 fibroblasts (passage 5) was added on top. The tube was centrifuged for 1 hr at 45 g. The cells remaining in the suspension were counted. The seeding efficiency was 68.4 ± 12.9%.

## Composite Mucosal Substitutes
Mucosal substitutes were prepared from the cells of five children with and five children without cleft palate (El-Ghalbzouri et al., 2002b). The cell-populated connective tissue was cultured for 1 wk, and then 200,000 keratinocytes (passage 3) were seeded into a stainless-steel ring (inner diameter, 1 cm) placed on top of the connective tissue. After incubation overnight in keratinocyte medium with 5% FCS, but without EGF (5% differentiation medium), the ring was removed, and culturing proceeded for an additional 3 days. Thereafter, the cultures were lifted to the air/liquid interface and cultured for 10 days in differentiation medium without FCS, but with extra L-serine (100 mM), L-carnitine (10 µM), bovine serum albumin-lipid mix (0.16%), and vitamin E (1 µM) (0% differentiation medium) (all from Sigma).

### Colony-forming Efficiency of Keratinocytes
Keratinocytes from each sample (passage 3) were grown on 3T3 feeder layers at a density of 1000 and 5000 cells per dish (diameter, 100 mm) in 5% differentiation medium. After 10 days, the cultures were fixed and stained with 1% Rhodamine B (Sigma) in 4% formaldehyde. The colony-forming efficiency was expressed as percentage of the plated cells (Jones and Watt, 1993). The proportion of terminally differentiated colonies was visually determined.

### Histology and Immunohistochemistry
Three biopsies of the children with and those without cleft palate, and all substitutes, were fixed in 4% paraformaldehyde for 4 hrs and embedded in paraffin. Six-micrometer sections were stained with hematoxylin and eosin. Immunohistochemical staining was performed on adjacent sections. After antigen retrieval (Table), the sections were washed in phosphate-buffered saline and incubated in 10% normal donkey serum (Sigma). Then, the sections were incubated with the primary antibodies (Table) at 4°C overnight, and with either goat or donkey anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) by the avidin-biotin-peroxidase method (Sigma). Finally, the sections were stained with diaminobenzidine (Sigma) and counterstained with Delafield’s hematoxylin.

For epithelial differentiation, 3 antibodies were used: anti-human keratin 5, keratin 10, and keratin 16. Anti-heparan sulphate was used to stain the basal membrane. This antibody was raised against heparan sulphate proteoglycan from rat glomeruli (van den Born et al., 1995). It recognizes N-unsubstituted glucosamine regions in the heparan sulphate chains. Ki67 was used as a proliferation marker. An antibody against decorin was selected to detect differences in decorin expression. In negative control samples, the primary antibody was replaced by normal donkey serum.

### RESULTS

#### Colony-forming Efficiency
The average colony-forming efficiency of cells from children with and without cleft palate was 8.7 ± 4.0% and 9.8 ± 3.2%, respectively (Fig. 1). The proportions of terminally differentiated colonies were 9.4 ± 3.3% and 8.8 ± 3.0%, respectively. The differences between the two groups were not significant (non-paired Student’s t-test, p > 0.05).

### Table. The Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Antigen Retrieval</th>
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<tbody>
<tr>
<td>Anti-keratin 5 (PKC103)</td>
<td>Euro-Diagnostica (Arnhem, The Netherlands)</td>
<td>Microwave 95°C, 10 min</td>
</tr>
<tr>
<td>Anti-keratin 10 (2202Mk10)</td>
<td>Euro-Diagnostica</td>
<td>Trypsin, 15 min</td>
</tr>
<tr>
<td>Anti-keratin 16 (LU0025)</td>
<td>Novocastra Laboratories Ltd (Newcastle upon Tyne, UK)</td>
<td>Microwave 95°C, 10 min</td>
</tr>
<tr>
<td>Anti-Ki67 (SP6)</td>
<td>Euro-Diagnostica</td>
<td>Microwave 70°C, 10 min; trypsin, 5 min</td>
</tr>
<tr>
<td>Anti-heparan sulphate (BA-2020)</td>
<td>Prof. Berden, Nijmegen, the Netherlands</td>
<td>Microwave 70°C, 10 min; trypsin, 5 min</td>
</tr>
<tr>
<td>Anti-decorin</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
<td>Microwave 70°C, 10 min; trypsin, 7 min</td>
</tr>
</tbody>
</table>
Histology

The native mucosa of the hard palate consisted of two layers: the epithelium and the connective tissue (Fig. 2, row 1, native mucosa). The epithelium of the hard palate was parakeratinized, and contained about 8-15 cell layers. The connective tissue consisted of an extracellular matrix containing cells (mainly fibroblasts), blood vessels, and neural elements. No differences were found between samples from children with and without cleft palate.

The keratinocytes from children with and without cleft palate and grown on connective tissue formed a well-keratinized stratified epithelium (Fig. 2, row 1, substitutes). The parakeratinized epithelia contained about 4-8 cell layers. The cornified layers were much thicker and looser than in native palatal mucosa, and epithelial ridges were not obvious.

Immunohistochemistry

Epithelial Markers

No differences were found in epithelial differentiation patterns between the substitutes with cells from children with and without cleft palate (Fig. 2, K5, K10, K16).

Keratin 5 was mainly expressed in the suprabasal layers, but not in the keratinized layer of the native palatal epithelium and the mucosal substitutes (Fig. 2, K5) from both children with and those without cleft palate. The staining intensity in the substitutes was higher than in the native tissue. Keratin 16 was mainly expressed in the suprabasal layers of the native epithelium, as well as in the cultured epithelium from persons with and without cleft palate (Fig. 2, K16). Again, the staining intensity was higher in the substitutes. Keratin 10 was expressed only in part of the cells in the superficial layers of the native epithelium (Fig. 2, K10). The expression pattern in the mucosal substitutes was similar.

Other Markers

No differences were found in cell proliferation (Fig. 3, Ki67), basal membrane (Fig. 3, HS), and decorin expression (Fig. 3, Dec). In the native mucosa, more parabasal cells were positive for Ki67 than in the mucosal substitutes, while more basal cells were stained in the substitutes (Fig. 3, Ki67). The number of proliferating cells in the substitutes from children with and without cleft palate were similar. Some positive cells were also observed in the underlying connective tissue.

Heparan sulphate staining was observed at the junction between the epithelium and the connective tissue, and around blood vessels in the native tissue (Fig. 3, HS). A similar staining pattern, although more intense, was observed in the substitutes. Heparan sulphate staining was similar before and after cell seeding (not shown).

In the native mucosa and all substitutes, decorin expression was distributed throughout the connective tissue (Fig. 3, Dec).

DISCUSSION

Reconstructions of the mucosa in the oral cavity after cleft palate repair are often complicated by a
lack of suitable graft tissue. Several researchers have constructed substitutes of normal palatal mucosa using de-epidermized connective tissue or collagen scaffolds to solve this problem (Cho et al., 2000; Ophof et al., 2002). In the present study, from children with cleft palate and from age-matched individuals, mucosal substitutes were constructed with keratinocytes and fibroblasts cultured on de-epidermized connective tissue. According to the methods described, from 4 to 6 composite cultures (3-5 cm²) can be prepared within 4-5 wks. This would be enough to cover the lateral denuded bony areas at the palate after surgical closure of the cleft.

The morphological characteristics of both types of mucosal substitutes were similar to those of normal oral mucosal epithelium in vivo, but they were generally thinner. The mucosal substitutes prepared from persons with cleft palate were similar to those from individuals without cleft palate, and to mucosal substitutes prepared by others (Cho et al., 2000). All substitutes possessed a cornified layer that was much thicker than in the native mucosa, which was also reported for skin substitutes (Lee et al., 2000). We suggest that this might be caused by the absence of mechanical abrasion in culture. The junction between epithelium and underlying connective tissue provides mechanical attachment. The epithelial ridges in the mucosal substitutes were much smaller, which might indicate that the attachment is weaker.

The expression of keratins was similar in substitutes from children with and without cleft palate, and both were similar to expression in native mucosa. This indicates that the differentiation pathway of cleft palate keratinocytes is not affected by reported genotypic differences (Blavier et al., 2001; Jugessur et al., 2003; Krapels et al., 2006). In general, the staining intensity for both keratins 5 and 16 was much higher in the substitutes than in the native mucosa. We suggest that the expression of keratins in the cultures is higher because of the stimulatory factors in the medium. Native mucosa and the mucosal substitutes expressed keratin 10 in the superficial cornified layers, but the expression was not continuous in 1 epithelial cell layer. This indicates that the expression of keratin 10 does not start at the same time in every differentiating keratinocyte. Alternatively, this might be related to the immaturity of the epithelium.

The colony-forming efficiency of cleft palate and normal keratinocytes was similar, but lower than in earlier reports on skin keratinocytes (Castro-Munozledo et al., 1997). The reason might be that the keratinocytes in our study were obtained from a very small biopsy and were passaged 3 times in culture to yield enough cells. In spite of this, the Ki67 staining in the basal layer suggests that the keratinocytes were still proliferating. The numbers of positive cells in substitutes from children with and without cleft palate were similar, but always less than in native mucosa. This suggests that the proliferative capacity of the keratinocytes decreases by culture, which was also shown for keratinocytes from masticatory mucosa (Izumi et al., 1999).

The extracellular matrix (ECM) proteoglycan decorin has important functions in organogenesis during embryonic development by binding and assembling other ECM molecules, such as collagens (Keene et al., 2000; Wiberg et al., 2001), fibronectin (Winнемoller et al., 1991), and thrombospondin (Winнемoller et al., 1992; Davies et al., 2001). It can also modulate the signaling pathway of TGF-β (Yamaguchi et al., 1990; Hildebrand et al., 1994), which is also important in organ development (Kingsley, 1994). In retinoic acid-induced cleft palate, decorin expression was increased (Zhang et al., 2003). In the present study, the expression of decorin was similar in both mucosal substitutes, and also similar to the levels in native mucosa. However, the protein was already abundantly present in the connective tissue before culture. Therefore, we cannot confirm earlier results showing that decorin expression is increased in cleft palate fibroblasts (Bodo et al., 1999).

In conclusion, the present results show that palatal cells from children with cleft palate have a capacity to regenerate palatal mucosa similar to that of palatal cells from children without cleft palate. The reported genetic and phenotypic differences between palatal cells from children with and without cleft palate apparently do not affect their regenerative capacity in vitro. Therefore, we conclude that palatal cells from children with cleft palate are able to regenerate an oral mucosa in vitro, which might be used for cleft palate repair.
ACKNOWLEDGMENTS

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REFERENCES


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