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Cancer progression is associated with increased expression of basement membrane proteins in three-dimensional *in vitro* models of human oral cancer

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ABSTRACT

Background: Although basement membrane was traditionally considered an inert barrier that tumour cells had to cross before invasion into the surrounding stroma, recent studies suggest that basement membrane components are not only degraded during tumour progression, but also newly synthesised at the invasive front.
Objective: This study aimed at evaluating (1) the expression of basement membrane proteins in human oral carcinogenesis and (2) the role that epithelial–mesenchymal interactions play on it, by using an *in vitro* oral cancer progression model.
Material and methods: *In vitro* three-dimensional (3D) organotypic cultures of normal, early neoplastic and neoplastic human oral mucosa were developed by growing primary normal human oral keratinocytes, dysplastic human oral keratinocytes (DOK cell line), and neoplastic human oral keratinocytes (PE/CA-PJ15 cell line) on type I collagen biomatrices, with or without primary fibroblasts isolated from normal human oral mucosa. The cultured tissues were immunohistochemically assessed for the expression of the major basement membrane proteins laminin-332, type IV collagen, and fibronectin.
Results: Expression of laminin-332, type IV collagen, and fibronectin was gradually more pronounced in neoplastic models when compared to normal mucosa models, and, with the exception of laminin-332, it was further enhanced by presence of fibroblasts. Deposition of type IV collagen at the epithelium–biomatrix interface occurred only in presence of fibroblasts, as well as the extracellular matrix deposition of fibronectin.
Conclusions: These findings, obtained in a 3D *in vitro* model that closely mirrors the *in vivo* human oral cancer progression, show an enhanced basement membrane protein expression during human oral cancer progression that is dependent on the epithelial–mesenchymal environment, respectively the existence of fibroblasts.

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1. Introduction

Basement membrane is the highly specialised structure of extracellular matrix between epithelium and underlying connective tissue that consists of a dense network of glycoproteins (such as laminin, nidogen and fibronectin), type IV collagen, and proteoglycans.[1] Although it was considered for decades a passive structure that separates epithelia from connective tissues only, later findings have shown that it is also able to influence the behaviour of the adjacent epithelium.[2–4] Basement membrane proteins have been found to affect epithelial cellular processes such as proliferation and differentiation in normal epithelia.[3–5] In neoplasia, breaks and defects of basement membrane were thought to be associated with the invasive behaviour of several types of tumours,[6,7] including oral neoplasia.[8–10] However, although historically considered as an inert barrier that tumour cells had to cross before they invaded the surrounding stroma, it has become recently clear that basement membrane is a rather dynamic structure in the neoplastic process.[11,12] It has been shown that basement membrane components are not only degraded during tumour progression, but also newly synthesised and deposited at the tumour invasive front. For example, overexpression of laminin-332 has been detected at the epithelial–stroma interface in invasive areas of colorectal, gastric and squamous cell carcinomas[13–16] and *in vitro* models of oral squamous cell carcinoma,[17] leading to the hypothesis that it could function as an extracellular ligand helping migration and invasion of tumour cells. Alterations in type IV collagen distribution in pre-malignant and malignant lesions of the oral epithelium have also been reported,[9,10] and its expression by neoplastic cells was found to be correlated with invasion in the connective tissue matrix in *in vitro* experimental models.[12,18] Increased expression of fibronectin was found as well to be associated with the invasiveness and the metastatic propensity of oral squamous cell carcinoma,[10,19] and the embryonic EDA and EDB fibronectin splice variants were found highly expressed in the active tumour stroma close to the invading oral squamous cell carcinoma nests.[20]

Taken together, these studies have shown that new deposition of major basement membrane proteins might be of importance for the invasive process, and suggest that the tumour stroma has a major role in this deposition. However, a more specific description of the dynamics of basement membrane and its dependency on epithelial–mesenchymal interactions during oral human tumour progression has not been studied. Thus, the aim of this study was to investigate the expression of laminin-332, type IV collagen, and fibronectin, and the role of epithelial–fibroblast interactions on their expression pattern during progression towards malignancy, using step-wise *in vitro* cell culture models of oral cancer progression.

2. Materials and methods

2.1. Tissue material

Normal human oral mucosa samples ($n = 17$) were obtained from redundant oral tissue removed during wisdom tooth extraction at Department of Oral Surgery, Faculty of Dentistry, University of Bergen, Norway. Informed consent was sought from the patients prior to obtaining the tissue material. The study was approved by the Ethical Committee of Western Norway and included tissues obtained only from clinically healthy donors.

2.2. Cell culture procedures

Primary normal oral keratinocytes (NOK) and fibroblasts (NOF) were isolated from normal human oral mucosa as described elsewhere.[21] NOK were routinely grown on plastic surfaces (Nunc, Naperville, IL, USA) with no feeding layers, in Keratinocyte-Serum Free Medium (KSFM, GibcoBRL, Grand Island, NY, USA) supplemented with 1 ng/ml human recombinant epidermal growth factor (GibcoBRL), 25 μg/ml bovine pituitary extract (GibcoBRL), 20 μg/ml L-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL), 100 μg/ml streptomycin (GibcoBRL), 0.25 μg/ml amphotericin B (GibcoBRL), 6 μg/ml fluconazole (Pfizer, Amboise, France). NOF were grown in Modified Eagle's Medium (Sigma, St. Louis, MO, USA) supplemented with 10% foetal calf serum (Sigma, St. Louis, MO, USA), 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 6 μg/ml fluconazole. The commercially available dysplastic keratinocyte cell line from the European Cell Culture Bank: human (Caucasian) dysplastic oral keratinocyte (DOK) cell line, accession no. 94122104 has been used in this study. This cell line was established from a tongue dysplasia. It was reported not to form tumours in nude mice, and considered to have a transformed but not fully malignant phenotype.[22] Later it was shown that the DOK cell line harbours p53 mutations.[23] DOK cells were routinely grown on plastic surfaces without feeding layers, in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% foetal calf serum, 20 μg/ml L-glutamine, and 5 μg/ml hydrocortisone. The oral cancer derived-PE/CA-PJ 15 cell line, accession no. 961211230, that has been previously successfully used in organotypic cultures of oral neoplasia, was also used.[16] It has been isolated from a well-differentiated buccal squamous cell carcinoma and it was reported to form tumours in nude mice with the histology similar to the native carcinoma.[17] PE/CA-PJ 15 were routinely grown in Iscove's Modified Dulbecco's Medium (Sigma) supplemented with 10% foetal calf serum and 20 μg/ml L-glutamine. DOK cells in passage 29 and PE/CA-PJ 15 cells in passage 9 were used for the experiments.

2.3. Cultivation of three-dimensional organotypic models of human oral mucosa

The multi-step model of oral epithelial tumour progression was obtained by growing normal (NOK, $n = 7$ cell strains isolated from different patients), early neoplastic/dysplastic (DOK) and neoplastic (PE/CA-PJ 15) oral keratinocytes on top of collagen type I biomatrices using a protocol well established in our laboratory.[24] In order to study the role of epithelial–mesenchymal interactions on basement membrane protein expression two types of organotypic cultures were prepared and used in this study: (1) organotypic monocultures in which each different type of keratinocytes was seeded on simple