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P-cadherin controls the differentiation of oral keratinocytes by regulating cytokeratin 1/10 expression via C/EBP-beta-mediated signaling

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ABSTRACT

P-cadherin belongs to the family of Ca^{2+}-dependent homophilic glycosylated cell adhesion molecules. In the normal oral epithelium it shows a strong expression in the basal cell layer which gradually decreases in the suprabasal cell layers. The exact role of P-cadherin during the development and homeostasis of the oral epithelium has not been elucidated, yet. Here, we show for the first time that P-cadherin controls differentiation by regulating cytokeratin (CK) 1/10 expression in primary oral keratinocytes (POK) from normal, but interestingly not in POKs from oral squamous cell carcinoma (OSCC) tissue. SiRNA knockdown of P-cadherin in normal POKs revealed a strong upregulation of CK1/10 expression on mRNA and protein level. In contrast, E-cadherin knockdown in normal oral keratinocytes did not show any influence on CK1/10 expression. Moreover, in comparison with normal control keratinocytes normal oral keratinocytes with reduced P-cadherin expression displayed an enhanced expression and a stronger nuclear staining of C/EBP-beta, a well-known regulator of CK1/10 expression in keratinocytes. Furthermore, after P-cadherin knockdown in normal POKs the promoter activity of a C/EBP-responsive luciferase construct was significantly higher than in normal POKs with regular P-cadherin expression. Additionally, we noticed a proliferation advantage in normal oral keratinocytes in contrast to keratinocytes with diminished P-cadherin expression. However, the inverted effect was seen in tumor derived primary oral keratinocytes. In summary, we show that P-cadherin contributes to the keratinocyte differentiation in the oral epithelium by influencing the CK1 and CK10 expression via C/EBP-beta-mediated signaling in normal but not in tumor derived oral keratinocytes from OSCC patients.

1. Introduction

It is known that cell-extracellular matrix and cell–cell interactions regulate early keratinocyte differentiation (Charest et al., 2009). Among various adhesion molecules the cadherins play an interesting role in this process. Besides intercellular adhesion, cadherin-mediated cell interactions stimulate signaling pathways that modulate the expression of differentiation related proteins, i.e. loricrin, profilaggrin and transglutaminase 1 (Larue et al., 1996) (Huber et al., 1996) (Holt et al., 1994) (Hines et al., 1999).

P-cadherin, an important epithelial protein which belongs to the family of glycosylated Ca^{2+}-dependent adhesion molecules, is specifically located in the membrane of the basal and suprabasal cell layers in normal oral mucosa (Lo Muzio et al., 2004) (Munoz-Guerra et al., 2005). With progression, the primary oral squamous cell carcinoma (OSCC) displays a strong overall P-cadherin staining in the malignant cells, whereas an increasing loss of P-cadherin can be observed in the cell membrane at the invasion front of OSCC (Williams et al., 1998) (Bauer et al., 2008). In our studies, we focus on the role of P-cadherin in normal and malignant oral keratinocytes. How P-cadherin is involved in tissue integrity and homeostasis is still elusive, thus our main goal is to determine the function of P-cadherin. In a recent study, we have demonstrated that the generation of truncated P-cadherin is involved in early steps of OSCC the most frequent malignant tumor of the oral cavity which arises from aberrant epithelial keratinocytes (Bauer et al., 2008). Malignant oral keratinocytes show a strong tendency to invade cervical lymph nodes and spread to distant sites relatively quickly (Baatenburg de Jong et al., 2001). Despite improved surgery, chemotherapy and radiation therapy over the last years the 5 year survival rate is still 50% (Argiris et al., 2008). This emphasizes the importance to explore novel molecular markers which could help to improve diagnosis and treatment of OSCC.

In this manuscript, we highlight the difference between P-cadherin and E-cadherin, another member of the prominent cadherin family which is expressed in the upper layers of the oral

Abbreviations: C/EBP, CCAAT-enhancer binding proteins; CK, Cytokeratin; OSCC, Oral squamous cell carcinoma

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epithelium (Munoz-Guerra et al., 2005). Both cadherins are important for maintaining cellular localization and tissue integrity (Shimoyama et al., 1989). By means of their extracellular domain they mediate homophilic interactions between cadherins of adjacent cells in a Ca^{2+}-dependent manner (Halbleib and Nelson, 2006). At low Ca^{2+}-concentrations cadherins are synthesized and are located mainly in perinuclear compartments but also at the cell membrane in an inactive state. By elevating Ca^{2+}-concentration, cadherins are transported to the cell surface, rapidly become activated and form adherens junctions (Wheelock and Jensen, 1992) (Hines et al., 1999) (Kam and Quaranta, 2009).

To maintain its structural integrity the oral epithelium is generated by a process of constant cell renewal (Dale et al., 1990). In the basal layer cells are produced by mitotic divisions and are pushed to the surface to replace the cells that are shed. While moving to the upper layers the keratinocytes undergo drastic changes which involve phenotypic aspects, changes in the expression of membrane-associated proteins and cytoskeletal components. Additionally, the differentiating keratinocytes alter their cytokeratin (CK) expression. CKs are the major structural proteins in epithelial cells. They exist as type I and type II heterodimers that assemble to form intermediate filaments and function to maintain cell and tissue integrity (Fuchs, 1988) (Fuchs, 1995). Depending on cell type and stage of differentiation distinct pairs of CKs are expressed. At the beginning of the terminal differentiation CK1 and CK10 assemble and build up the intermediate filament in the suprabasal cell layers (Dale et al., 1990) (Steinert and Roop, 1988). It is known that various extra signals like calcium and Wnt molecules as well as transcription factors influence the expression of the CKs and, consequently, the keratinocyte differentiation in the oral epithelium. Besides various transcription factors, CCAAT-enhancer binding proteins (C/EBP) are considered to be potent regulators of CK1/10 expression in keratinocytes (Eckert et al., 1997) (Lopez et al., 2009) (Maytin et al., 1999) (Zhu et al., 1999).

In this study, we show that P-cadherin which is expressed in the basal and suprabasal cell layers is an important component in the oral epithelium which helps to control CK1/10 expression and keratinocyte differentiation.

2. Materials and methods

2.1. Isolation of normal and OSCC primary keratinocytes

Primary keratinocytes from normal oral mucosa and OSCC tissue which were collected with patient consent and ethics approval were isolated as previously described (Izumi et al., 2009). Briefly, normal oral mucosal and OSCC tissue from the same patient undergoing oral surgery procedures were digested overnight with 0.04% trypsin solution (Invitrogen, Karlsruhe, Germany) diluted in phosphate buffered saline (PBS, Sigma, Steinheim, Germany) at 4 °C. After stopping trypsinization with 0.0125% trypsin inhibitor (Invitrogen) the mucosa was separated from the submucosa and minced by scalpel. Dissociated oral keratinocytes were cultured in cell culture flasks coated with coating matrix which includes collagen (1:100) (Invitrogen) diluted in dilution medium (Invitrogen) for 30 min. To sort putative basal keratinocytes efficiently, a serial passive filtration system using nylon filters of two different pore sizes (Partec GmbH, Görlitz, Germany) was used. The 30 μm filter removed the differentiated cells, while the 20 μm filter allowed smaller, putatively undifferentiated cells to pass through, since their size was less than 20 μm (Barrandon and Green, 1987).

2.2. Cells and cell culture

Isolated normal primary oral keratinocytes (normal POK) and primary oral keratinocytes from OSCC tissue (OSCC POK) were maintained in EpiLife medium (contains 0.06 mM calcium chloride) (Invitrogen) supplemented with 1% EpiLife defined growth supplement (Invitrogen), 25 μg/ml Gentamycin (Invitrogen) and 0.375 μg/ml Fungizone (Invitrogen). The human OSCC cell lines PCI1, PCI13 and PCI68 were kindly provided by Prof. T. Whiteside (University of Pittsburgh Cancer Institute, Pittsburgh, PA). The OSCC cell lines were maintained in Dulbecco's modified Eagle's medium (Pan-Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (Gibco, Karlsruhe, Germany), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco).

2.3. Transient transfections

Transient transfections of normal POKS, OSCC POKs and OSCC cells lines, respectively, were conducted using X-tremeGene HP DNA transfection reagent (Roche, Penzberg, Germany) and Dharmafect-1 (Thermo Scientific, Bonn, Germany), respectively, according to the manufacturer's instructions. The cells were transfected with small interfering RNA (siRNA) specific against human P-cadherin, E-cadherin and C/EBP-beta (Thermo Scientific), respectively. As a control scrambled (scr) siRNA (Thermo Scientific) was used. Briefly, cells were seeded the day before transfection in medium without antibiotics in 6-well cell culture plates (BD Transduction Laboratories, Philadelphia, USA). Subsequently, the cells were transfected with 2 μg siRNA against P-cadherin (siRNA Pcad), against E-cadherin (siRNA Ecad), against C/EBP-beta (siRNA C/EBP-beta) and scr siRNA, respectively. After 48 h, total cellular RNA was isolated for quantitative real-time polymerase chain reactions (qRT-PCR). For each experiment, mRNA knockdown was determined and a knockdown efficiency was achieved between 60% and 95%. Three days after siRNA transfection cell lysates were prepared for immunoblot analyses and immunofluorescent stainings were determined.

Additionally, to analyze CK expression in keratinocytes cultivated in medium with high calcium chloride concentration, normal POKs maintained in EpiLife medium supplemented with 1 mM calcium chloride (Merck, Darmstadt, Germany) were transfected with siRNA.

2.4. RNA isolation and quantitative real-time polymerase chain reaction

Total cellular RNA from cells was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of 1 μg RNA to complementary DNA (cDNA) was performed using transcriptor high fidelity cDNA synthesis kit (Roche) according to the manufacturer's protocol. cDNA was amplified using the Brilliant III ultra fast quantitative polymerase chain reaction master mix (Stratagene Agilent Technologies, Santa Clara, CA.) in combination with TaqMan UPL probes (Roche). Real-time PCR primers were obtained from TibMolBiol (Berlin, Germany). Beta-actin messenger RNA (mRNA) was used for normalization (beta-actin: 5′-ATTGGCAATGAGCGGTTC-3′ and 5′-TGAAGGTAGTTTCGTGGATGC-3′. C/EBP-beta: 5′-CGCTTACCTCGGCTACCA-3′ and 5′-ACGAGGAGGACGTGGAGAG-3′. CK1: 5′-TCGACAATGTCAAGAAGCAGA-3′ and 5′-GTCTTCCTTGGCCTGCTG-3′. CK5: 5′-GTTGGACCAGTCAACATCTCTG-3′ and 5′-CTGCTACCTCCGGCAAGA-3′. CK10: 5′-CCATCGATGACCTTAAAAATCAG-3′ and 5′-GCAGAGCTACCTCATTCTCATACTT-3′. CK14: 5′-GTCCCAGCTCAGCATGAAA-3′ and 5′-GCAGTAGCGACCTTTGGTCT-3′).

2.5. Human stem cell PCR array

Normal POKs were transfected with siRNA against P-cadherin and with scr siRNA. Two days after transfection total cellular RNA was isolated using the RNeasy mini kit. Reverse transcription of 1 μg RNA to cDNA was performed using RT first strand kit