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Modulation of gingival epithelial phenotypes by interactions with regionally defined populations of fibroblasts

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Background and Objective: The unusual structure and functions of junctional epithelium, together with its pattern of migration in periodontal disease, raise interesting questions about the factors associated with the maintenance of its unique phenotype. To explore the effects of regionally differing fibroblast populations on the growth and patterns of differentiation of oral epithelia, this study used an organotypical *in vitro* model in an attempt to detect interactions occurring between populations of human oral fibroblasts and keratinocytes.

Material and Methods: Keratinocytes and fibroblasts, isolated from the gingival region and periodontal ligament, were characterized by their patterns of growth and by their expression of known differentiation markers. Changes in cell behaviour and phenotypic marker expression were examined during *in vitro* passage as an indication of the maintenance of *in vivo* phenotypic traits. Using early passage cells, organotypical cultures were generated and patterns of epithelial growth and expression of phenotypic markers were examined.

Results: Phenotypically different populations of junctional and oral-gingival keratinocytes, and of oral-gingival and periodontal ligament fibroblasts, were successfully isolated, cultured and characterized. In the organotypic culture system, oral-gingival fibroblasts were found to have a markedly greater ability than periodontal ligament fibroblasts to support and maintain the growth of either type of epithelium. Shifts of epithelial phenotype were induced by different fibroblasts.

Conclusion: Periodontal and gingival fibroblast subpopulations have differential effects on the growth and patterns of differentiation of oral and junctional epithelia. By modulating the epithelial phenotype, regionally differing fibroblasts can influence the stability and behaviour of the gingival attachment apparatus in health and disease.

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The mammalian dentition has many unusual structural and functional properties, one of which is the unique pattern of attachment of an erupted tooth to the adjacent gingiva (1). Epithelia with three distinct phenotypes are present in the gingival region: the oral-gingival epithelium, which is similar in structure to keratinizing epithelia found in other regions of the oral cavity; the oral-sulcular epithelium, which is similar to nonkeratinizing oral epithelia; and the junctional epithelium, which provides physical attachment to the tooth surface and

has a unique phenotype (2). The stratifying squamous epithelium forming the junctional epithelium is continuous with the oral-sulcular epithelium and oral-gingival epithelium but differs from them histologically and ultrastructually (3,4) and in its differential expression of a wide range of molecules such as cytokeratins, blood group antigens, integrins, intracellular adhesion molecule-1, type VIII collagen, lectins, desmoplakins and matrix metalloproteinases (5–16). An early defining change of advancing periodontal disease is migration of the apical junctional epithelium from its normal termination at the cemento–enamel junction towards the root apex. It has been suggested that such migration is normally inhibited by the presence of intact periodontal ligament fibres and that junctional epithelium migration occurs only secondarily after their inflammatory destruction (17,18). Other observations suggest that stabilization of the gingival attachment apparatus depends also on unusual phenotypic properties of the junctional epithelium itself (19–21). As yet, however, whether the junctional epithelium responds to inflammatory stimuli in the same way as other epithelia remains unclear and even the origin of the junctional epithelium that is associated with fully erupted teeth is uncertain. During tooth eruption there is proliferation and transformation of the reduced enamel epithelium into a stratifying epithelium (1), but this epithelium may be subsequently replaced by downgrowth of the oral-gingival epithelium (1,3). After its removal by gingivectomy, regeneration of junctional epithelium occurs, possibly as a result of phenotypic change of either the oral-gingival epithelium adjacent to the oral aspects of the wound or from rests of Malassez at the radicular surface (19,22). The formation around titanium implants of an epithelium expressing an apparently normal junctional epithelium phenotype indicates the likelihood of its reformation from the oral-gingival epithelium (23).

Cytokeratins, the major structural proteins of epithelial cells, form a family of over 20 different polypeptide gene products whose patterns of expression vary both with the epithelium of origin and with the stage of differentiation of individual epithelial cells. The consistency of these patterns provides a sensitive, but robust, method for defining regionally differing epithelial phenotypes, including those of the oral mucosa (24–26). In general, the basal cells of all stratified oral epithelia express cyokeratin 5 and cytokeratin 14. With the onset of differentiation and movement suprabasally, cells of nonkeratinizing epithelia express cytokeratin 4 and cytokeratin 13, and cells of keratinizing epithelia express cytokeratin 1 and cytokeratin 10. Human and murine junctional epithelia show the expression of cytokeratins 5 and 14, typical of a stratified epithelium, but lack expression of normal suprabasal markers, such as cytokeratins 1, 4 and 10 and show suprabasal expression of cytokeratins 8, 18 and 19, cytokeratins usually found in simple epithelia (2).

The phenotypes expressed by adult epithelia are typically determined through reciprocal interactions with adjacent connective tissues during development (23,27,28). Several families of signalling molecules are associated with epithelial–mesenchymal interactions in embryonic tissues and in adult tissues such as hair (29). Less is known about the roles of interactions occurring between adult mucosal tissues (30,31) but experimental recombination of the epithelial and subepithelial connective tissues components of adult skin and mucosae has shown that tissue interactions continue to be involved with the maintenance and repair of epithelia during later life, that the adult epithelial phenotype is to some extent plastic, and that expression of new phenotypic patterns can be induced by recombination with subepithelial connective tissues from other regions (32–35). Fibroblasts form the major cell type residing in gingival and periodontal connective tissues and synthesize an array of biological mediators capable of influencing epithelial behaviour. They also show markedly different properties, depending on their region of origin, and functionally heterogenous populations of fibroblasts can exist within the same anatomical region (30,31,36). Emphasis was initially placed on the role of subepithelial fibroblasts in determining regional variations in the epithelial phenotype (30,31,36,37), but it is also apparent that epithelial cells synthesize a wide variety of cytokines that have paracrine effects on subepithelial fibroblasts (19,27,28).

Cell and tissue interactions, particularly those potentially occurring in the gingival region, are difficult to analyse *in vivo* and the patterns of structural organization that characterize regionally differing epithelia are largely lost when epithelial cells are isolated and grown under typical *in vitro* conditions (38). However, some *in vitro* models have been able to address questions of how epithelial–mesenchymal exchanges may influence the growth and differentiation of epithelia (39–41). Such 'organotypic' culture systems, in which epithelial cells are grown on collagen matrices containing fibroblasts, mimic *in vivo* interactions and allow persistence of at least some of the *in vivo* cell interactions associated with epithelial maintenance (42–45). Consequently, such cultures can lead to the re-expression of *in vivo*-like patterns of epithelial differentiation and kinetic behaviour (42,43,46) and allow investigation of the roles played by cell interactions and other physiological processes in oral tissues (39,42,43,46,47). The present study exploited the opportunity provided by organotypic cultures to construct recombinations of fibroblasts and epithelial cells that allow comparison of differences in the abilities of oral-gingival fibroblasts and periodontal ligament fibroblasts to support and maintain the oral-gingival epithelium and junctional epithelium phenotypes.

Material and methods

Tissue collection

Informed consent was obtained from patients for collection of samples of gingiva, ≈ 5 mm $\times$ 3 mm, attached to teeth at the time of routine tooth extraction. Samples showing little or no signs of clinical inflammation were

collected from patients with an age range of 13–44 years. Tissue was stored at 4°C in a supplemented medium termed FAD (48), consisting of 3 : 1 (v/v) Dulbecco's modified Eagle's medium : Ham's F12 with 10% fetal bovine serum, epidermal growth factor (10 ng/mL), penicillin/streptomycin (100 IU/mL), adenine (0.089 mm), hydrocortisone (400 ng/mL), insulin (5 μg/mL) and cholera toxin (10^{-10} M). The tissue was vortexed in three changes of medium to remove loose surface material and, for further control of microbial contamination, specimens were incubated overnight in medium additionally supplemented with amphotericin B (0.25 μg/mL), nystatin (24 mL/L) and Plasmocin© (25 μg/mL), an inhibitor of Mycoplasma.

Cell isolation and amplification

Keratinocyte and fibroblast populations were derived as described by Igarashi *et al.* (49). Each sample of attached gingiva was microdissected, as previously described in detail by Gao & Mackenzie (2), to separate the junctional epithelium complex from the bulk of the tissue, and then to remove the crestal region to leave the oral-gingival epithelium attached to its supporting connective tissues. Tissues were then treated with 0.25% trypsin overnight at 4°C to separate the epithelium from the underlying connective tissue components. Each epithelial sample was then further dissociated into a cell suspension by mincing and brief treatment with 0.25% trypsin/EDTA at 37°C. Using the basic techniques of Rheinwald & Green (50), with minor modifications described previously (51), cells were pelleted by centrifugation, resuspended in medium and plated onto a mitomycin-C-treated 3T3 feeder layer. Keratinocytes were grown in the basic FAD medium and, when colonies were well established, passaged by treatment with trypsin for 10 min at 37°C and split at a 1 : 10 dilution ratio for amplification in T75 flasks with 3T3 feeders.

Populations of oral-gingival fibroblasts were obtained by suspending minced fragments of the subepithelial gingival connective tissues in a collagen gel to allow cell outgrowth. Fibroblasts were harvested from gels by treatment with 0.1% collagenase at 37°C before plating for amplification in T75 flasks. When reaching 80–90% confluency, fibroblasts were replated in flasks at a dilution ratio of 1 : 6. Populations of periodontal ligament fibroblasts were similarly obtained from connective tissues scraped from the mid-third region of the tooth root. Fibroblast cultures were maintained in medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin/streptomycin (100 IU/mL). Unless otherwise stated, all cell culture consumables were obtained from Invitrogen™ Life Sciences (Paisley, UK) and other reagents and chemicals from Sigma-Aldrich (Dorset, UK).

Alkaline phosphatase expression

Fibroblast cultures were fixed in 4% paraformaldehyde for 10 min and then washed in three changes of phosphate-buffered saline over 20 min with Tris(hydroxymethyl)aminomethane buffer (TRIS)-buffered saline. The alkaline phosphatase detection substrate, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma B0274; Sigma-Aldrich), was prepared according to the manufacturer's instructions and samples were incubated for up to 2 h. The reaction was halted by rinsing in double-distilled water and specimens were mounted in aqueous Crystal/mount™ (Biomeda Corp., Foster City, CA, USA). Specimens were viewed using a Nikon Eclipse TS100 inverted phase microscope and photographed using a Nikon Coolpix 950 digital camera (Kingston, UK).

Organotypic cultures

Organotypic cultures were prepared as previously described by Igarashi *et al.* (49) with preparative procedures performed on ice. Briefly, collagen matrices containing 5×10^5 fibroblasts were prepared by the addition of 10× Dulbecco's modified Eagle's medium/fetal bovine serum/collagen (0.1% acetic acid digestion of rat-tail tendon at 4 mg/mL) (1 : 1 : 8; v/v/v). Following mixing and neutralization of this mixture by the dropwise addition of 1 N NaOH, fibroblasts suspended in 1 mL of fibroblast medium were added. The mixture was poured into 24-well plate wells (1.0 mL/well) and allowed to gel for 20 min at 37°C. Dulbecco's modified Eagle's medium was then added to cover each gel for incubation overnight at 37°C and in 5% CO_2. The medium was removed 24 h later and the gels were plated with 1×10^6 keratinocytes (at passages 2 or 3) in FAD medium and the keratinocytes were allowed to adhere for 24 h. A nylon mesh of 100-μm pore size (Tetko Inc., Depew, NY, USA) was prepared by coating with collagen, which was then cross-linked with 0.1% glutaraldehyde and extensively washed. Gels were placed onto pieces of mesh, epithelium uppermost, and supported on stainless steel grids in six-well culture plates with FAD medium added to just touch the underside of the mesh. Cultures were then maintained for 14 d at the air/liquid interface with the medium changed every 3 d.

Immunohistochemistry

Half of each organotypic culture was fixed in 4% paraformaldehyde and wax embedded for sectioning and haematoxylin and eosin staining. The other half of each culture was orientated and mounted in embedding medium (Cryomatrix™; Thermo Shandon, Runcorn, UK) and snap-frozen in isopentane chilled with liquid nitrogen for frozen sectioning. Sections were cut at 7 μm, collected on poly-L-lysine-coated slides, fixed in ice-cold acetone/methanol (1 : 1) for 10 min, washed in phosphate-buffered saline for 5 min and blocked with 2% bovine serum albumin for 30 min. Anti-cytokeratin monoclonal antibodies were used as undiluted supernatants and commercial antibodies used at previously determined optimal concentrations (Table 1). Staining was carried out in a humid chamber at 4°C overnight and, following three 10-min washes with phosphate-buffered saline, the appropriate fluorescein isothiocyanate-conjugated secondary antibody (Dako, Ely, UK) was applied for 2 h at room

Table 1. Monoclonal antibody staining patterns of cells and tissues for markers of epithelial differentiation

	In vivo		*In vitro*		Organotypic					
Marker	JE	OGE	JE colonies	OGE colonies	OGE/OGF	JE/PDLF	JE/OGF	OGE/PDLF	mAb	Concentration
CK1	–	++	–	+	++	–	+	+	LHK1[a]	Supernatant
CK10	–	+++	–	+	+++	–	++	+	LHP2[a]	Supernatant
CK4	–	+	–	–/+	++	–	+	–/+	6B10[b]	1 : 50
CK13	–	+	–	–/+	++	+	++	+	1C7/2D7[b]	1 : 50
CK6	–	++	+/+++	+/+++	+++	+++	+++	+++	HK6[a]	Supernatant
CK16	–	++	+/+++	+/+++	+++	+++	+++	+++	LL025[a]	Supernatant
CK8	++	–	+/++	+–	+	++	+/++	–/+	LE41[a]	Supernatant
CK18	+	–	++	+–	–	++	+/++	–	LE61[a]	Supernatant
CK5	+++	+++	+++	+++	+++	+++	+++	+++	AF138[c]	1 : 500
CK14	+++	+++	+++	+++	+++	+++	+++	+++	LL002[a]	Supernatant
CK19	+++	–	+++	–/+	+	+++	++	++	LP2K[a]	Supernatant
ICAM-1	++	–	++	–	–	++	–	–	CD54[d]	1 : 40
DP I+II	+	+	+	–/+					DP2.15[g]	10 μg/mL
DBA	++	–	+/++	–	–	++	+	++	Lectin[e]	5 μg/mL
MMP-7	+++	–	+/+++	+/++					Ab-3[f]	(1 : 100)

Staining of specimens was classified as: +++, strong positive staining; ++, positive staining; +, weak positive staining, –/+, variable weak staining; – no staining.

Antibody sources were as follows: [a]Gifts of Prof. I. Leigh (University of Dundee, UK); [b]Neomarkers; [c]Berkeley Antibody; [d]Dako; [e]Vector labs; [f]Oncogene; [g]Cymbus Biotech.

S, supernatant.

CK, cytokeratin; DBA, *Dolichos biflorus* agglutinin; DP, desmoplakin; ICAM-1, intercellular adhesion molecule-1; JE, junctional epithelium; mAb, monoclonal antibody; MMP, matrix metalloproteinase; OGE, oral-gingival epithelium; OGF, oral-gingival fibroblasts; PDLF, periodontal ligament fibroblasts.

temperature. Samples were viewed at standard magnifications on an Olympus Provis AX70 fluorescent microscope (Olympus UK Ltd, London, UK) at 420–490 nm and photographed using a Nikon DXM1200 digital camera.

Results

Fibroblast characterization

Fibroblast populations were successfully generated from the superficial lamina propria of eight specimens of oral-gingival connective tissues and from seven specimens of periodontal connective tissues (periodontal ligament fibroblasts). From six of the patient samples, paired cultures of oral-gingival fibroblasts and periodontal ligament fibroblasts were obtained. All fibroblast populations had the distinctive spindle-shaped appearance typical of fibroblasts, and no consistent morphological differences were apparent between oral-gingival fibroblasts and periodontal ligament fibroblasts. Below passage 4, oral-gingival fibroblasts and periodontal ligament fibroblasts both showed good growth, each approaching confluence within 5–7 d after splitting at a 1 : 6 dilution ratio. However, at higher passages growth rates deteriorated, with growth ceasing for periodontal ligament fibroblasts at about passage 7 and for oral-gingival fibroblasts at about passage 9. With cessation of growth, cells became larger, flattened and developed multiple cell processes (Fig. 1A,B). Primary and early passage (passages 2 or 3) cultures of oral-gingival fibroblasts contained a few cells staining weakly for alkaline phosphatase but the equivalent periodontal ligament fibroblast cultures contained an abundance of strongly reactive cells (Fig. 1C,D). Staining of individual cells was readily identified in cell smears developed from early passage 1 or 2 samples and counts indicated 0–11% of positive cells in oral-gingival fibroblasts populations compared with 51–88% in periodontal ligament fibroblast populations. To determine how alkaline phosphatase expression changed with increasing passage number, cultures stained at each passage were scored as positive for alkaline phosphatase expression if any positive cells were present. Alkaline phosphatase activity was absent in 50% of oral-gingival fibroblast cultures by passage 3 and from all oral-gingival fibroblast cultures by passage 4. All periodontal ligament fibroblast cultures retained alkaline phosphatase expression up to passage 5, and three of seven periodontal ligament fibroblast cultures retained alkaline phosphatase expression up to passage 7.

Keratinocyte characterization

Cryosections of gingival samples stained with a panel of monoclonal antibodies against the markers of interest confirmed the presence of differential patterns of expression in the epithelia of origin. Oral-gingival epithelium differed from junctional epithelium in its expression of cytokeratins 1, 6, 10 and 16, and junctional epithelium differed from oral-gingival epithelium in its expression of cytokeratins 8, 18 and 19, intercellular adhesion molecule-1, *Dolichos biflorus* agglutinin and matrix metalloproteinase-7 (Table 1). Actively growing cultures of keratinocytes were successfully generated from 11 specimens of junctional epithelium and from six specimens of oral-gingival epithelium. In coculture with 3T3 feeder cells, junctional epithelium and oral-gingival epithelium cells formed colonies with a

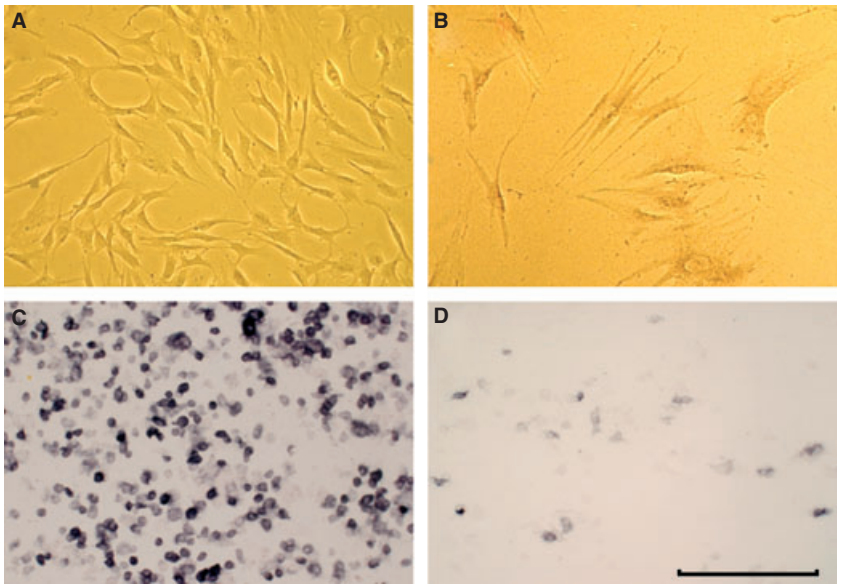

Fig. 1. Fibroblast morphologies and alkaline phosphatase expression. (A) At low passage (P1), oral-gingival fibroblasts show the spindle-shaped morphology typical of proliferative fibroblast cultures. (B) At high passage (P9) the same strain of oral-gingival fibroblasts approaches the end of its replicative life span and shows an increase in size, granular cytoplasm and a flattened morphology with multiple cell processes. Cell smears, prepared at similar cell densities from paired cultures of periodontal ligament fibroblasts (C) and oral-gingival fibroblasts (D) illustrate major differences in the number and intensity of cells reactive for alkaline phosphatase. (Scale bar for panels A and B= 75 μm; scale bar for panels C and D = 150 μm.).

range of morphologies corresponding to the holoclones, meroclones and paraclones, as previously described by Barrandon & Green for epidermal cell cultures (52). No major differences were noted in the early growth patterns of oral-gingival epithelium and junctional epithelium cultures, and the majority of colonies initially had smooth colony outlines and consisted of tightly packed cells, an appearance corresponding to that of holoclones. With further passage, however, there was an increase in the number of colonies consisting of larger, flattened cells and with morphologies corresponding to meroclones and paraclones. Such changes were apparent during a single passage; for example, examination of cultures of low-passage (passage 2) oral-gingival epithelium and junctional epithelium 2 d after plating showed a majority of colonies with holoclone morphologies, but by day 6 the proportion of holoclones had decreased for both types of cultures (Fig. 2). Counts of the number of cells per colony type showed a large, rapid increase in holoclones and a smaller increase in meroclones, an observation in keeping with the sustained

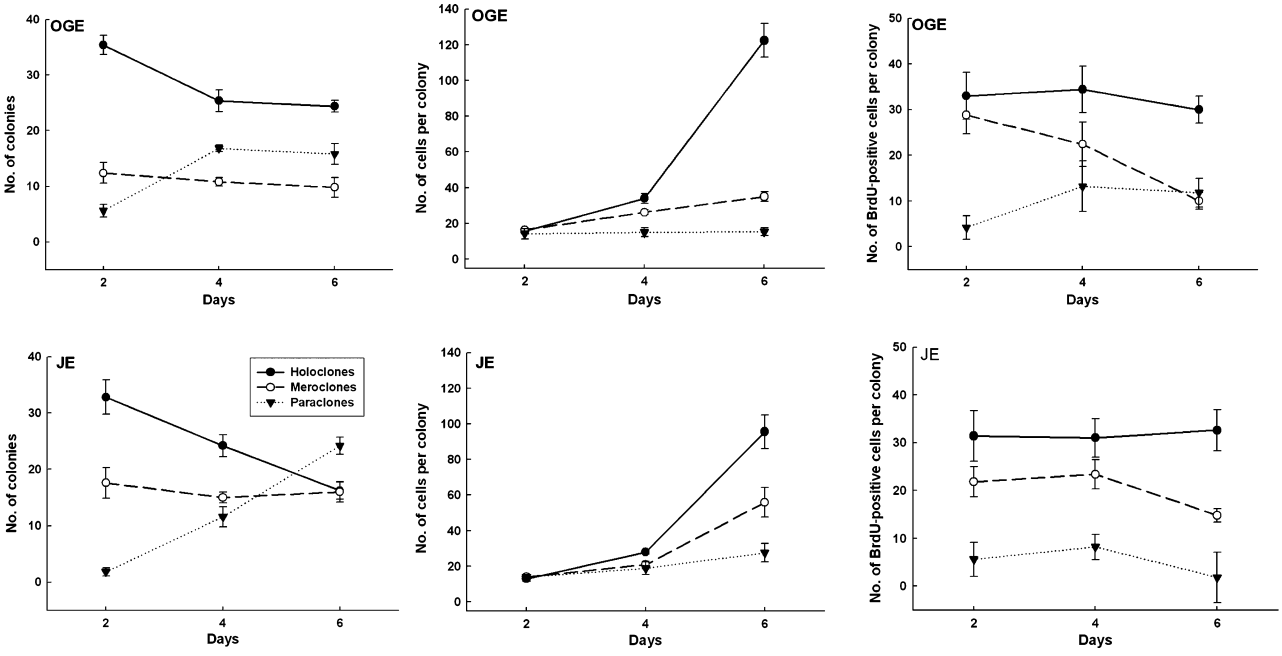

Fig. 2. Changes with time in epithelial colonies. The plots show, for oral-gingival epithelium and junctional epithelium colonies, counts of holoclone, meroclone and paraclone colonies per unit area (left panels), the total number of cells in each colony type (centre panels), and the percentage of cells labelled with 5-bromo-2-deoxyuridine (right panels). Counts were made 2, 4 and 6 d after plating. Initially high levels of holoclones decreased with time as the proportion of paraclones increased. Holoclones showed a marked increase in cell number over the 4-d period and a higher proportion of 5-bromo-2-deoxyuridine-labelled cells. BrdU, 5-bromo-2-deoxyuridine.

higher rate of cell proliferation in holoclones, as indicated by 5-bromo-2-deoxyuridine incorporation.

To test the degree to which phenotypic differences between *in vivo* epithelia were maintained *in vitro*, epithelial colonies were stained with monoclonal antibodies against the panel of markers listed in Table 1. Junctional epithelium *in vitro* retained *in vivo*-like patterns of expression, except that cytokeratins 6 and 16 were expressed at higher levels. Oral-gingival epithelium also maintained an *in vivo*-like profile but gained some expression of cytokeratin 19 and, occasionally, low levels of cytokeratins 8 and 18. Despite these changes, the expression of cytokeratins 1, 8, 10, 18 and 19, and of intercellular adhesion molecule-1, desmoplakin, and binding of the lectin *D. biflorus* agglutinin, provided consistent markers distinguishing the epithelial phenotypes.

Characterization of organotypic cocultures

Twelve separate series of organotypic cultures prepared from oral-gingival epithelium, junctional epithelium, oral-gingival fibroblasts and periodontal ligament fibroblasts demonstrated a consistent pattern of morphological differences. Oral-gingival epithelium cells plated on collagen gels populated with oral-gingival fibroblasts proliferated to form a stratified epithelia consisting of multiple cell layers with morphological evidence of suprabasal differentiation (Fig. 3A). Junctional epithelium cells plated on the surface of gels populated with periodontal ligament fibroblasts formed only a single layer of cells, typically with little

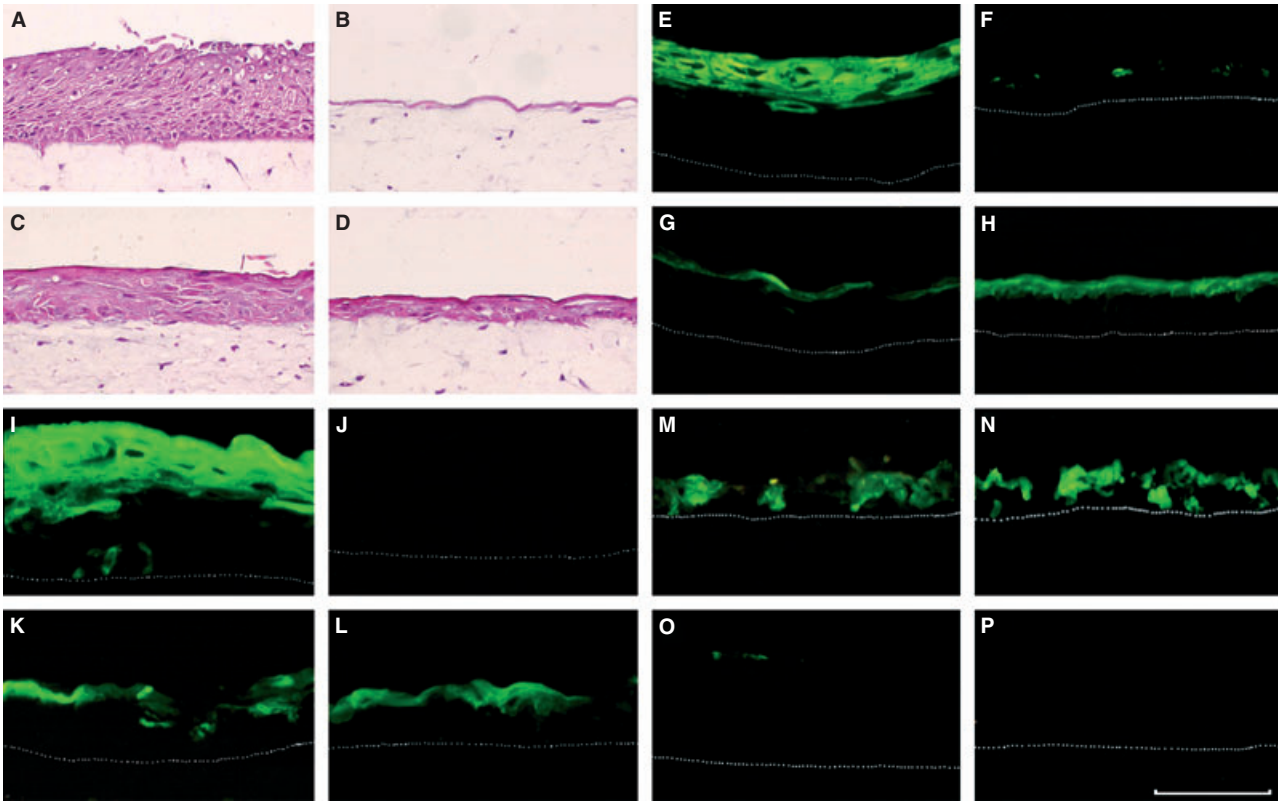

Fig. 3. Morphology and expression patterns of organotypic cultures 14 d after construction. (A) Haematoxylin and eosin-stained section of an organotypic culture of oral-gingival epithelium with oral-gingival fibroblasts. A thick well-formed epithelium consisting of many layers of stratifying cells has formed and shows morphological differences between basal and suprabasal cells. (B) A culture formed from junctional epithelium and periodontal ligament fibroblasts shows a thin epithelium with little evidence of stratification. (C) A culture of oral-gingival epithelium with periodontal ligament fibroblasts shows less growth and stratification than oral-gingival epithelium growing with oral-gingival fibroblasts. (D) A culture of junctional epithelium with oral-gingival fibroblasts shows enhanced growth compared to growth with periodontal ligament fibroblasts. Panels E–H show cryostat sections of organotypic cultures of oral-gingival epithelium/oral-gingival fibroblasts (E), junctional epithelium/periodontal ligament fibroblasts (F), oral-gingival epithelium/periodontal ligament fibroblasts (G) and junctional epithelium/oral-gingival fibroblasts (H) stained for cyokeratin 4. The culture of oral-gingival epithelium/oral-gingival fibroblasts shows high suprabasal expression and lack of expression of junctional epithelium/periodontal ligament fibroblasts. Growth of oral-gingival epithelium on periodontal ligament fibroblasts markedly reduces expression but growth on oral-gingival fibroblasts induces cytokeratin 4 in junctional epithelium. A similar pattern of induction and loss is seen for cytokeratin 10: (I) oral-gingival epithelium/oral-gingival fibroblasts; (J) junctional epithelium/periodontal ligament fibroblasts; (K) oral-gingival epithelium/periodontal ligament fibroblasts; and (L) junctional epithelium/oral-gingival epithelium. Cultures of junctional epithelium/periodontal ligament fibroblasts show expression of cytokeratin 8 (M) and intercellular adhesion molecule-1 (N) but cultures of oral-gingival epithelium/periodontal ligament fibroblasts do not show induction of these markers (O,P). (Scale bar for panels A–D = 150 μm; scale bar for panels E–P = 75 μm.).

evidence of stratification (Fig. 3B). The expression patterns of differentiation markers in such 'homotypic' cultures are listed in Table 1 and illustrated in Fig. 3. In general, the staining patterns of epithelia in oral-gingival epithelium/oral-gingival fibroblast cultures conformed to the patterns found for oral-gingival epithelium *in vivo* with the suprabasal expression of cytokeratins 1, 6, 10 and 16 typical of keratinizing oral epithelia, and some expression of cytokeratins 4 and 13, a pattern typical of gingival epithelia. Differences from the *in vivo* pattern were seen in the moderate suprabasal staining of oral-gingival epithelium/oral-gingival fibroblast cultures for cytokeratin 19 and their occasional weak staining for cytokeratin 8. In junctional epithelium/periodontal ligament fibroblast cultures, staining patterns for cytokeratins 8, 14, 18 and 19, and for *D. biflorus* agglutinin and intercellular adhesion molecule-1, corresponded to the *in vivo* tissue-staining patterns. The stronger staining of junctional epithelium/periodontal ligament fibroblasts for cytokeratins 6 and 16 differed from the *in vivo* pattern but conformed to the expression of these cytokeratins by colonies of junctional epithelium cells cultured on plastic.

'Heterotypic' cultures of oral-gingival epithelium grown with periodontal ligament fibroblasts showed markedly less growth than oral-gingival epithelium grown as homotypic cultures with oral-gingival fibroblasts and there was formation of fewer stratified layers of epithelial cells (Fig. 3C). Conversely, growth of junctional epithelium in heterotypic recombination with oral-gingival fibroblasts resulted in an epithelium of increased thickness compared with the single layer of keratinocytes seen in junctional epithelium/periodontal ligament fibroblast cultures (Fig. 3D vs. 3B). Small, but consistent, shifts of expression of phenotypic markers were seen in heterotypic cultures, with junctional epithelium cells grown on oral-gingival fibroblasts showing some gain of expression of cytokeratin 4 and loss of cytokeratin 19, and oral-gingival epithelium grown on periodontal ligament fibroblasts showing reduced expression of cytokeratins 1, 4 and 10, gains of expression of cytokeratin 19 and minor gains of cytokeratin 8.

Discussion

Previous studies have indicated that junctional epithelium expresses a highly unusual epithelial phenotype and have suggested potential roles for the junctional epithelium in stabilizing the dento–gingival attachment (4,19,21). A significant relationship between the form and function of junctional epithelium is indicated by its basic similarity of anatomical organization and phenotypic expression in humans, rodents and other animals (18). Further support for a concept of the functional significance of the unique junctional epithelium phenotype is provided by its consistent regeneration after its removal by gingivectomy (4). However, despite the potential importance of junctional epithelium in maintaining a healthy dentition, it remains unclear what mechanisms lead to the normal stability of the junctional epithelium attachment, its altered migratory behaviour during active periodontal disease and the epithelial plasticity necessary for its regeneration (3,18,19). It has been suggested that maintenance of the sulcular region relies on interactions between the gingival epithelia and underlying connective tissues with regionally differing functional properties (19,53). Regional differences in cytokine production have been reported (27,37,53,54) but detailed *in vivo* studies have been hindered by the complexity of gingival structure. *In vitro* models indicate that the phenotype and behaviour of gingival epithelia are strongly influenced by growth substrates (55,56) but influences of paracrine-acting mesenchymal factors on the *in vivo* properties of epithelia remain uncertain. Clear roles for epithelial–mesenchymal interactions in the regeneration and maintenance of epidermal and mucosal structure have been shown using *in vivo* transplantation (57) as well as *in vitro* organotypic culture systems (41–43,46). Studies focusing on oral mucosa have shown that organotypic cultures can regenerate essentially normal epithelial structure and that fibroblasts have important roles in its establishment (58,59). The present investigation, which set out to see whether organotypic methods could help unravel the nature of interactions occurring between gingival tissues, demonstrates: (a) that the basic cellular phenotypes of fibroblast populations isolated from around the tooth appear to persist *in vitro*, at least for several passages; (b) that there are marked differences in the abilities of oral-gingival fibroblasts and periodontal ligament fibroblasts to support epithelial growth; (c) that intrinsic differences between oral-gingival epithelium and junctional epithelium cells persist *in vitro*; and (d) that fibroblasts influence the phenotype expressed by an adjacent epithelium but were not, at least in this system, able to induce a complete phenotypic change.

Adequate isolation of regionally defined populations of fibroblasts was achieved using mechanical and enzymatic microdissection of tissue specimens. Gingival and periodontal fibroblasts have been reported to differ morphologically (60), but we were unable to detect consistent morphological differences between pairs of oral-gingival fibroblasts and periodontal ligament fibroblast cultures generated from individual patients. There were, however, consistent differences between oral-gingival fibroblasts and periodontal ligament fibroblast populations in their patterns of growth and marker expression. Periodontal ligament fibroblast cultures consistently showed less growth; there was failure of all periodontal ligament fibroblast cultures to passage beyond passage 7, whereas all oral-gingival fibroblast cultures passaged at passage 7 and five of eight passaged successfully at passage 9. When growth arrest eventually occurred, both types of fibroblasts showed morphological changes characteristic of senescence (Fig. 1B). Early passage cultures of periodontal ligament fibroblasts and oral-gingival fibroblasts were also clearly distinguished by their degree of alkaline phosphatase expression, with all periodontal ligament fibroblast populations showing reactiv-

ity for alkaline phosphatase in more than 50% of cells. The reactivity of oral-gingival fibroblast specimens ranged only from 0 to 11%, confirming the reports by Somerman *et al.* (61) and Piche *et al.* (60) that oral-gingival fibroblasts exhibit much lower alkaline phosphatase activity than periodontal ligament fibroblasts. The fraction of periodontal ligament fibroblast cells expressing alkaline phosphatase was higher during early passages and, in the absence of other clear markers, the maintenance of alkaline phosphatase expression was taken as an indication of the extent of *in vitro* phenotypic stability of periodontal ligament fibroblasts. All periodontal ligament fibroblast lines maintained alkaline phosphatase expression up to passage 5 and organotypic cultures were therefore constructed using periodontal ligament fibroblasts (and by analogy also oral-gingival fibroblasts) at passage 3 to avoid the potential effects of further proliferative phenotypic change before the cultures stabilized.

Keratinocyte cultures also maintained some of the basic phenotypic differences present between their *in vivo* tissues of origin. Immunofluorescence staining of *in vitro* colonies gave consistent and well-defined binding patterns for each of the antibodies used and the staining patterns observed were in essential agreement with previous reports (2,51). The principal changes resulting from *in vitro* growth on plastic were that colonies of oral-gingival epithelium cells showed reduced expression of *in vivo* differentiation markers (cytokeratins 1, 10 and 13) and acquired some expression of cytokeratin 19. Junctional epithelium colonies showed increased expression of cytokeratins 6 and 16. Thus, although keratinocytes growing as colonies on plastic with feeder cells have markedly different patterns of spatial organization from those present *in vivo*, they largely maintained phenotypic differences in their patterns of keratin expression, at least when tested at low passage. Differences in their expression of intercellular adhesion molecule-1 and *D. biflorus* agglutinin binding were also maintained. Analysis of colony morphologies and of the growth potentials of junctional epithelium and oral-gingival epithelium cultures indicated the presence of other *in vitro* differences. At each passage, cells were plated into T75 flasks at a relatively high dilution to allow morphological classification of individual colonies together with an assessment of colony sizes and cell labelling indices (Fig. 2). Barrandon & Green (52) have classified the colonies formed by individual keratinocytes into three types: round coherent colonies, consisting of small cells that can be extensively passaged, were termed holoclones and shown to give rise sequentially to meroclone and paraclone colony types, characterized by increasing cell size and a decreasing ability of their cells to passage. Initially, a high proportion of holoclones, thought to represent the stem cell component of the population, was present in both types of cultures. Total cell counts at early passages indicated similar growth rates for oral-gingival epithelium and junctional epithelium cultures, but, with increasing passage, junctional epithelium cultures showed less rapid growth and greater numbers of abortive, paraclone-like colonies. These results are in keeping with those of Gao & Mackenzie (2), who found that junctional epithelium showed less long-term *in vitro* growth than oral-gingival epithelium. As shown in Fig. 2, oral-gingival epithelium and junctional epithelium cultures initially showed similar proportions of holoclone colonies, rates of increase in holoclone size with time and rates of holoclone labelling with 5-bromo-2-deoxyuridine. However, whereas oral-gingival epithelium populations maintained rapidly proliferating holoclone colonies up to and beyond passage 5, junctional epithelium cells showed a rapid decrease in their ability to generate holoclone-like colonies and formed an increasing proportion of paraclones, the cell population entering a phase of terminal differentiation (50).

Fibroblasts play critical roles in maintaining epithelial differentiation, and epithelial cultures lacking fibroblast influences typically show less growth and reduced expression of regional differentiation markers (46,59). The construction of organotypic cultures from early cell cultures provided the opportunity to evaluate how the presence of fibroblasts influenced the *in vitro* growth and differentiation of gingival epithelia. Preliminary studies showed that neither oral-gingival epithelium nor junctional epithelium was consistently able to form a stratifying epithelium on collagen in the absence of fibroblasts (data not shown) and subsequent studies were therefore limited to cultures incorporating fibroblasts. Differences between epithelial growth in 'homotypic' cultures (i.e. junctional epithelium with periodontal ligament fibroblasts, and oral-gingival epithelium with oral-gingival fibroblasts) were extremely consistent. Oral-gingival epithelium formed a relatively thick, well-stratified epithelial structure and junctional epithelium formed an atrophic epithelium with little stratification. The pattern of epithelial growth observed in 'heterotypic' cultures indicated that despite their isolation and growth *in vitro*, intrinsic differences persisted between each cell type. Fibroblasts differed in their growth support, and cultures of oral-gingival epithelium with periodontal ligament fibroblasts showed a marked reduction in epithelial thickness compared to oral-gingival epithelium grown with oral-gingival fibroblasts. Conversely, cultures of junctional epithelium with oral-gingival fibroblasts showed a marked increase in epithelial stratification compared to junctional epithelium grown with periodontal ligament fibroblasts. However, some intrinsic differences persisted between the two epithelia: junctional epithelium failed to reach the same degree of stratification as oral-gingival epithelium when grown on oral-gingival fibroblasts; and oral-gingival epithelium formed a better stratified epithelium than junctional epithelium when grown on periodontal ligament fibroblasts.

The patterns of staining for cytokeratins in cultures of oral-gingival epithelium with oral-gingival fibroblasts showed a less regular relationship between differentiation and stratification than *in vivo* tissues, with expression of differentiation markers occurring at relatively high levels in the

epithelium. Generally, however, patterns of marker expression were similar to those of the parent tissue, the principal change being limited to the increased expression of cytokeratin 19. This change was also found for oral-gingival epithelium cells growing on plastic (Table 1) and is perhaps related to the presence of retinoids in the fetal bovine serum used to supplement the growth media (58). Cultures of junctional epithelium with periodontal ligament fibroblasts, although atrophic in appearance, maintained patterns of marker expression similar to those of junctional epithelium *in vivo* and the only consistent change was the increased expression of cytokeratins 6 and 16. Although growth with heterotypic fibroblasts resulted in clear morphological differences between epithelia, the effects of heterotypic fibroblasts on epithelial phenotypes were less clear. Heterotypic cultures of junctional epithelium grown with oral-gingival epithelium showed some induction of expression of cytokeratins 4 and 10, and oral-gingival epithelium grown with periodontal ligament fibroblasts showed induction or enhancement of expression of cytokeratins 8, 18 and 19, of intercellular adhesion molecule-1 and of *D. biflorus* agglutinin binding. Fibroblast-induced shifts towards new epithelial markers appropriate for the region of fibroblast origin suggest some influence on epithelial differentiation, but only a partial response was seen. This may have resulted from inadequacies of the *in vitro* conditions, but some observations indicate that the response may be limited biologically. For example, only partial shifts of prior phenotypic properties are seen when tissue recombinants are transplanted *in vivo* (57). Organotypic cultures demonstrate an influence of oral fibroblasts on the keratin expression of skin keratinocytes, but no influence of dermal cells on mucosal keratinocytes and no further changes were detected when such organotypic cultures were grafted to immune-deficient mice (44).

Further studies are needed to determine the nature of the signalling molecules that mediate reciprocal epithelial–fibroblast interactions in periodontal tissues in both health and disease. The junctional epithelium phenotype has previously been attributed to differences in the ability of subepithelial fibroblasts to support epithelial migration and differentiation (21) with intrinsic differences in fibroblast production of paracrine-acting growth factors playing a central role in such differences. However, additional secondary changes in fibroblast expression of epithelial mitogens could be exerted by various environmental stimuli and thus lead to alteration of the epithelial phenotype with acquisition of new patterns of behaviour, phenomena that are not dissimilar to those seen in chronic and advancing periodontal disease. The production of two growth factors – keratinocyte growth factor and hepatocyte growth factor – by mesenchymal cells lying close to epithelial structures, and the distinctive specificity of these factors for epithelial cells, suggests that they may be of particular importance in regulating the epithelial phenotype. The continuous interaction between epithelia and their supporting connective tissues appears to be maintained by a feedback mechanism in which epithelial release of factors, such as interleukin-1, stimulates the production of keratinocyte growth factor and hepatocyte growth factor by adjacent fibroblasts (41). As indicated by *in situ* hybridization, keratinocyte growth factor is normally produced by subepithelial fibroblasts but not by periodontal ligament fibroblasts *in vivo* (62). However, keratinocyte growth factor is produced by periodontal ligament fibroblasts when there is apical inflammation (54), and the production of similar quantities of keratinocyte growth factor and hepatocyte growth factor by periodontal ligament fibroblasts and oral-gingival fibroblasts when growing *in vitro* (53) illustrates that the levels of cytokine release depend on fibroblast activation as well as on their intrinsically different properties. Involvement of molecules such as interleukin-1, both in epithelial signalling pathways and in inflammation, indicates the possibility of cross-talk between these normally separate processes and suggests that subtle changes, such as the inflammatory activation of an invasive epithelial phenotype, may play a more important role in advancing periodontal disease than previous explanations of frank inflammatory destruction of the periodontal ligament. The present study indicates that fibroblasts have crucial effects on the proliferation and differentiation of gingival epithelia reconstituted *in vitro*. It also shows that organotypic culture methods can provide an experimental system for determining the roles of subepithelial fibroblasts, both in maintaining normal epithelial phenotypes and in modulating shifts from junctional epithelium stability to migration seen in advancing periodontal disease.

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