From the
Institute of Environmental Medicine, Division of Biochemical Toxicology and
Experimental Carcinogenesis, Karolinska Institutet, Stockholm, Sweden
and the Institute of Odontology, Division of Basic Oral Sciences,
Karolinska Institutet, Stockholm, Sweden

MODELING OF MULTI-STEP ORAL CARCINOGENESIS IN VITRO: ASSESSMENT OF GROWTH, DIFFERENTIATION AND APOPTOSIS MARKERS

Annette Hansson

Stockholm 2003

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-445-3

To My Nearest and Dearest

ABSTRACT

Human oral mucosa, especially the buccal epithelium, is worldwide a common site for cancer. Cancer development frequently results in inactivation of tumor suppressor p53, a central regulator of growth and programmed cell death, and deregulated expression of structural elements like cytokeratins. The overall aim of this study was to investigate if the multi-step process of carcinogenesis can be modeled and studied from a mechanistic stand point utilizing cultured normal (NOK), immortalized (SVpgC2a) and malignant (SqCC/Y1) human buccal keratinocytes.

Organotypic epithelia of the respective cell lines, as derived from serum-free culture on a collagen gel containing oral fibroblasts, showed morphological features ranging from normal tissue to carcinoma *in situ*. The respective epithelia showed sharp differences in immunochemical expression of keratins. NOK expressed many of the same keratins as buccal mucosa, whereas loss of keratins in SVpgC2a and their retention in SqCC/Y1 showed similarities to oral dysplasia and well-differentiated squamous cell carcinoma. Assessment of tissue homeostatic functions demonstrated that NOK exhibited a terminal squamous differentiation (TSD) and apoptosis-capable phenotype, that responded to fibroblast-mediated proliferation with increased apoptosis and to elevation of Ca^{2+} by induction of TSD. In contrast, SVpgC2a and SqCC/Y1 exhibited hyper-proliferative, TSD-deficient and hyper-apoptotic phenotypes that failed to respond to the above stimuli. Immunochemical expression of tumor suppressor p53 was scattered in NOK, heterogeneous in SVpgC2a and negative in SqCC/Y1. Exclusively for NOK, p53 expression increased with proliferation and decreased with TSD, moreover, expression of Bax, a gene associated to apoptosis in many cell types, correlated with TSD. Further evaluation of NOK and SVpgC2a in various conditions for up to 17 days consistently showed several-fold higher proliferation and apoptosis rates in SVpgC2a. Micro-array analysis of NOK and SVpgC2a in monolayer culture confirmed the respective keratin protein profiles to the mRNA level, and indicated expression of keratins not previously reported for buccal epithelium. Under sparse or confluent culture, SVpgC2a exhibited relatively higher cloning ability and growth rate as well as lower responsiveness to contact inhibition than NOK. Apoptosis and TSD were regulated in NOK in response to increasing cell density whereas SVpgC2a showed resistance. Cultures of NOK showed obligatory dependence for the growth supplement pituitary extract, whereas SVpgC2a showed independence, and thus, SVpgC2a could be cultured at chemically defined conditions. Immunochemical assessments in NOK showed increased Bax expression under conditions that increase TSD and decrease apoptosis, providing further evidence for the dissociation of Bax expression from apoptosis in keratinocytes.

A composite *in vitro* model for malignant transformation of oral epithelium is described. Characterization of NOK, SVpgC2a and SqCC/Y1 demonstrated that the multi-step process of malignant transformation of buccal keratinocytes clearly associates with alterations in basic cellular functions and mechanisms that regulate tissue homeostasis and build-up of the cytoskeleton. Overall, standardized, highly defined culture conditions, different cell densities and co-culture models provide useful means of investigating mechanisms underlying oral cancer development.

LIST OF MANUSCRIPTS

I Annette Hansson, Balvinder K Bloor, Ylva Haig, Peter R Morgan, Jan Ekstrand and Roland C Grafström
Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture.
Oral Oncology. 2001, 37: 419-430

II Annette Hansson, Balvinder K Bloor, Zsolt Sarang, Ylva Haig, Peter R Morgan, Hans-Jürgen Stark, Norbert E Fusenig, Jan Ekstrand and Roland C Grafström
Analysis of proliferation, apoptosis and keratin expression in cultured normal and immortalized human buccal keratinocytes.
Eur J Oral Sci. 2003, 111: 34-41

III Annette Hansson, Xi Zheng, Ylva Haig, Balvinder K Bloor, Peter R Morgan, Hans-Jürgen Stark, Norbert E Fusenig, Åsa Elfwing and Roland C Grafström
Growth, programmed cell death and gene expression related to p53 function in epithelia regenerated with cultured normal, immortalized and malignant human buccal keratinocytes.
Manuscript

IV Ylva Haig, Annette Hansson, Xi Zheng, Balvinder K Bloor, Peter R Morgan, Åsa Elfwing and Roland C Grafström
Growth, programmed cell death and gene expression related to p53 function in normal and SV40T antigen-immortalized human buccal keratinocytes.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine
CFE	Colony forming efficiency
CGR	Clonal growth rate
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EMHA	Epithelial medium with high levels of amino acids
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
IFs	Intermediate filaments
kD	Kilo Dalton
NOK	Normal oral keratinocytes
PBS	Phosphate buffered saline
PEX	Pituitary extract
PCD	Programmed cell death
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SV40T	Simian Virus 40 large T antigen
TSD	Terminal squamous differentiation
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling

INTRODUCTION

Carcinogenesis

Oral squamous cell carcinoma (SCC) in humans is a major cause of morbidity and mortality worldwide [Moore, 2000]. SCC of the head and neck is a major health problem and in some part it remains one of the most common forms of cancer including areas in India and South East Asia [Moore, 2000]. The majority of lesions in the mouth, arise from oral epithelium witch include cancer from the gingiva, alveolar ridge, floor of the mouth, buccal mucosa, hard and soft palate, uvula and others regions of the oral cavity [Moore, 2000]. In South East Asian countries the buccal mucosa represents the primary site for cancer development, as compared to the tongue and the floor of the mouth in Western countries. Epidemiological studies indicate a multi-factorial etiology for malignant transformation in oral epithelium [La Vecchia, 1997]. Etiological factors most probably involved in oral carcinogenesis are tobacco smoke, smokeless tobacco, betel quid and areca nut chewing, alcohol, diet and nutrition intake, and genetic susceptibility [Das, 2002]. Tobacco and alcohol seem to be the most important risk factors often coupled with a low consumption of fruit and vegetables [La Vecchia, 1997]. Accordingly, the most cited risk factors for patients with tumors of the oral cavity are, tobacco and alcohol [Moore, 2000]. Infections with human papilloma virus may also play a part in developing oral SCC [Sugerman, 1997] [Sand, 2002]. Recent studies have demonstrated that incidence of oral cancers increase globally, particularly among young men, with the same types of exposure to alcohol and tobacco, and low intake of fruit and vegetables, as older patients. Inside Western and Eastern Europe the incidence of oral cancer has increased about 3-fold and 8-fold within one generation consistently involving persons at younger age [Mackenzie, 2000]. The survival rate is among the lowest of major cancers and has not improved significantly in the past two decades.

Cancer is a genetic disease that require numerous mutations or accumulation of genetic changes to occur including activation of oncogenes and inactivation of tumor suppressor genes [Moore, 2000]. Tumor formation and development in the oral mucosa is primarily of squamous type, and clearly a multi-step process involving the transition from a normal to a malignant cellular state. There is no sharp border between neoplasia and preneoplasia. These steps in the mutation theory of carcinogenesis reflect genetic alterations that drive the progressive transformation of normal human cells into malignant copies. Mutations in proto-oncogenes and tumor suppressor genes can occur early in some tumor progression pathways and late in others. As a consequence apoptosis, sustained angiogenesis, and unlimited replicative potential can appear at different times during these various sequences [Hanahan, 2000]. Programmed cell death (PCD) is expected to eliminate unwanted tumor cells and apoptosis represents a major source of this wearing away. The ability of tumor cells to expand in number is determined not only by the rate of cell attrition but also the rate of cell proliferation. An additional suggestion for carcinogenesis is were proliferation is the default state of all

cells and neoplasia is a defect of structural design. Carcinogens would act initially by disrupting the normal interactions that take place among cells in the parenchyma and stroma of an organ [Hanahan, 2000] [Sonnenschein, 2000]. The role of the differences noted in morphology, biochemistry and molecular mechanisms in malignancy of oral epithelium are only partly understood. The specific order and number of necessary actions remains unknown, but certain steps are believed to be important for cancer to develop, such as gain of proliferative signaling, inhibition of growth inhibitory signals, avoidance of PCD, immortalization, angiogenesis, and the ability to invade tissue [Das, 2002].

Oral mucosa

The oral mucosa is a target for exogenous agents and toxicological actions, e.g. tobacco, chemicals, nutrients and dental materials. The highly specialized, stratified epithelia is an efficient barrier that protects the organism/body from physical and chemical damage, such as infections, pathogens, tissue dehydaration, and heat loss. The oral mucosa is the lining that form the primary structural barrier between the internal and external environments. Like epidermis the keratinized layer reduces the permeability of the oral mucosa, although palatal mucosa is approximately 10 times more permeable than the skin [Winning, 2000].

Structure of oral mucosa

The oral mucosa consists of an epithelium supported by connective tissue termed the lamina propria. Tremendous regional variations exist, not only in the composition of the lamina propria, but also in the form of the epithelial-connective tissue junction and type of covering epithelium. It presents variations in the thickness and in the prevalence and type of keratinisation. Keratinizing epithelia such as the oral gingiva, that have to withstand more severe physical and chemical forces, than the buccal mucosa, produce a toughened structure, the cornified cell envelope. This envelope is a major component of the epithelial barrier at the tissue surface [Presland, 2001]. Functional demands and tissue features are reflected by the structure and biology of tissues and cell products that make up the oral mucosa. These functionally different types of oral mucosa can be divided into three groups. The lining mucosa, a very thin, non-keratinised or parakeratinised epithelium are located in the soft palate, ventral surface of the tongue, floor of the mouth, and in the buccal and labial mucosa. The thick and orthokeratinized masticatory mucosa includes gingiva and hard palate. The specialized mucosa is represented by the dorsal surface of the tongue, consisting of non-keratinised interpapillary epithelium interspersed with keratinised filiform papillae [Squier, 2001]. In parakeratinized epithelium, nuclei are retained in the superficial layer, in contrast to the orthokeratinized where no nuclei are seen.

The stratified and squamous mucosa consists of several cell layers that undergo constant renewal and repair, to maintain this critical defense barrier [Potten, 2001]. Stratum basale is a single layer of undifferentiated keratinocytes that is anchored to the basal lamina. The basal cells are proliferative, and divide either to a new stem cell, which maintain the population of dividing cells, or to a transit cell, which differentiate,

mature and migrate through the layers to finally fade away into the oral cavity. Basal cells adhere to the underlying basal lamina via integrins, and decreased adhesion is a key event in epidermal differentiation [Watt, 1998]. Stratum spinosus contains cells with desmosomes and stratum granulosum is characterized by the presence of intracellular granules. Dead flattened cells occupy stratum corneum, containing squames of cross-linked protein. Stratum lucidum is a transitional layer between the living layers and dead tissue in the cornified layer. It normally takes about 1-3 weeks to renew buccal epithelia, compare with 4-10 weeks in the epidermis [Winning, 2000].

Specialized cell junctions, desmosomes and hemi-desmosomes, maintain cohesion between the cells and control the permeability of the epithelium [Mackenzie, 1983] [Squier, 2001]. These cell adhesion structures are important in bonding keratinocytes to one another and to the basal lamina/basement membrane, and connect the keratin cytoskeleton to the cell surface. The basal lamina separates epithelial elements from the surrounding stroma. Laminin-receptors on the cell surface acts like a bridge, which ensure the stability of connection and communication between the basal lamina and the epithelial cells. The extra cellular matrix glykoprotein laminin 5 is essential to epidermal cell attachment. Like the epithelial cells, tumor cells have the same surface receptors, subsequently laminin plays an important role for adhesion of tumor cells. Underneath the epithelium, the superficial dense connective tissue, lamina propria, overlies the more inferior submucosa. The most common mesenchymal cell type fibroblasts produce matrix rich in macromolecules, e.g. collagen. Collagen has many forms divided in types from I-X. The most common type I is found in skin, tendon and bone. Type II exist in cartilage and type IV is present in the basal lamina. Keratinocytes, the epithelial cells, express a set of structural proteins, which assemble into filaments and function to maintain cell and tissue integrity.

Cytoskeleton

The cytoskeleton of all mammalian cells is composed of a filament system for organization and movements. The cytoskeleton of eukaryotic cells consists of three main groups of filamentous proteins [O'Guin, 1987]. These are the microfilament, 5-6 nm in diameter (composed of actin), which provide the contractile forces within cells. The 25 nm microtubules (composed of tubulin), plays a role in orientation and polarization. The 8-10 nm intermediate filaments (IFs) provide a functional cytoskeleton and reflect the epithelial phenotype and its commitment to terminal squamous differentiation (TSD) [Smack, 1994].

Intermediate Filaments (IFs) are divided into six different sequence types based on three different domains structures. Among other, the IFs include acidic-keratins, neutral-basic keratins, vimentin, nestin and neurofilaments. Vimentin, desmin, glialfilament, and peripherin are all type III IFs [Smack, 1994]. Of the type III IFs, vimentin is the most widely expressed, being involved in a diverse range of functions from RNA processing and interactions with nucleic acids to protein degradation. Type III IFs desmin is restricted to striated and smooth muscle, glialfilament to glial cells and astrocytes, and peripherin to neurones. Type IV IFs include the neurofilaments, which are widely distributed in neurons, and α-internexin found in prenatal neurons. Type V IFs constitute the nuclear lamins and minor lamins whose tissue expression is restricted

to the nuclear lamina. Nestin is the only member of the type VI IFs group and is expressed in embryonal and neuroepithelial stem cells [Smack, 1994]. Normal oral mucosa show abundant expression of fibroblast produced vimentin in dermis and no expression in the epithelium. Although, co expression of IFs and vimentin has been found in lesions having accumulations of inflammatory cells in the sub epithelial cell layer and in epithelial cell under various pathological conditions, including premalignancy. In lesions with lymphocyte infiltration of sub epithelial compartment vimentin has been expressed in basal epithelial cell layers, and are therefore not an exclusive mesenchymal cell [van der Velden, 1999]. Expressed in many mesenchymal cell types during development, there is also evidence that vimentin associates with stress response proteins [Evans, 1998]. Most IFs in human epithelia are members of the cytokeratin group, a complex family encoded by at least 20 genes in humans, in contrast to hard keratins in hair and nail and a further 10 gene products characteristic of trichocytes [Moll, 1982].

Classes of keratins

Keratins are expressed in a tissue-specific manner in different combinations of pairs that distinguish stages or types of differentiation (Table I). Keratins are divided into two major types, initially identified by their molecular weight ranging from 40 to 68 kDa and isoelectric points from 5.2 to 7.8 [Moll, 1982]. Each keratin exists in several isoelectric variants of different isoforms of both type I and type II keratins [Smack, 1994]. Type I keratins, which comprise keratins K9 to K20 (and the trichokeratins, Ha1 to Ha4 and Hax) and range from 40 to 64 kD and type II keratins include K1 to K8 (and the trichokeratins, Hb1 to Hb4 and Hbx) and vary from 52 to 68 kD [Moll, 1982]. Type II keratins are large with neutral or basic isoelectric points while type I keratins are small and more acidic [Su, 1994].

In all epithelial cells keratins are usually expressed as a pair showing considerable specificity among epithelial tissues. Keratins are also differentially expressed along the different pathways of epithelial cell development and differentiation. In stratified squamous epithelium, as keratinocytes undergo differentiation from the basal layer to the suprabasal layers, cells express a range of keratins and other cytoskeletal proteins at different stages of the maturation. It is this variation that gives rise in heterogeneity as well as tissue specificity. The underlying reasons for a high degree of tissue specificity in keratin expression remain unclear. Their diversity may be due to different structural and functional requirements, implicating roles for the specific filament formation with their respective keratin partners. As keratins are markers of epithelial TSD, any alteration in their expression may be of biological significance. Keratin expression is often altered in diseases and expression of structural elements like the cytokeratin family of proteins is commonly deregulated in oral cancers. It possibly will be of use to examine altered expression of normal keratins for involvement in these processes. The importance of keratins in the maintenance of tissue structure is emphasized by the fact that many hereditary epidermal disorders are caused by mutations in keratin genes [Moll, 1982] [Morgan, 1994] [Smack, 1994] [Su, 1994] [Blumenberg, 1997].

Differentiation-specific keratins

Differentiation-specific keratins, K4/K13 and K1/K10 are early markers of differentiation. K4 and K13 are keratins expressed primarily in suprabasal cells of non-keratinizing stratified squamous epithelium such as esophageal, exocervical, anal canal, buccal and lingual epithelium. K4 and K13 were originally thought to represent markers of non-keratinisation, but subsequently K4 and K13 were detected as major constituents of fetal epidermis, although in adult epidermis they are present in small clusters above the basal cell layer [Moll, 1982]. Moreover K4 and K13 have been identified in small groups of cells in attached gingiva and the hard palate [Morgan, 1994]. Under tissue culture conditions, both K4 and K13 can be invariably synthesized by a number of epithelial cell lines [Bloor, 1998].

The largest cytokeratin members, K1 and K10, are keratins found mainly in keratinising stratified squamous epithelia. K1 and K10 are regarded as markers for keratinisation and these keratins are predominantly expressed in differentiated cells in the basal layer of epidermis, although in less than 1% of the cells. In addition to epidermis, K1 and K10 are expressed in other keratinising epithelia, including gingiva and filiform papillae of dorsal tongue. At a restricted level, K1 and K10 have also been identified in zones of the suprabasal layer, in non-keratinizing buccal epithelium [Morgan, 1994] [Bloor, 1998] [Bloor, 2001].

Expression of K2e, K11 and K9 is found in regions of epidermis [Moll, 1982] [Langbein, 2001]. K2 was originally thought to be a break-down product of K1, but two different human genes encoding K2 have now been identified. These genes are of almost identical charge and size but are differentially expressed. One gene encodes for the epidermal form (K2e), and in addition to K11, is found in regions of epidermis, which are particularly exposed to friction, such as palmoplantar epidermis. K2e can also be detected in the exocervix and the anal canal [Moll, 1982]. A second gene encoding K2 (K2p) is expressed in the palate and gingiva but not in epidermis [Langbein, 2001]. Although K2 and K11 are characteristic of type II and type I keratins, they are not expressed as a keratin pair. K9 is the largest type I keratin and expression is mainly restricted to sweat glands, albeit in small clusters of cells [Moll, 1982] [Langbein, 2001].

Corneal-type keratins

K3 and K12 are regarded as markers for corneal-type differentiation as they are predominantly expressed in the cornea [Moll, 1982] [Blumenberg, 1997] [Kivela, 1998]. The structural integrity of the cornea is believed to be maintained of K12 [Blumenberg, 1997]. K3 and K12 are co-expressed with minor amounts of K4 and K13 in suprabasal cells of corneal epithelium, along with K5, K14 and K19 in basal cells. Although expressed in small amounts, these keratins have been identified in other stratified epithelia, such as the lip. K3 has also been detected in the suprabasal layers of gingiva and sulcular epithelium [Juhl, 1989].

Basal specific keratins

Keratins 5 and 14 are defined as primary keratins or basal specific keratins, because they are usually the first to be expressed in basal cells of stratified squamous epithelia, including epidermis and oral epithelium [Blumenberg-97] [Freedberg, 2001]. From

analysis of localization and function, the keratins are typically basal cell-specific markers although their expression has additionally been detected in the suprabasal compartment of normal epidermis [Blumenberg-97] [Freedberg, 2001].
K15 and K17 are small type I keratins that are expressed in pairs with different type II keratins. Both are closely related to K14 as defined by peptide mapping and gene sequencing [Moll, 1982]. K15 is among one of the earliest markers of developing fetal epidermis indicating the beginning of stratification [Moll, 1982]. In adult epidermis however, this keratin represents a minor component in basal cells and in the basal layer of esophageal epithelium, K15 is expressed in a major amount. K17, occasionally classified as a high cell turnover keratin, is expressed in hair follicles, basal cells of pseudostratified epithelia such as that of the trachea and bronchus, in transitional epithelia of the urinary bladder, in basal cells of sweat glands and in myoepithelial cells of sebaceous glands [Moll, 1982] [Freedberg, 2001]. Under tissue culture conditions, K17 synthesis can be induced in epithelial cells [Freedberg, 2001].
Predominantly expressed in simple epithelia, K19 can be identified in basal cells of non-keratinising epithelia. Due to epithelial differentiation and maturation, as basal cells differentiate, one or more pairs of other keratins are superimposed on the basal specific keratins.

High cell turnover keratins
High cell turnover or hyper proliferation keratins, K6/K16 and K17, are markers of activation, because they are expressed during wound healing in suprabasal cells of interfollicular epidermis [Blumenberg-97] [Freedberg, 2001]. K6 is usually the first to appear in hyper proliferation, but pairing of K6 and K16 is complicated by the existence of different isoforms, K6a, K6b, K6c and K6d [Blumenberg, 1997] [Freedberg, 2001]. K6/K16 are expressed constitutively in suprabasal cells of palmar and plantar epidermis but also in several sites of the oral cavity including gingiva and tongue. Additionally, high cell turnover keratins have been detected above the level of rete processes in the non-keratinizing buccal mucosa, as well as in the cornea [Su, 1994]. As with K4 and K13, high cell turnover keratins can be synthesized by cultured epidermal keratinocytes [Bloor, 1998].

Simple epithelial keratins
Simple epithelial keratins K7, K8, K18, K19 and K20 are known as early markers of differentiation during embryonic development, and can also be detected in different stratified adult squamous epithelia, albeit at a low level [Morgan, 1994] [Su, 1994]. K8 and K18 are identified in simple epithelia, such as the intestinal mucosa, but subsequently these keratins have been detected in basal keratinocytes of esophagus and parabasal cells of the palate, gingiva and lip. K8 and K18 are usually the first to appear in early embryonic epithelia, dominating the cytoskeletal character of early differentiation [Morgan, 1994] [Su, 1994]. K7 is a small type II keratin expressed predominantly in simple epithelia and shapes a keratin pair with K19 in luminal cells. However in glandular epithelial cells, K7 is co-expressed with K20 [Morgan, 1994] [Su, 1994].
K19, the smallest keratin, is expressed in both simple and stratified epithelia. K19 is co-expressed with K7 and K8 in glandular epithelia, such as that of the breast and prostate,

in epithelia lining the gall bladder and urinary bladder, but also in the endometrium. K19 does not have a specific partner but often accompanies K8 and K18 during embryonic development. K19 can also be co-expressed with K15 in basal cells of the esophagus and exocervix [Morgan, 1994]. Detectable in a few keratinocytes of attached gingiva and hard palate, K19 is found to be restricted to sparsely scattered basal cells [Lindberg, 1990]. In buccal epithelium, K19 is identified in basal cells, but also irregularly expressed in suprabasal cells [Morgan, 1994]. Heterogeneously detection of K19 in basal cells of the lateral and ventral surfaces of tongue has been reported. Although regarded as a simple epithelial keratin, K19 has a widespread distribution and might function to balancing the over-production of basic keratins to retain an equimolar state [Blumenberg, 1994]. K20 simple epithelial keratin is detected in gastric epithelium, in the epithelium lining the urinary bladder, and in Merkel cells [Moll, 1982].

A reduction in the differentiation-specific keratins localized to suprabasal cells at the center of tumor islands has been associated to development of oral SCCs. Another feature of altered keratin expression in SCCs is the anomalous expression of simple keratins. Detection of these keratins in SCCs shows an inversely intensity and frequency, varying with the degree of differentiation [Morgan, 1994] [Su, 1994]. These studies on oral SCCs suggest that keratin gene expression in oral epithelia undergo aberrant regulation during development and progression of malignancy. Many studies analyzed the alterations that take place in keratin expression during malignant transformation in tissue samples whereas few studies have relied on cell cultures. Keratins have been conventionally identified at the protein level, using biochemical, immunoblotting and immunochemical procedures. Keratin genes, in company with other housekeeping genes are regulated both at the mRNA and protein levels. Few studies have attempted to correlate the distribution of mRNA with that of protein. The loss of normal regulatory control at any of these levels may have a profound effect on cell function.

Table I

Classes and specific pairing of type I and type II keratin filaments, and their distribution in different epithelial tissues.

Type I keratins acidic		Distribution in epithelia	Type II keratins neutral to basic	
Size (kD)	Number		Number	Size (kD)
64	K9	suprabasal cells of palmoplantar epithelia		
56.5	K10	suprabasal cells of keratinising stratified epithelia	K1	68
56	K11	suprabasal cells of keratinising stratified epithelia	K2e/K2p	66
55	K12	suprabasal cells of corneal epithelium	K3	64
51	K13	suprabasal cells of non-keratinising stratified epithelia	K4	59
50	K14	basal cells of keratinising epithelia	K5	58
50	K15	basal cells of keratinising and non-keratinising epithelia		
46	K17	basal cells of keratinising and non-keratinising epithelia		
40	K19	basal cells of non-keratinising epithelia		
48	K16	suprabasal cells of high cell turnover stratified epithelia	K6	56
40	K19	simple epithelial cells	K7	54
45	K18	simple epithelial cells	K8	52
46	K20	simple epithelial cells		

Table modified from [Morgan, 1994] [Smack, 1994] [Watt, 1998].

Growth and death regulation of human keratinocytes

Homeostasis in continually renewing epithelia, like oral mucosa, is maintained by cellular proliferation, death and differentiation [Sugerman, 1995] [Compagni, 2000] [Su, 2000]. The mitoses in the basal compartment formed by the innermost cell layers will be balanced by PCD, an active normal removal of cells. The term PCD is derived from developmental biology, genetically programmed to occur during development and differentiation. These programmed, domed cells die on schedule even if they are transplanted elsewhere in the embryo [Teraki, 1999].

Apoptosis

One form of PCD, apoptosis, was first described as a necrotic process of cell death, morphologically separated from necrosis. Involving histological changes, including the obvious cell shrinkage it was originally called "shrinkage necrosis" for this reason. The suitably name apoptosis comes from a Greek world describing the falling off of petals from a flower or leaves from a tree [Teraki, 1999].

Morphological and biochemical description of apoptosis comprise nuclear condensation, DNA-fragmentation, dismantling of cell ultra structure, blebbing of plasma membrane and formation of membrane-bound fragments, termed apoptotic bodies. Apoptosis in normal tissue does not generally affect tissue function, as remnants of the dead cells are fagocytosed and internally degraded by neighboring cells. Removal occurs before lysis, which prevents the release of potentially toxic and immunogenic intracellular contents from the apoptotic cells into the surrounding tissue [Teraki, 1999]. Degradation and engulfing generally take place within lysosomes via the activation of endogenous Ca^{2+}/Mg^{2+}-dependent endonucleases and a family of intracellular proteases called caspases [Polakowska, 1994] [Reed, 1999] [Teraki, 1999]. Apoptosis plays an essential role in tissue homeostasis to ensure that new cell production is balanced by cell loss [Metcalfe, 1997]. It plays an important role during morphogenesis of the embryo where organs re-shape themselves by carefully eliminating unwanted cells, but also an important defense mechanism following exposure to foreign antigen. Apoptosis removes unnecessary, damaged or harmful cells via a genetically controlled mechanism in both normal and diseased processes. An apoptosis frequency of a few % of the total cell number can result in the complete deterioration and removal of a tissue, or cell culture model, within a time frame of days to weeks [Colombel, 1995]. Failure to undergo apoptotic cell death might be involved in the pathogenesis of a wide variety of human diseases, including cancer, autoimmune diseases, and viral infections [Teraki, 1999]. Inefficient or suppression of apoptosis can result in accumulation of cells which are normally removed and inhibition of cell death can lead to cancer and persistent viral infection. Excessive cell death can lead to impaired development and degenerative diseases. Oral lichen planus and erythema multiforme are diseases with a known involvement of enhanced apoptosis [Dekker, 1997] [Chrysomali, 1997]. Most somatic cells have limited replicative lifespan and those with excessive DNA damage are removed before cell division to avoid malignant transformation. Malignancy provides an evidence of apoptosis, when cells accumulate in an uncontrolled manner, as malignant cells live longer and divide at a faster rate than normal.

The ability to undergo apoptosis is fundamental and appears to be common to all mammalian cell types. To eliminate senescent and, especially DNA-damaged cells from the body, apoptosis requires the activation of a genetic program [Kamer, 1999]. The activation of the apoptotic pathway is regulated by many different intrinsic and extrinsic signals, where some are activators while others are inhibitors. Several specific genes have been identified and the regulation is conserved in groups as divergent as nematodes, viruses and mammals [Vaux, 1995]. As the genetic control and biochemical mechanisms of apoptosis become better understood, development of novel approaches in disease processes can be predictable.

Terminal squamous differentiation

An alternative form of active PCD is TSD or developmental cell death. As epithelial cells undergo TSD, they move from the basal cell layer through the superior layers to the surface of the epithelium, where the cells become enlarged and flattened. Once differentiation is complete, desquamation enables cells to be shed from the superficial layers of the epithelium. Involucrin a non-keratin, and other proteins are substrates for cross-linking by transglutaminase activity resulting in the formation of a heat- and detergent-resistant envelope just inside of the plasma membrane. During terminal stages in keratinocyte differentiation, involucrin becomes cross-linked to other proteins to form part of the cornified envelope. Involucrin is synthesized early during TSD and its expression is therefore considered to reflect commitment to this process [Blumenberg, 1997] [Presland, 2000]. TSD is the normal choice during maturation in different epithelial strata, whereas apoptosis is likely to provide a protective mechanism that eliminates harmful cells at any point, during epithelial differentiation. TSD takes place only in the suprabasal layers, whereas apoptosis occurs throughout the epithelium.

Necrosis

Necrosis represents cell death, which is typically unplanned to structures and neighboring cells. Passive and pathological form of cell death are resulting from acute cellular injury induced by physical or chemical distress and may result from sudden deprivation of blood supply to any part of the body. Necrosis occurs from any kind of cell trauma and involves group of cells that die by a pathological response involving Ca^{2+} influx which induce swelling and lysis of the cytoplasm and organelles. A necrotic cell loses its membrane integrity, which is followed by an acute inflammatory response in surrounding tissue. It release cellular debris to adjacent tissue and changes the environmental [Wyllie, 1980] [Majno, 1995] [Teraki, 1999].

Unless the cells are severely injured and die by necrosis, TSD and apoptosis lead to the elimination of cells primarily by desquamation and phagocytosis, respectively. The processes of proliferation and loss of cells by TSD or apoptosis are normally in a dynamic steady state, whereas imbalances can lead to development of pathologic states, including cancer [Colombel, 1995] [Sugerman, 1995] [Polverini, 1999] [Compagni, 2000] [Su, 2000]. Most apoptosis work in vitro has so far involved non-epithelial models that potentially regulate PCD in manners different to epithelial cells. Nearly all keratinocyte studies have primarily involved tumor cells with acquired deficiencies in

PCD without consideration of the results in the normal state [Weinberg, 1995]. In this respect, limited information exists concerning the choice between induction of TSD and apoptosis in human epithelial tissues, including both normal and tumor tissue. Modeling of physiological keratinocyte PCD, therefore requires extensive kinetic analysis as well as the utilization of methods which are applicable to single or few cells. Under the current lack of conclusive knowledge of mechanisms of keratinocyte PCD, genes potentially involved in keratinocyte apoptosis should also be considered for induction of TSD. Assessments of apoptosis in non-keratinocyte cell types, or cancerous cells alone, may provide a simplified, and potentially faulty view of keratinocyte PCD.

Gene families involved in regulation of apoptosis

Stimulation versus inhibition of oncogenes and tumor suppressor genes are involved in apoptosis regulation. Oncogenes stimulate appropriate cell growth under normal conditions, as required for the continued cell turnover and replenishment of the epithelia. Proto-oncogenes can be altered by point mutation, amplification or rearrangement. Structural alteration of proto-oncogenes leads to a quantitative or qualitative expression change of the corresponding protein product. Oncoproteins undermine signal transduction pathways at the cell surface, in the cytosol and the nucleus. Cells with a mutation in an oncogene continue to grow, or refuse to die, even when they are receiving no growth signals. Tumor suppressor genes keep cell numbers down, either by inhibiting progress through the cell cycle and thereby preventing cell birth, or by promoting PCD. When several of these tumor suppressor genes are rendered non-functional through mutation, the cell becomes malignant [Weinberg, 1994]. Further, in the absence of tumor suppressor gene products, oncogenes contribute to human cancer formation by supporting accelerated proliferation, de-regulating cell cycle control or blocking apoptosis [Reed, 1999]. Blockage of normal cell death from inactivation of genes that induce apoptosis or activation of genes that block apoptosis, provides a parallel to how alterations in proto-oncogenes and tumor suppressor genes dysregulate growth [Weinberg, 1994] [Sugerman, 1995] [Polverini, 1999] [Reed, 1999].

The involvement and interplay of these factors in apoptosis is far from understood. Regulation of apoptosis in normal and cancerous tissue involves various gene families. A mixture of family members may interact in this process by supporting or inhibiting the implementation of the death programs. p53 and p53 regulated genes, has a central roll in cell cycle control, cell death and defense reactions after DNA damage or other types of stress [Choisy-Rossi, 1998].

Information derived primarily from studies of cell types other than epithelial cells, indicate that bcl-2 gene family blocks, whereas wild-type p53 can stimulate apoptosis. Comparisons of normal, precancerous and cancerous epithelial tissue have indicated altered apoptosis frequencies and dysregulated expression of apoptosis-related genes in parallel analysis. In oral lesions including SCC, apoptosis seems to decrease as the histological abnormality increases [Birchall, 1996]. Studies as to the possible role of p53 and other apoptosis-related genes in oral mucosal tissue homeostasis are therefore desirable.

p53

The tumor suppressor p53 controls numerous proceedings that can result in variable outcomes, involving maintained genomic integrity by controlling cell cycle progression and cell survival. p53 are the most frequently chromosomal mutation in human cancer and one of the most important and studied genes [Levine, 1991]. Since p53 is a key element for maintaining genetic stability, a mutation in p53 gene itself predispose for cancer formation. Wild-type p53 protein can be activated following diverse cellular stress conditions, e.g. DNA-damage agents, viral infections, irradiation, hypoxia, oncogene activation, oxidative stress, hyperthermia, cytotoxic drugs, and in response to chemotherapeutic agents [Pluquet, 2001]. p53-knockout mice do not develop apoptosis in the epidermis after UV irradiation, because the ability of p53 to persuade apoptosis as a potent tumor suppressor [Teraki, 1999]. Through independent pathways, p53 protein can induce cell growth as well as cell death, but the mechanism is still not well characterized [Nylander, 2000]. Activated p53 protein mobilizes regulate and induce expression of other genes responsible for cell cycle arrest, trigger of apoptosis and DNA repair [Ravi, 1999] [Pluquet, 2001]. A negative regulator of p53 is the MDM2 protein. Over expression of MDM2 has a tumor-promoting effect similar to that of mutated p53. Normally MDM2 protein targets p53 for proteasome-mediated degradation, and thus stabilizes p53 [Asker, 1999]. When cell suffer DNA damage, p53 protein accumulates and blocks entry into S phase, for replication. This delay permits repair of damaged DNA in G1 phase, but if that fails p53 trigger apoptosis [Asker, 1999] [Kaelin, 1999]. Weather a cell would undergo growth arrest or apoptosis following p53 activation appears to depend on a variety of factors, such as environmental conditions and the cell type [Nylander, 2000]. In the normal cell there is always a small amount of p53, since it breaks down consistently in the proteasome. When DNA damage occurs, p53 break down stops, and p53 concentration increases and thus apoptosis accelerate. Wild-type p53 normally has a short half-life (6-20 min) and protein retained in the tissue can be detected. This retained wild-type protein is presumably inactive. In mutant forms or by binding to virus protein such as SV40 large T, p53 can remain in tissue for an extended time, with a half-life for up to 6 h [Ravi, 1996] [Nylander, 00].

p21

Over expression of the p21 protein (also named CIP1/WAF1) induces cell cycle arrest. Since p21 plays an important role in arresting cell cycle, loss of function in p21 may favor tumor growth [Harada, 2000]. p21 expression is up regulated by wild-type p53 and may be associated with cellular senescence and apoptosis. The p53 gene stimulates p21expression, which in turn inhibits cyclin- complex formation, phosphorylation and DNA replication as an alternate route to apoptosis [el-Deiry, 1998]. p21 expression can be increased by serum starvation, contact inhibition and senescence without being accompanied by an increase in p53 expression.

Bax

p53 increases expression of the pro-apoptotic protein Bax, whose gene has p53 response element [Gallaher, 2001]. Although Bax is homologous with Bcl-2, mainly within two highly conserved regions called the Bcl-2 homology (BH) 1 and 2 domains,

these proteins do not work together, but instead, are antagonists [Polakowska, 1994] [Evan, 1998] [Vogelstein, 2000] [Adams, 2001]. Activation of Bax promotes apoptosis, through dimerization with Bcl-2 proteins [Chao, 1998] [Tomkova, 1999]. Despite the capacity of these molecules to form heterodimers, they could each be capable of independent regulation of PCD. Pro-apoptotic Bax, at high and low calcium, have been linked to TSD. High calcium can possibly induce keratinocyte differentiation, and Bax show a stronger expression in cells with high calcium [Maruoka, 1997] [Tomkowa, 1999]. Although, from work in fibroblast, cells of hematopoetic origin, carcinoma cells, etc, this gene would clearly be defined as an apoptosis-inducing gene [Evan G, 1998] [Asker, 1999].

Bcl-2

The p53 gene induces the production of other apoptosis promoting proteins, such as Bax, but also represses the expression of apoptosis inhibitors, such as the Bcl-2 protein. Inactivation or mutation of the p53 gene may therefore result in over expression of Bcl-2 and down regulation of Bax leading to defective apoptosis [Polakowska, 1994] [Evan, 1998] [Ravi, 1999] [Vogelstein, 2000]. Proto-oncogene bcl-2, has ability to inhibit apoptosis, by prolonging the survival of cells and prevents cells from entering the apoptotic pathway, so loss of Bcl-2 expression may lead to onset of apoptotic cell death [Polakowska, 1994]. Thus, Bcl-2 prevents PCD instead of promoting proliferation [Evan, 1998] [Polverini, 1999]. Genes that inhibit Bcl-2 can induce apoptosis in tumor cells. Many of these proteins are co-expressed in the same cells and the ratio of anti-apoptotic versus pro-apoptotic protein levels, may determine the cell to respond to apoptotic signal [Teraki, 1999]. In normal human stratified squamous epithelium, the Bcl-2 proto-oncogene, expression is restricted to the basal cell layer, and not in suprabasal layers. Compared to normal oral epithelia the malignant keratinocytes in basal cell carcinoma express high levels of Bcl-2, but in oral SCC decreased Bcl-2 expression, a lower Bcl-2/Bax ratio and increased apoptosis have been found [Loro, 1999]. Bcl-2 expression, is generally associated to proliferative and regenerative keratinocytes in the basal layer of normal mucosa, were proliferation takes place [Gandarillas, 2000]. Consistent with this, Bcl-2 might be a stem cell marker, thus cells whose major function is to give rise to new cell populations are Bcl-2 positive [Polakowska-94] [Harada, 1998]. Expression of Bcl-2 in the major proliferative compartment may function in protection of basal cells from cell death.

Inactivation of p53 in oral SCC, increased p53 expression in apoptosis-prone oral lichen planus and erythema multiforme lesions, as well as differential Bcl-2 and Bax expression in oral SCC versus normal tissue may currently represent the strongest indications for altered apoptosis gene regulation in oral disease [Jordan, 1996] [Ravi, 1996] [Raybaud-Diogene, 1996] [Ito, 1999] [Loro, 1999] [Xie, 1999] [Yao, 1999] [Loro, 2000] [Ralhan, 2000].

Cell culture technique to study oral functions

Cell cultures are used as a tool for studies of cell physiology and transformation, as compared to animal-based models [Mori, 1999]. The morphology and biochemistry of cells in laboratory animals differ from the human equivalent and commonly both quantitative and qualitative differences are found in the metabolism of carcinogens between human and animal cells. Furthermore, rodent cells undergo immortalization and malignant transformation more frequently than human cells [Kulkarni, 1995]. The phenotypic changes associated to transformation are likely to differ in epithelial and mesenchymal cells and human epithelial cells generally show higher capability for metabolism of carcinogens than fibroblasts from the same tissue. These results emphasize the value of functional keratinocyte models of human origin for *in vitro* studies of metabolism and toxicology in carcinogenesis [Kulkarni, 1995] [Hedberg, 2001]. An experimental approach to study normal biology as well as tumorigenesis of human epithelia, involves culture of normal and transformed cells *in vitro*. A cell culture technique should not only provide a number of cells with desired proliferative ability and longevity to allow experimentation, but also permit retained expression of tissue type-specific markers and functions. Specific characteristics of the oral epithelium can be used to assess the differentiated state of both tissue samples and cultured cells. Therefore an important feature of oral epithelial cell cultures is that they retain the ability to undergo TSD [Prime, 1990].

Variable growth conditions have been applied to growth of normal, immortal and malignant oral cell types. The majority of methods employ serum supplementation in the media as well as the use of feeder-layers. However, recent studies have utilized serum-free media for generation of organotypic epithelia although the serum-free period is limited to the generation of the epithelium [Kang, 2000]. Serum is a potent differentiation-inducing agent causing decreased growth and migration, increased cell area, involucrin expression and cross-linked envelope formation, of otherwise serum-free cultures of oral epithelial cells [Kasperbauer, 1990] [Sundqvist, 1991]. The defined approach with serum-free conditions offers several advantages, and less experimental variability. It provides the possibility to identify factors that directly regulate cell death, proliferation and differentiation, ease of isolation of cellular products, delayed TSD and senescence in the absence of serum, and utilize selective growth conditions for different cell types.

Epithelial growth and differentiation can be studied in an organized, three-dimensional, tissue-like state using organotypic cultures. Organotypic cultures based on epithelial cells grown to confluence on extra cellular matrices and lifted to the air-liquid interface, can differentiate into a multilayered epithelium [Fusenig, 1994] [Tomakidi, 1997] [Stark, 1999]. Information on growth regulation with this model has been obtained primarily from the squamous epithelia of the skin and with skin fibroblasts or fibroblasts provided from mouse [Fusenig, 1994] [Schoop, 1999] [Maas-Szabowski, 2001] [Werner, 2001]. Inclusion of oral fibroblasts and/or fetal bovine serum (FBS) in the gels offers a dermal equivalent, and an opportunity to study epithelial-mesenchymal interactions in the oral cavity [Liu, 1991]. The cell

culture medium EHMA (epithelial medium with high levels of amino acids) was developed for serum-free culture of oral keratinocytes, but it is also applicable to keratinocytes from other squamous epithelia, including the epidermis [Sundqvist, 1991]. EMHA, was reconstituted from MCDB 153 medium [Boyce, 1986], and supplemented with growth factors, hormones and pituitary extracts (PEX). PEX is the only undefined supplement, in the otherwise defined EHMA.

Normal oral keratinocytes

Normal oral keratinocyte (NOK) cultures derived from non-pathologic tissue express many features of normality including limited life spans and capability of undergoing TSD. For processing of primary NOK cell cultures, human buccal tissue was selected from non-smoking, non-cancer patients.

SVpgC2a cell line

Epithelial cell have been spontaneously or experimentally transformed into immortalized lines. Keratinocyte immortalization has been accomplished by transfection of oral epithelial cells with the E6/E7 genes from HPV16 or HPV18 and the T antigen from simian virus SV40. Some of these lines give rise to premalignant cells with irregular proliferation and loss of TSD, which occasionally indicate malignant transformation [Oda, 1996]. The E6 and E7 proteins form complexes with the p53 and Rb products, respectively, leading to inactivation of the latter [Ludlow, 1993]. Because the HPV E6 protein, unlike SV40T which complexes with both the p53 and Rb proteins, also catalyzes degradation of the p53 protein, SV40 T- and HPV E6/E7-transfected lines offer complementary systems of transformation. Such lines are easily grown in high cell numbers, although they retain many properties of normal cells. In the absence of well-characterized cell lines from dysplastic tissue, cell lines, which have been immortalized by DNA tumor virus oncogenes, may currently be the best available *in vitro* models for a preneoplastic state [Grafström, 1990] [Vaccariello, 1999]. In effort to develop *in vitro* models that represent this preneoplastic stages, buccal keratinocytes were transfected with a plasmid coding for SV40T [Kulkarni, 1995]. This attempt resulted in stable integration of the SV40T antigen and apparently the immortalized cell line SVpgC2a. The SVpgC2a cell line express wild-type p53, retains a non-tumorigenic phenotype during extended culture, and fails to produce tumors in athymic nude mice [Kulkarni, 1995]. For DNA sequencing of p53 in the SVpgC2a cell line, exons 4 to 9 of p53 were analyzed by DNA amplification followed by automated sequencing [Berg, 1995]. The SVpgC2a cell line has been characterized in a number of studies [Liu, 1997] [Farmer, 2001] [Hedberg, 2001] [Vondracek, 2001] [Dressler, 2002] [Vondracek, 2002].

SqCC/Y1 cell line

A commonly studied buccal carcinoma line, SqCC/Y1 obtained from a verrucous carcinoma, has been adapted to grow in the serum-free culture conditions developed for NOK [Liu, 1997] [Zou, 1999] [Farmer, 2001] [Hedberg, 2001] [Vondracek, 2001] [Dressler, 2002]. This serum-free strain of the buccal SqCC/Y1 cell line, reproducibly generates tumors following subcutaneous injection in athymic nude mice [Kulkarni, 1995]. The SqCC/Y1 cells cannot generate functional p53 protein, due to the single

p53-allele present are rearranged and carries two missense mutations in exon 5 [Reiss, 1992]. Dominant-negative or loss-of-function mutations of the p53 tumor suppressor gene are frequently found in SCC of the head-and-neck region and an in vitro malignant cell line ought to have that same property.

The composite application of NOK, SVpgC2a and SqCC/Y1 cells, representing normality and various stages of oral cancer, were developed to study aspects of the complex carcinogenesis process.

AIM OF THE STUDY

The overall aim was to investigate if the multi-step process of carcinogenesis can be modeled and studied from a mechanistic stand-point utilizing normal (NOK), SV40 T antigen-immortalized (SVpgC2a) and malignant (SqCC/Y1) human buccal keratinocytes.

Specific sub-aims

To investigate if the serum-free conditions developed for monolayer culture of NOK, SVpgC2a and SqCC/Y1 are applicable to organotypic culture of these cells.

To investigate the longevity of organotypic epithelia from the above cell types under various culture conditions, including the influences of Ca^{2+} and serum, as well as the impact of epithelial-mesenchymal interactions.

To determine if organotypic structures derived from the respective cell types show histology or genetic changes similar to normal tissue or different states of the cancer process.

To profile cytokeratin expression in the respective cell types by assessment of both mRNA and protein.

To study basic aspects of tissue homeostasis in the respective cell types, involving gain of cells by proliferation and loss of cells by induction of TSD and apoptosis.

To study apoptosis under influences of stimuli which regulate keratinocyte proliferation and TSD.

To investigate if known differences in p53 protein expression among the cell types would influence genes typically regulated by p53-mediated transcription, including p21, bax and bcl-2, under stimuli that induce proliferation or TSD.

To study the phenotypic expression of typical criteria of cell transformation in SVpgC2a in order to define to what extent the immortalized state expresses properties associated to normality or full malignancy.

To study the impact of immortality on cell density-dependent regulation of proliferation, TSD, apoptosis and expression of the above genes.

To study the requirement for PEX in NOK and SVpgC2a in efforts to define the role of this undefined growth supplement, and to culture cells without it, under chemically defined conditions.

MATERIAL AND METHODS

Table II Modus operandi

Cell Type	Overview of techniques	Manuscript
NOK SVpgC2a SqCC/Y1	**Organotypic culture in EMHA with 1.0 mM Ca^{2+} on collagen with fibroblasts, evaluation after 10 days of culture** Morphology / H&E staining Keratin expression / Immunochemistry	I
NOK SVpgC2a	**Organotypic culture in EMHA with 1.0 mM Ca^{2+} with or without FBS on collagen with fibroblasts and monolayer culture in EMHA, evaluation after 10 and 17 days of culture** Morphology / H&E staining Net growth / Microscopic quantitation of cell layers and cell numbers Keratin expression / Immunochemistry, Micro-array Proliferation / BrdU detection Apoptosis / Morphologic features, caspase-3 detection	II
NOK SVpgC2a SqCC/Y1	**Monolayer culture in EMHA and organotypic culture with or without fibroblasts and at 0.1 or 1.0 mM Ca^{2+} evaluation after 10 days of culture** Morphology / H&E staining Net growth / Microscopic quantitation of cell layers and cell numbers Proliferation / BrdU detection Apoptosis / Morphologic features, TUNEL-assay TSD / Involucrin immunochemistry Expression of p53, p21, Bax and Bcl-2 / Immunochemistry p53 gene sequence / DNA sequencing	III
NOK SVpgC2a	**Monolayer culture involving different cell densities including maintenance at confluency** Net growth / Microscopic quantitation of cell numbers Saturation density / Microscopic quantitation of cell numbers Colony forming efficiency / Quantitation by stereo microscopy Clonal growth rate / Quantitation by stereo microscopy Apoptosis / Morphologic features TSD / Involucrin immunochemistry Expression of p53, p21, Bax and Bcl-2 / Immunochemistry	IV

RESULTS AND DISCUSSION

Morphology of monolayer cultures (paper II-IV)

Morphological analysis of normal, SV40T-immortalized and malignant human buccal keratinocytes in monolayer culture clearly revealed certain consistent differences in their histological characteristics. NOK showed a pleomorphic, polygonal and epithelial–like shaped cells, except for some cells that had increased surface area indicative of TSD. The SVpgC2a cell population exhibited cells with polygonal and elongated shapes. SqCC/Y1 also showed a polygonal shape but with distinct cell borders. When maintained at confluence, SVpgC2a appeared smaller in size compared to NOK, and included signs of multi-nucleation, vacuolization and TSD.

Morphology of organotypic cultures (paper I-III)

Oral keratinocytes from NOK, SVpgC2a and the SqCC/Y1 cell lines were cultured to regenerate organotypic epithelia *in vitro*. Organotypic cultures grown at the air-liquid interface of collagen gels showed morphological features ranging from normal tissue to carcinoma *in situ*. In the presence of serum-free EMHA with 1 mM Ca^{2+} on collagen gels, containing human oral fibroblasts, NOK formed an organized multi-layered stratified epithelium, after 10 days. Epithelia generated with NOK demonstrated several morphological features of normal non-keratinized buccal epithelium *in vivo*, including elongated basal cells and typical features of compartmentalization. The basal compartment consisted of a single layer of generally elongate cells. The suprabasal compartment contained cells of variable morphology but with dense nuclear chromatin in the uppermost layers. There appeared to be a lack of intercellular cohesion amongst some cells of the lower layers, a feature that may be attributed to inconsistent desmosome formation. Some discrete keratohyalin granules in the upper one-third of the suprabasal compartment were presence, although keratinization was minimal. Immortalized cells consistently generated additional layers than NOK. Epithelia from SVpgC2a demonstrated comparatively homogeneous morphology and loss of typical compartmentalization, and moreover, smaller and more compact cells than in epithelium grown from NOK. Although small, the cells had a basaloid morphology throughout. There was a degree of nuclear hyperchromatism and anisonucleosis at all levels. Prickle cell formation was minimal and there were numerous apoptosis throughout. Multi-layered epithelia generated from the tumor cell line consistently generated additional layers than NOK. SqCC/Y1 cultures showed similar cell morphology throughout the multiple layers with no obvious basal cell compartment. The cells were larger than those of the immortalized epithelium but similar in size to the cells of the normal cultures. Malignant organotypic cells also showed limited prickle formation and also a considerable degree of size variation. Individual cell keratinization was often present. Nuclei were of variable size and staining characteristics, and sometimes bizarre in appearance. Mitoses were more frequently encountered amongst these cultures, together with apoptosis, although the latter were less abundant than in the SVpgC2a epithelia. In some SqCC/Y1 cultures, groups of cells grew into the collagen matrix in a manner reminiscent of SCC. Clearly morphological differences were noted between the cell lines.

A study of extended analysis of NOK and the SVpgC2a cell line were performed in attempt to improve the incomplete epithelial differentiation and maturation noted in NOK. The prolonged incubation periods from culture on collagen gels were made for up to 17 days. FBS is known to induce keratinocyte differentiation in monolayer cultures and for further analysis, the organotypic epithelia were derived using media with or without FBS. To provide a protocol that would allow future experimental utilization of established organotypic epithelia without serum for a week, the protocol with initial exposure to serum for 7 days was followed by 3 days without serum in efforts to minimize possible influences of residual. Multi-layered epithelia were formed at the air-liquid interface of collagen gels under all conditions tested. For both types of cells, epithelia grown for 17 days consistently showed supplementary cell layers than those grown for 10 days, and the different variants of serum exposure commonly produced epithelia with 1 to 2 additional layers. No change in cell morphology was noticed and the lack of intercellular cohesion in NOK epithelia appeared to resist.
Organotypic epithelia generated with or without fibroblasts in the collagen gel and with variable Ca^{2+}-levels were made in effort to further assess potential differences among all three cell lines. Of several conditions tested the inclusion of fibroblasts in the supportive collagen gel and elevation of Ca^{2+}-concentration, from 0.1 to 1 mM, in serum-free EMHA provided the preferred condition for all cell types. Consistent with previous protocols the organotypic culture of NOK formed an epithelium of 5 to 6 cell layers, whereas epithelia formed by SVpgC2a and SqCC/Y1 cell lines contained an additional 7 to 8 layers, after 10 days. The generation of organotypic epithelia from 10 days showed that the variable conditions from this protocol supported proliferation, including the stimulatory effect of oral fibroblasts.

Formation of a basal lamina (paper II)

Formation of a basal lamina was indicated by collagen IV and laminin 1, in organotypic cultures generated from NOK and SVpgC2a. Collagen IV and laminin 1 protein expression was detected in both cell lines with higher staining intensity at 17 days compared to 10 days of culture. The variable culture conditions did not detectably influence the immunochemical expression of these structural markers in either cell type. Monolayer cultures from NOK and SVpgC2a, were additionally investigated at the mRNA level using micro-array. The results confirmed an expression for collagen IV and laminin 1 in both cell types. SVpgC2a showed fairly higher abundance of collagen transcripts. Among the six known isofoms for collagen IV, isoforms three and four were indicated as absent or not detected in either NOK or SVpgC2a.
Properties and functions are different in buccal mucosa from those in the skin epithelia. For reconstruction and studies of mucosal changes, there is a need to evaluate all different kinds of epithelia [Cho, 2000] [Chung, 1997]. The morphological results above demonstrate a new model of buccal cancer progression. Although the expression of histological signs were limited as compared to the *in vivo* situation, the composite spectrum of features detected in the cell lines ranged from normal epithelium to carcinoma *in situ* or even invasive carcinoma. Assessment of the histology alone of organotypic regenerated epithelia indicated that a comparative analysis of the cell lines may serve as a standardized laboratory tool for studies of multi-step oral carcinogenesis.

Expression and distribution of keratins in organotypic epithelia (paper I-II)
Staining for keratin proteins indicated differences among the organotypic cultures. The organotypic epithelia of NOK, SVpgC2a and SqCC/Y1 were compared with that of intact buccal epithelium, severe oral epithelial dysplasia and well-differentiated oral SCC, respectively. Organotypic epithelia were cultured for 10 days in EMHA with 1mM Ca^{2+} and fibroblasts in the collagen gel. Immunochemical staining, on paraffin embedded and frozen sections, demonstrated expression of a range of cytokeratins in these cell lines. Analysis of the cytokeratins indicated obvious dissimilarities among the different organotypic epithelia.

Typical differentiation-specific keratins were present in the upper suprabasal compartment of NOK and SqCC/Y1, although to a lesser extent in the latter. As expected K4 and K13 showed a higher rate of synthesis than K1 and K10 in NOK, since these keratins are only synthesized in trace amounts in buccal mucosa. Conservation of differentiation keratins in SqCC/Y1 is in agreement with previous studies on monolayer culture and well-differentiated oral SCCs [Bloor, 1998] [Zou, 1999] [Bloor, 2001]. No detection of differentiation-specific keratins were made in epithelia from SVpgC2a, additionally cultured either with or without FBS. Partial or complete loss of suprabasal expression of differentiation-specific keratins may relate to the inability of filaggrin to re-organize and pack keratin filaments necessary for differentiation [Eckert, 1997] [Izumi, 2000]. As disturbed differentiation is a major feature of severe oral dysplasia, absence of differentiation keratins may be reflected in transformed buccal cells. Lack of differentiation keratins might signify a loss of epithelial maturation, and imitate a system comparable to pre-malignancy, such as severe oral epithelial dysplasia [Bloor, 1998] [Bloor, 2001].

High cell turnover keratins related to activation and cell stress were present in both NOK and SqCC/Y1 but absent in SVpgC2a. Distributed uniformly in the suprabasal layers of keratinizing and non-keratinizing oral epithelia [Morgan, 1991]. K6 and K16, were up regulated *in vitro* to include basal cells, from NOK and SqCC/Y1. Enhanced synthesis of high cell turnover keratins may result from tissue culture conditions, or due to increased proliferative activity in the basal compartment, similar to that in hyperproliferative conditions [Leigh, 1995]. A deficiency of K6 and K16 in SVpgC2a conflicts with their heterogeneous distribution in severe oral dysplasia, possibly because cells in SVpgC2a may represent a more basaloid phenotype [Morgan, 1991]. However, further studies of K6 and K16 gene expression on dysplastic and transformed epithelium may aid our understanding of the processes involved in pre-malignancy.

Basal cell-specific keratins were expressed in all cell types although SVpgC2a showed absence of K14. A reduction in expression of K5 and K14 in SVpgC2a may represent early events of carcinogenesis, a situation similar to that reported in pre-cancerous lesions [Vaidya, 1998] [Waseem, 1999]. The retention of K6 and K16 in SqCC/Y1 may be linked to the ability of different cells to proliferate at different rates and show less proliferation than oral epithelial dysplasia or hyperplasia.

Representatives of simple keratins were expressed in all cell types. Simple epithelial keratins are not usually present in non-keratinizing mucosal epithelia [Morgan, 1987] [Bosch, 1988], although their synthesis is induced in cultured keratinocytes [DiazGuerra, 1992]. In this organotypic epithelium, expression of simple keratins may result in an embryonic phenotype of keratinocyte differentiation. In the presence of excess K18 and K19 filaments, low levels of K7 and K8 imply that other type II keratins may have paired with K18 and K19 to counterbalance the uneven concentration of type I and II keratins in NOK. Increased expression of K8 and K18 seen in SqCC/Y1, has been associated with decreasing differentiation in oral SCC [Su, 1994] [Su, 1996]. Expression of K8 and K18 in SVpgC2a also supports findings on oral dysplasia, in which these simple keratins are enhanced with decreasing epithelial stratification [Su, 1994] [Morgan, 1994]. A rapid degradation of K8 and K18 protein might be occurring in normal epithelia but be suppressed in dysplasia and malignancy. Lack of K19 in immortalized epithelium is consistent with the situation in oral dysplasia. K20 was absent in both NOK and SqCC/Y1, but was expressed heterogeneously in all cells in SVpgC2a. The expression of K20 has not been previously described for oral keratinocytes, and may represent an important regulatory element in transformed keratinocytes, and hence in dysplasia.

By profiling keratin expression, it showed that organotypic cultures of NOK expressed many of the same keratins as intact buccal tissue, and that the loss of keratins in SVpgC2a and their retention in SqCC/Y1 had several features in common with the respective keratin profile of oral epithelial dysplasia and well-differentiated oral squamous cell carcinoma. The experiments also demonstrated that immortalization and malignant transformation was associated with loss of keratins associated to TSD. Discordance between keratin expression *in vivo* and *in vitro*, may be due to the specificity of the antibody, or the effect of serum, whereas this study employed serum-free medium [Oda, 1998].

Keratin mRNA expression in monolayer cultures (paper II)

Keratins were evaluated by a micro-array analysis from serum-free monolayer cultures of normal and immortalized cells. For both NOK and SVpgC2a, differentiation-specific keratin K4 and K1 was absent while K13 and K10 mRNA was transcribed. This discordance in keratin gene expression was previously observed at both the mRNA and protein levels in various oral epithelia, involving cultured cells as well as tissue [Lindberg, 1990] [Sexton, 1993] [Brysk, 1995] [Neugebauer, 1996] [Bloor, 1998] [Oda, 1998]. Epidermal-type keratin, K2a was expressed in one sample while absence of K9 was not unexpected, as this keratin is characteristic of palmar and plantar keratinocytes during morphogenesis [Moll, 1982]. K2a expression is not typical of buccal keratinocytes, but this keratin may have been spontaneously induced during tissue culture conditions as seen for other keratins in cultured cell lines. Intensely expressed high cell turnover keratin, K6a, K16 and K17 in NOK, is in accord with many other studies. In contrast to NOK, high cell turnover keratins were absent in SVpgC2a. Most basal specific keratins were transcribed at a high level in both NOK and SVpgC2a samples, although K15 mRNA expression was lacking in the immortalized cells.

Simple epithelial keratins, K7, K8, K18 and K19 were present in NOK. Spontaneous induction of simple keratins in normal keratinocytes may be a feature of culture conditions or a switch towards a simple phenotype [Morgan, 1994]. K19 and K20 was absent in SVpgC2a, whereas K7, K8 and K18 were intensely expressed. Also evident from analyses in the organotypic cultures, the notable loss of transcripts for differentiation and high cell turnover keratins, and increase in transcripts for simple keratins, are consistent with a basaloid phenotype, in immortalized cells. K12, specific for corneal-type differentiation was absent in all samples. Most of the hair keratins were absent, although some were transcribed at a low level from both cell types. Low-level mRNA expression of hair keratins has not been reported previously for buccal keratinocytes. However, proteins for both acidic and basic hair keratins have been detected in normal dorsal tongue [Langbein, 2001]. Different probes can detect different sites on the gene and that might give an explanation to the disparate results achieved for K7, where one probe showed high expression and one was absent.

NOK and SvpgC2a exhibited a range of keratins, evident from analysis at the mRNA level. NOK generally showed more than 5-fold higher transcripts levels in many keratins, than SVpgC2a line. The results confirmed that NOK expressed keratins in a tissue-like manner and that SVpgC2a showed a keratin profile more commonly associated to a dysplastic epithelium. The differences between NOK and SVpgC2a were stated and keratin types not previously reported for buccal epithelium were also implicated, including those associated to epidermis and hair. The SVpgC2a cell line may be a useful model of a dysplastic state on the basis of the consistent formation of a disorganized epithelium without the upward sequential differentiation pattern and maturation that characterized NOK. The overall results provided further evidences that models of normal and preneoplastic epithelia could be generated with NOK and SVpgC2a cell lines.

Cell growth and cell death

Proliferation (paper II-III)

Analyzes on proliferation relative to apoptosis were primarily made from organotypic cultures generated with and without FBS. Comparison was made from different protocols with NOK and SVpgC2a. Results from BrdU incorporation showed that SVpgC2a was several-fold more active than NOK. Proliferation was detected primarily in the basal layer of NOK whereas SVpgC2a showed proliferation throughout the epithelium. SVpgC2a showed increased proliferation and sustained growth under certain protocols with serum. Serum may act to support keratinocyte growth indirectly by promoting mesenchymal-epithelial changes, but serum may also decrease growth by inducing TSD, the latter process being partially defective in SVpgC2a [Grafström, 1997]. It seems conceivable that the proliferative and anti-proliferative actions of serum were generally balanced in NOK, whereas proliferation was favored in SVpgC2a with certain protocols. Moreover serum apparently stimulated the migration of SVpgC2a cells into the collagen matrix, in some cultures. This effect was not noticed with NOK. Related to 10 days, the proliferative procedures showed a tendency to decrease when cultures were apprehended for 17 days.

Further studies of proliferation relative to apoptosis were made in epithelia regenerated with NOK, SVpgC2a, and the malignant SqCC/Y1 cell line. The organotypic epithelia were cultured with or without fibroblasts in the collagen gel and with variable Ca^{2+}-levels in efforts to assess potential differences among the respective cell lines in regulation of fundamental tissue homeostatic processes. Fibroblasts as mesenchymal element can modulate and influence the phenotype of epithelial cells *in vitro* in accordance with their origin. A more malignant epithelial phenotype can be obtained using tumor fibroblast instead of normal, in organotypic cultures [Atula, 1997]. For all our studies, normal oral fibroblasts were used as mesenchymal component. Incubation of cells with BrdU during the final period of culture clearly indicated capability of expanding and differences in the proliferative activity among cell lines. At all conditions, SVpgC2a and SqCC/Y1 showed several-fold higher proliferation than normal cultures. In all cell types, incorporation of fibroblasts in the collagen gel significantly increased proliferation, while elevation of Ca^{2+} had no effect.

Vimentin expression (paper II)

Expression of vimentin was analyzed by immunochemistry, in organotypic epithelia generated with and without serum and a culture period for 10 and 17 days. The NOK and SVpgC2a cell lines were evaluated from these protocols. Both cell lines exhibited activated, proliferative states as implied from vimentin expression. In 10 day-cultures the vimentin antibody stained basal and some parabasal cells and from the prolonged culture period, vimentin generally showed a decreased staining in epithelial compartment but were still visible in some basal cells. In both cell types, the majority of vimentin-positive cells were situated beneath the basal lamina indicative of strongly stained fibroblasts. Transcripts coding for vimentin, were also detected in both NOK and SVpgC2a monolayer cultures. Vimentin was present at many-fold higher levels than the other transcripts. Induction of vimentin may be connected with increased cell motility, since vimentin expression has been reported in highly malignant tumors of the head and neck region with a poor clinical prognosis. Vimentin together with strong proliferation has been observed at the invasion front of assessed carcinomas and might reflect a permanently activated state in response to surrounding tumor stroma [Richard, 1990] [Tomakidi, 1997]. Vimentin expression in epithelia, could be an indicator of a hyper-proliferative state, also associated to stress response and activity [Evans, 1998] [Runembert, 01]. Further, vimentin deficient cells exhibit a slower rate of proliferation and DNA synthesis [Wang, 2000]. Migratory growth of keratinocytes into the collagen matrix may reflect an activated state, although in the present study, this effect was noted selectively with SVpgC2a.

Apoptosis in organotypic epithelia (paper II-III)

Assessment on apoptosis was exploit after 10 and 17 days, from organotypic epithelia cultured with or without FBS. Apoptosis was detected throughout the epithelium in cells derived both from NOK and SVpgC2a. Frequency of apoptosis, evaluated from morphological characteristics and caspase-3 immunoassay, were independent of the variable growth conditions. Although one exception was noticed in SVpgC2a, showing significantly increased apoptosis from prolonged culture under a protocol with serum

followed by a serum-free period. SVpgC2a showed significantly higher indices for apoptosis than NOK under five tested conditions.
Further, apoptosis was scored from organotypic epithelia derived from all three cell lines, with or without fibroblasts, at serum-free conditions with different concentrations of Ca^{2+}. Apoptosis was detected from morphological criteria in H&E stained sections and from TUNEL assay. In NOK, desquamation of apoptotic cells was noted in the upper suprabasal layers, whereas the TUNEL assay detected desquamation of TSD cells without morphological signs of apoptosis. Organotypic epithelia generated from transformed cells did not exhibit this phenomenon, possible due to reduced ability for TSD [Grafström, 1997]. Both SVpgC2a and SqCC/Y1 epithelia were clearly capable of apoptosis without undergoing TSD. Numbers of fragmented nuclei detected by TUNEL were equal to morphological scoring of apoptosis in all organotypic epithelia, with the exception of a few desquamating cells in NOK. SVpgC2a showed a 4 to 10-fold higher apoptosis frequency than normal epithelia, and the SqCC/Y1 line demonstrated apoptosis to a 2 to 4-fold higher level, compared with NOK. Inclusion of fibroblasts significantly elevated apoptosis in NOK whereas elevated Ca^{2+} had little effect. SVpgC2a showed relatively higher apoptosis frequency than SqCC/Y1, but neither cell type increased the action in respond to fibroblast incorporation.

Terminal squamous differentiation in organotypic epithelia (paper III)
Collagen with or without fibroblasts, and serum-free conditions with different concentrations of Ca^{2+} was used to evaluate the influences of TSD. Involucrin was applied as a marker for cellular commitment to TSD. Immunochemical expression from involucrin was visualized throughout the suprabasal compartment, but absent in the basal layer in NOK. Involucrin was also expressed in a low number of apoptotic cells. That indicate, although rarely, that a cell committed to TSD may take the alternate route of apoptosis, but apoptosis does not require induction of TSD. It is currently unclear to what extent TSD and apoptosis share biochemical features [Mitra, 1997]. In SVpgC2a, involucrin was predominantly absent, whereas in SqCC/Y1, scattered staining was detected in the upper suprabasal layers. NOK consistently exhibited higher expression of the TSD marker involucrin than the transformed lines, and elevated Ca^{2+} significantly increased the differentiation. Ionized calcium is the most common signal transduction element in a wide range of cells including keratinocytes. A calcium gradient is present *in vivo* where the levels are higher in the outermost, more differentiated layers. In high calcium medium keratinocytes undergo differentiation and form intercellular networks. Using media with low or high calcium concentration, keratinocytes can proliferate for a limited time period but finally become senescent and degenerated [Fusenig, 1994]. Transformed cells showed a minimal change in differentiation at various conditions, and a failure to respond to elevated Ca^{2+}. The results linked the immortal and malignant buccal phenotypes to aberrant regulation of tissue homeostatic processes.

Utilized epithelia regenerated in vitro provides new information on the influences of FBS, fibroblasts and Ca^{2+} on oral keratinocytes, demonstrating firstly that regulation of proliferation, TSD and apoptosis is inter-connected in many ways. Distinct states of proliferation, TSD and apoptosis and different responses to growth and death stimuli

characterized NOK, SVpgC2a and SqCC/Y1. This supports their usefulness in studies of homeostasis, including carcinogenesis. The differences in proliferation and TSD markers are similar to those shown for normal and transformed keratinocyte lines from other squamous epithelia [Merrick, 1992] [Tsunenaga, 1994] [Delvenne, 1995]. Although the rates of proliferation and apoptosis in NOK, SVpgC2a and SqCC/Y1 were higher than in normal and dysplastic oral tissue [Birchall, 1997]. Parallel increases of proliferation and apoptosis in NOK under a proliferative stimulus may reflect an effort to maintain tissue homeostasis. Apoptosis may also protect against transformation and increased proportion of aberrant cells under conditions of promoted growth. Elevated Ca^{2+} selectively stimulated TSD of NOK but not of SVpgC2a and SqCC/Y1 and altering Ca^{2+} levels failed to influence apoptosis in all cell types. The lack of a significant coupling between proliferation and PCD in SVpgC2a and SqCC/Y1 implies that the immortal and malignant phenotypes may be deficient in this regard. The carcinogenesis model constituted by these cell lines thus include a hyper-proliferative, TSD-deficient and hyper-apoptotic phenotype for the immortalized stage, incapable of regulating TSD and apoptosis. Further a malignant stage showing a hyper-proliferative phenotype, with some ability for TSD although with lowered ability for apoptosis. Additional studies are obviously needed to delineate the similarity and complex relationship in epithelial homeostasis, from normal and transformed cells.

Expression of p53 and p53-regulated genes

Organotypic epithelia (paper III)

Organotypic cultures at different conditions, were generated from NOK, SVpgC2a and SqCC/Y1. Immunochemically research was assessed on p53, p21, Bax and Bcl-2 protein expression in respective epithelia. Analysis of the frequencies of the proteins in each of the cell types demonstrated different expression patterns.

p53

In NOK, p53 was dispersed in a random, tissue-like manner in basal and parabasal cells. The frequency of p53 positive cells was increased in the suprabasal compartment by the presence of fibroblasts in the collagen gel. For p53, it is possible that the condition with fibroblasts might exert certain stress that promotes protein stabilization. Contradictory the TSD-inducing effect of elevated Ca^{2+} was coupled with decreased p53 expression. p53 protein is presumably functional in normal oral keratinocyte lines. The scattered tissue-like expression of p53 in regenerated NOK-epithelium indicated the functionality of p53. In SVpgC2a, p53 was heterogeneously distributed and showed a strong, intense expression. Neither fibroblasts nor elevated Ca^{2+} influenced the p53 expression in SVpgC2a. Anomalous expression of p53 in SVpgC2a is of interest since the progression of oral cancer has been widely associated with aberrant p53 expression or p53 mutation [Edington, 1995] [Raybad-Diogine, 1996] [Nylander, 2000]. DNA sequencing confirmed the wild type p53 genotype of SVpgC2a, implying that functional inactivation of p53 would occur by complex formation with SV40T antigen [Grafström, 1990] [Kulkarni, 1995]. Expression of p53 was missing in SqCC/Y1, as a result of a single rearranged p53 allele that cannot generate functional p53 protein [Grafström, 1990] [Kulkarni, 1995].

p21

A minority of cells expressed p21, in SVpgC2a and SqCC/Y1, showing small differences between the various epithelia. The intensity of expression for p21 provided no consistent pattern from the different conditions implicated. p21 has been detected in differentiating layers of normal oral epithelium and associated with the initiation of differentiation [Matsuta, 1997] [Harada, 2000]. NOK presented a slightly stronger expression from p21 in the suprabasal cells, in accord that expression of p21 can induce cell cycle arrest.

Bax

Differences in immunochemical expression of p53 protein among the cell lines associated to the differences in Bax expression. In NOK, Bax protein was visualized in many cells in the suprabasal compartment, and the expression level of Bax clearly increased superficially with all culture conditions. The effect from fibroblast-mediated increase in proliferation and apoptosis was coupled with decreased Bax expression, and the TSD-inducing effect of elevated Ca^{2+} was coupled with an increased Bax level. For Bax in NOK, the high frequency of expression as well as the existence of a basal to suprabasal gradient that was promoted by Ca^{2+} implied a coupling to TSD and not to apoptosis. Only the normal cells showed an increased Bax expression in the presence of elevated Ca^{2+}. Based on the ability of p53 to transactive expression of Bax the results supported a role for p53 in Ca^{2+}-mediated TSD-induction in normal keratinocytes. Linking Bax and TSD was previous suggested, showing a lack of Bax in keratinocytes with low calcium medium, but expressed in differentiated cells whit high calcium [Maruoka, 1997]. Bax expression in SVpgC2a epithelia was predominantly absent and different conditions were without effect. A deficiency in Bax regulation, parallel with loss of TSD in immortalized cells was expound. The SqCC/Y1 epithelia showed small differences in expression of Bax, from the different conditions applied. In SCC, as well as in normal oral epithelia, the proportion of Bax-positive cells is higher in the suprabasal than in the basal cell layer [Loro, 1999]. Confirming the *in vivo* situation, Bax were expressed in the suprabasal compartment of both SqCC/Y1 and NOK models.

Bcl-2

Considered to be a stem cell marker the Bcl-2 expression is generally associated with a basal location [Gandarillas, 2000], whereas its immunochemical detection in apoptotic cells is likely to reflect a cleavage product [Fadeel, 1999]. Other studies have verified difficulties to detect Bcl-2 expression *in vitro* but not *in vivo*, although it is unknown why [Maruoka, 1997]. The week or absent expression for Bcl-2 in this study, showed no consistent pattern with the different conditions in the organotypic cultures. As shown for NOK, occasional cells were Bcl-2 positive in the basal layer although cells with characteristics of apoptosis also stained for Bcl-2. Anti-apoptotic molecules can work by dimerizing with pro-apoptotic molecules to inhibit their function, therefore the relative expression levels of Bcl-2 and Bax may determine if cells survive or undergo cell death, respectively. The expression of Bcl-2 at a low and constant level would

support that changes in Bax normally regulate the biological outcome of the Bcl-2/Bax ratio [Gross, 1999].

Sub-confluent and confluent monolayer cultures (paper IV)

Further evaluations in immunochemical expression of proteins encoded by the p53, p21, bax and bcl-2 genes were analyzed in sub-confluent and confluent monolayer cultures. To provide a comparison between cells that are proficient and deficient in p53-function, the assessment included normal and immortalized cells.

p53

Assessment of expression frequency showed that a minority of NOK expressed p53 and confluence somewhat influenced and lowered the frequency of positive cells. In SVpgC2a, strong expression of p53 was seen in the majority of cells, comparable to levels shown in earlier studies of the organotypic cultures.

p21

In NOK, a few cells expressed p21, without observed changes in the staining intensity. Post-confluence in NOK cultures, showed an increase in the frequency of p21 expression. The low frequency of p21 remained unchanged with confluence in SVpgC2a. The gradual and density-dependent increase of p21 expression in only a fraction of NOK, and the parallel absence of p21 regulation in SVpgC2a, agree with its involvement in regulation of growth arrest and TSD of keratinocytes [DiCunto, 1998] [Gandarillas, 2000].

Bax

Assessment of expression frequency showed that most cells expressed Bax from the different states of confluence in NOK. The high frequency of Bax expression remained consistent with the conditions, although the staining intensity increased at confluence. The expression pattern for Bax differed between NOK and SVpgC2a. The frequency of Bax expression in SVpgC2a increased to some extent with confluence although without altered intensity. For normal cells, Bax expression increased in growth-inhibited, confluent conditions that induced TSD and decreased apoptosis. Thus, the elevated Bax expression was associated with keratinocyte TSD but not with apoptosis, since confluence significantly increased TSD while decreasing apoptosis. In agreement, Ca^{2+} exposure induces Bax expression in differentiating oral carcinoma cells in monolayer culture [Tomkova, 1999], and the suprabasal compartment of organotypic epithelia regenerated from NOK. The results provide further support for a dissociation of Bax expression from keratinocyte apoptosis in NOK and are likely relevant for epithelia other than buccal.

Bcl-2

For both NOK and SVpgC2a, the expression of Bcl-2 protein was detected to a low extent from the immunochemical staining. Confluence somewhat increased the frequency of Bcl-2 expression in NOK, but levels of Bcl-2 remained unchanged with confluence in SVpgC2a. Based on the concept that the relative expression levels of Bcl-2 and Bax determines if cells survive or undergo cell death, a role for Bax in regulating

TSD agrees with the fact that over-expression of Bcl-2 blocks induction of oral keratinocyte TSD [Harada, 1998] [Gross, 1999]. The Bcl-2 expression remained low and relatively constant with the various confluent conditions in both NOK and SVpgC2a. This further supported that changes in Bax would normally regulate the biological outcome of the Bcl-2/Bax ratios in keratinocytes, as implicated from the organotypic epithelia.

A composite view on the processes and genes that regulate tissue homeostasis in squamous epithelia such as buccal epithelium is lacking, and the roles of genetic alterations associated to p53 function inferred during oncogenesis need further investigation. The present study utilize a technique to culture cells and regenerate epithelia, from different culture conditions to compare expression of p53, p21, Bax and Bcl-2 proteins. In organotypic epithelia, fibroblasts included in the collagen gel and variable Ca^{2+} in the growth medium demonstrated specificity among the cell types in regards to protein expression. In monolayer cultures, different states of confluence, also provided protein expression particular for the assorted cells. Pre-confluent to post-confluent monolayer cultures in the presence or absence of PEX did not influence the protein expression in SVpgC2a. The organotypic models did not generate epithelia when cultured in PEX-free media, and assessments from this condition were excluded.

Previous studies would agree with a possible role for p53 in regulating gene expression in growth-inhibited keratinocytes undergoing TSD also without the involvement of external stress or DNA damage [Weinberg, 1995] [Shin, 1996] [Pluquet, 2001]. The existence of p53-independent regulation of the above genes implies a need for studies on mechanism, including the p53 homologues p63 and p73, transcription factors known to influence ectodermal differentiation [Levrero, 2000] [Irwin, 2001]. Furthermore, involvement of additional Bcl-2 family members should be considered in confluence-induced TSD of keratinocytes [Gross, 1999] [Polverini, 1999] [Reed, 2000]. In reference to the exploration of p53 function, this model provided new results, associated with acquisition of immortality and loss of p53 function similarly to the proposed scenario *in vivo* [Edington, 1995]. Therefore, cultures of human normal and transformed oral epithelial cells are applicable to study various aspects of the involvement of the p53 gene in oral carcinogenesis.

Influence of cell density (paper IV)

Available information on phenotypic alterations of transformed cells is obtained primarily from culture of mesenchymal and hematopoetic cell types. Generally, the immortal, non-malignant stage has been analyzed to a lesser degree than the malignant stage. Hence, the additional studies of tissue homeostatic mechanisms including proliferation, TSD and apoptosis, were assessed in NOK and SVpgC2a to elucidate alterations occurring in the transition from normality to an early stage of cell transformation. Reports on successful culture of most cell types without supplementation of extracts or serum are scarce, and the potential for serial culture of SVpgC2a at chemically defined conditions may be advantageous in further applications of this cell line [Grafström, 1990]. The requirement for PEX, including its presumed

growth-supporting effect, has not been studied in normal and immortalized oral keratinocytes. Measurements in sub-confluent and confluent mass cells cultures was determined in NOK and SVpgC2a at 70 and 100% confluence and in cultures kept at confluence for additionally 3 and 6 days. Pre-confluent to post-confluent monolayer cultures was analyzed in conditions with or without PEX as an alternative approach to the regenerated epithelia.

Morphology and net growth

At these conditions, both cell types grew from a stage of single, separated cells into a tightly packed mass of cells, showing signs of multi-focal growth at confluence. SVpgC2a cells were more numerous at all states of confluence, both with and without PEX. The morphological differences were most apparent at low density. NOK was pleomorphic at all stages, exhibiting differences in shape and size, whereas SVpgC2a was mostly uniform. When maintained at confluence, SVpgC2a appeared smaller in size, in contrast to NOK that expanded.

Saturation density

SVpgC2a exhibited almost 10-fold higher saturation density than NOK at confluency, and these cells expanded significantly until 3 days post-confluence. Growth inhibition of SVpgC2a required longer time and higher cell numbers relative to NOK. The number of SVpgC2a cells increased 3 to 4-fold when maintained at confluence for 3 days in media with or without PEX. Insignificant further expansion in cell density at confluence plus 6 days, indicated that the cultures had reached their full saturation density at these conditions, partly due to enlargement of the cell surface area and partly due to detachment of cells. The growth behavior of NOK at different cell densities, roughly imitated perturbed tissue states *in vivo*, not previously reported.

Colony forming efficiency

Colony forming efficiency (CFE) were determined in conditions with or without PEX for both NOK and SVpgC2a. The removal of PEX essentially abolished growth and almost abrogated CFE of NOK, whereas SVpgC2a only exhibited a minor lowering of CFE. The CFE observed in sub confluent NOK decreased at confluency and by maintenance at confluency. SVpgC2a exhibited significantly higher CFE than NOK at all evaluated conditions. Growth to a confluent state increased CFE for SVpgC2a, and moreover, SVpgC2a tolerated confluency without loss in CFE. The lowered CFE of SVpgC2a noted in defined PEX-free media was not significantly influenced by differences in cell density.

Clonal growth rate

Analysis of clonal growth rate (CGR) at different cell densities demonstrated marked differences in the requirement for PEX in NOK and SVpgC2a. NOK could not tolerate confluency without loss of growth capacity whereas growth to high density increased the cloning ability of SVpgC2a. SVpgC2a exhibited significantly higher CGR, than normal cells, at all conditions. Confluent NOK exhibited pronounced contact inhibition as indicated from decreased cell numbers and loss of population doublings per day. Removal of PEX markedly decreased CGR in NOK. In contrast, confluent SVpgC2a

cells showed sustained growth and elevated cloning ability. SVpgC2a provided partial or total independence from the PEX-related effects. In SVpgC2a removal of PEX did not influence CGR, that was similar in both conditions. For SVpgC2a, the high cloning ability, in part explain why this line is easily expanded into high cell numbers under conditions of monolayer culture in EMHA.

Programmed cell death

From the fact that relationship between TSD and apoptosis is not clear, loss of TSD features has been shown in immortalized keratinocytes from many tissues other than buccal, and apoptosis is still largely undefined. Commitment to cell death by TSD or apoptosis was analyzed in sub-confluent and confluent mass cultures of NOK and SVpgC2a. Conditions without PEX were also evaluated in monolayer cultures from SVpgC2a cells.

Detection of apoptosis

The frequency of apoptosis in sub-confluent cultures was about 3-fold lower in NOK than in SVpgC2a. In NOK, confluent cultures and cultures maintained for 3 and 6 days at confluence, the apoptotic frequency significantly decreased. The finding that confluent normal keratinocytes exhibit decreased apoptosis has not previously been demonstrated. This result suggests a coupling between growth and apoptosis regulation. Decreased apoptosis may relate to the increased commitment of the cell population to TSD, or in addition, decreased growth may lead to fewer errors and apoptotic deletions of defective cells. SVpgC2a showed lack of change in apoptosis in both sub-confluent and confluent conditions. For SVpgC2a, a lack of considerable changes was noted in apoptotic frequency with all conditions, including the reduced value noted at 6 days under conditions of decreased growth. The presence or absence of PEX had no influence on apoptosis frequency of SVpgC2a. Accordingly, the high apoptosis frequency of SVpgC2a may reflect high proliferation as well as inability to undergo death by TSD, thus showing a shift from TSD to apoptosis.

Detection of terminal squamous differentiation

The state of confluency induced growth arrest and TSD in normal cells. Frequency of expression, calculated from involucrin positive cells, was significantly increased in the cell population of NOK maintained for additionally 3 and 6 days post-confluence. For normal keratinocytes, the transition from a proliferative, sub-confluent to a growth-inhibited, confluent stage is known to induce TSD [Poumay, 1995] [Poumay, 1999]. The confluent NOK exhibited pronounced contact inhibition as indicated from decreased cell numbers and loss of cloning ability concomitant with commitment to TSD, similarly to other keratinocytes [Poumay, 1995] [Poumay, 1999]. In each cell culture grown to various states of confluency, the TSD frequency was more than 10-fold higher in NOK than in SVpgC2a. For SVpgC2a, the transition from sub-confluent and confluent conditions occurred without significant change in TSD, or apoptosis, it retained an expression frequency of about the same level. The presence or absence of PEX showed no influence on involucrin expression of SVpgC2a.

Assessment of growth of NOK and SVpgC2a at variable densities, and in conditions with or without PEX, demonstrated that immortalization of oral keratinocytes involved a spectrum of phenotypic alterations. SVpgC2a exhibited increased cloning ability, increased growth rate, decreased requirement for supplementation with PEX, decreased responsiveness to contact inhibition of growth, lack of commitment for TSD and inability to regulate apoptosis. For SVpgC2a, the high cloning ability, growth rate and saturation density and failure to undergo normal TSD further more explain why this line is easily expanded into high cell numbers under conditions of monolayer culture in EMHA. The results indicated that the immortal, non-malignant state of SVpgC2a expressed phenotypic properties commonly associated to tumor cells. Previous lack of definition of characteristics associated to keratinocyte immortalization implied that the results may be generally applicable to other tissues than oral mucosa, including that chemically defined conditions may be applied to monolayer culture of immortalized keratinocytes. In contrast, EMHA excluded from PEX, provided obliterated growth in both monolayer cultures and organotypic cultures generated from NOK. Keratinocyte culture is likely promoted by several components in PEX, since various singly identified factors, e.g. growth factors and hormones, are unable to fully replace the extract in various experimental systems [Asami, 1984] [Halper, 1992] [Tanigaki-Obana, 1994].

CONCLUSIONS

A compound *in vitro* model for step-wise malignant transformation of oral epithelium is described where altered regulation of growth, cell death, differentiation and p53-expression is imperative.

Normal, experimentally immortalized and malignant human buccal keratinocyte lines derived in monolayer culture can generate organotypic epithelia under standardized culture conditions. These conditions include media with or without serum, at variable Ca^{2+}-levels, and with or without influences of oral fibroblasts.

Organotypic epithelia derived from NOK, SVpgC2a and SqCC/Y1 provide a model that exhibits histological features that span the progression from normal to malignant tissue.

Organotypic epithelia regenerated with NOK, SVpgC2a and SqCC/Y1 cell lines exhibit distinct phenotypes with respect to the expression of markers of proliferation, TSD and apoptosis.

Profiling of keratin expression, including both mRNA and protein, showed that NOK expressed keratins typically found in normal tissue, whereas SVpgC2a and SqCC/Y1 showed features typical of severe epithelial dysplasia and well-differentiated oral SCC, respectively. These results confirm that altered keratin expression is a feature of malignant transformation.

The role of inactivated tumor suppressor p53 function was implicated based on the presumed functionality of p53 in normal cells, non-functionality of p53 in SVpgC2a and loss of p53 in SqCCY/1. Assessment of gene expression linked p53 and Bax as candidates for participating in keratinocyte TSD in normal epithelium among other genes.

Investigation of the impact of cell density demonstrated that immortality is coupled to altered regulation of cell proliferation, TSD and apoptosis as well as the expression of proteins associated to these processes. These results also show that SVpgC2a is easily expanded into large cell numbers since it escapes various pressures that normally would induce growth-arrest and TSD in culture.

Assessment of cloning efficiency and growth in mass culture under serum-free conditions demonstrated the respective dependence and independence for PEX of NOK and SVpgC2a. Differently to NOK, it is therefore possible to cultivate SVpgC2a under chemically defined conditions.

These results suggest that the above cell lines can serve in further studies aimed at investigating the changes that occur in normal and transformed states of oral buccal epithelium.

ACKNOWLEDGEMENTS

I Like to Gratitude Everyone that Helped me with this Work!

No One Mentioned and No One Forgotten!

THANK YOU ALL!

REFERENCES

Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci. 2001, 26: 61-6

Asami O, Nakamura T, Mura T, Ichihara, A. Identification of trypsin inhibitor in bovine pituitary extracts as a survival factor for adult rat hepatocytes in primary culture. J Biochem. 1984, 2: 299-309

Asker C, Wiman KG, Selivanova G. p53-induced apoptosis as a safeguard against cancer. Biochem Biophys Res Commun. 1999, 265: 1-6

Atula S, Grenman R, Syrjanen S. Fibroblasts can modulate the phenotype of malignant epithelial cells in vitro. Exp Cell Res. 1997, 235: 180-7

Berg C, Hedrum A, Holmberg A, Ponten F, Uhlen M, Lundeberg J. Direct solid-phase sequence analysis of the human p53 gene by use of multiplex polymerase chain reaction and alpha-thiotriphosphate nucleotides. Clin Chem. 1995, 41:1461-1466

Birchall M, Winterford C, Tripconi L, Gobe G, Harmon B. Apoptosis and mitosis in oral and oropharyngeal epithelia: evidence for a topographical switch in premalignant lesions. Cell Prolif. 1996, 29: 447-56

Birchall MA, Schock E, Harmon BV, Gobe G. Apoptosis, mitosis, PCNA and bcl-2 in normal, leukoplakic and malignant epithelia of the human oral cavity: prospective, in vivo study. Oral Oncol. 1997, 33: 419-425

Bloor BK, Su L, Shirlaw PJ, Morgan PR. Gene expression of the differentiation-specific keratins (4/13, and 1/10) in normal human buccal mucosa. Lab Invest. 1998, 78: 787-795

Bloor BK, Seddon SV, Morgan PR. Gene expression of differentiation-specific keratins in oral epithelial dysplasia and squamous cell carcinoma. Oral Oncol. 2001, 37: 251-61

Blumenberg M, Tomic-Canic M. Human epidermal keratinocyte: keratinization processes. EXS. 1997, 78:1-29

Bosch, F X, Leube, R E, Achtstatter, T, Moll, R, Franke, W W. Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization in situ. J Cell Biol. 1988, 106: 1635-48

Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol. 1983, 81: 33s-40s

Brysk MM, Arany I, Brysk H, Chen S-H, Calhoun KH, Tyring SK. Gene expression of markers associated with proliferation and differentiation in human keratinocytes cultured from epidermis and from buccal mucosa. Arch Oral Biol. 1995, 40: 855-862

Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. Annu Rev Immunol. 1998, 16: 395-419

Cho KH, Ahn HT, Park KC, Chung JH, Kim SW, Sung MW, Kim KH, Chung PH, Eun HC, Youn JI. Reconstruction of human hard-palate mucosal epithelium on de-epidermized dermis. J Dermatol Sci. 2000, 22:117-24

Choisy-Rossi C, Reisdorf P, Yonish-Rouach E. Mechanisms of p53-induced apoptosis: in search of genes which are regulated during p53-mediated cell death. Toxicol Lett. 1998, 102-103: 491-6

Chrysomali E, Lozada-Nur F, Dekker NP, Papanicolaou SI, Regezi JA. Apoptosis in oral erythema multiforme. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1997, 83:272-80

Chung JH, Cho KH, Lee DY, Kwon OS, Sung MW, Kim KH, Eun HC. Human oral buccal mucosa reconstructed on dermal substrates: a model for oral epithelial differentiation. Arch Dermatol Res. 1997, 289: 677-85

Colombel MC, Buttyan R Hormonal control of apoptosis: the rat prostate gland as a model system. Methods Cell Biol. 1995, 46: 369-85

Compagni A, Christofori G. Recent advances in research on multistage tumorigenesis. Br J Cancer. 2000, 83: 1-5

Das BR, Nagpal JK. Understanding the biology of oral cancer. Med Sci Monit. 2002, 8:RA258-67

el-Deiry WSRegulation of p53 downstream genes. Cancer biology. 1998, 8: 345-357

Dekker NP, Lozada-Nur F, Lagenaur LA, MacPhail LA, Bloom CY, Regezi JA Apoptosis-associated markers in oral lichen planus. J Oral Pathol Med. 1997, 26: 170-5

Delvenne P, Al-Saleh W, Gilles C, Thiry A, Boniver J: Inhibition of growth of normal and human papillomavirus-transformed keratinocytes in monolayer and organotypic cultures by interferon-γ and tumor necrosis factor-α. Am J Pathol. 1995, 146: 589-598

Diaz-Guerra, M, Haddow, S, Bauluz, C, et al. Expression of simple epithelial cytokeratins in mouse epidermal keratinocytes harboring Harvey ras gene alterations. Cancer Res. 1992, 52: 680-7

Di Cunto F, Topley G, Calautti E, Hsiao J, Ong L, Seth PK, Dotto P. Inhibitory function of p21 (CIP/1, WAF1) in differentiation of primary mouse keratinocytes independent of cell cycle control. Science. 1998, 280: 1069-1072

Dressler D, Sarang Z, Szondy Z, Engelhart K, Grafstrom RC. Expression of retinoid-related genes in serum-free cultures of normal, immortalized and malignant human oral keratinocytes. Int J Oncol. 2002, 20: 897-903

Eckert RL, Crish JF, Robinson NA. The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. Physiol Rev. 1997, 77: 397-424

Edington KG, Loughran OP, Berry IJ, Parkinson EK. Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss. Mol Carcinog. 1995, 13: 254-65

Evan G, Littlewood T. A matter of life and cell death. Science. 1998, 281: 1317-22

Evans RM. Vimentin: the conundrum of the intermediate filament gene family. Bioessays. 1998, 20: 79-86

Fadeel B, Hassan Z, Hellstrom-Lindberg E, Henter JL, Orrenius S, Zhivotovsky B. Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. Leukemia. 1999, 13: 719-728

Fambrough D, McClure K, Kazlauskas A, Lander ES. Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. Cell. 1999, 97: 727-741

Farmer I, Freysdottir J, Dalghous AM, Fortune F. Expression of adhesion and activation molecules in human buccal epithelial cell lines and normal human buccal epithelium in situ. J Oral Pathol Med. 2001, 30: 113-20

Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M. Keratins and the keratinocyte activation cycle. J Invest Dermatol. 2001, 116: 633-640

Fusenig NE. Epithelial-mesenchymal interactions regulate keratinocyte growth and differentiation *in vitro*. In The keratinocyte handbook, Cambridge University Press. 1994, pp.71-94

Gallaher BW, Hille R, Raile K, Kiess W. Apoptosis: live or die--hard work either way! Horm Metab Res. 2001, 33: 511-9

Gandarillas A. Epidermal differentiation, apoptosis, and senescence: common pathways? Exp. Gerontol. 2000, 1: 53-62

Grafström RC. Carcinogenesis studies in human epithelial tissues and cells in vitro: emphasis on serum-free culture conditions and transformation studies. Acta Physiol Scand. 1990, 140 (suppl 592): 93-133

Grafström RC, Noren U, Zheng X, Elfwing Å, Sundqvist K. Growth and transformation of human oral epithelium in vitro. In Recent Results in Cancer Research. 1997, pp.275-306

Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999, 15: 1899-911

Halper J, Parnell PG, Carter BJ, Ren P, Scheithauer BW. Presence of growth factors in human pituitary. Lab Invest. 1992, 5: 639-45

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000, 100: 57-70

Harada H, Mitsuyasu T, Seta Y, Maruoka Y, Toyoshima K, Yasumoto S. Overexpression of bcl-2 protein inhibits terminal differentiation of oral keratinocytes in vitro. J Oral Pathol Med. 1998, 1: 11-17

Harada K, Ogden GR. An overview of the cell cycle arrest protein, p21(WAF1). Oral Oncol. 2000, 36: 3-7

Hedberg JJ, Hansson A, Nilsson JA, Hoog JO, Grafstrom RC. Uniform expression of alcohol dehydrogenase 3 in epithelia regenerated with cultured normal, immortalised and malignant human oral keratinocytes. Altern Lab Anim. 2001, 29: 325-33

Irwin MS, Kaelin WG. p53 family update: p73 and p63 develop their own identities. Cell Growth Differ. 2001, 12: 337-349

Ito T, Fujieda S, Tsuzuki H, Sunaga H, Fan G, Sugimoto C, Fukuda M, Saito H. Decreased expression of Bax is correlated with poor prognosis in oral and oropharyngeal carcinoma. Cancer Lett. 1999, 140: 81-91

Izumi K, Terashi H, Marcelo CL, Feinberg SE. Development and characterization of a tissue-engineered human oral mucosa equivalent produced in a serum-free culture system. J Dent Res. 2000, 79: 798-805

Jordan RC, Catzavelos GC, Barrett AW, Speight PM. Differential expression of bcl-2 and bax in squamous cell carcinomas of the oral cavity. Eur J Cancer B Oral Oncol. 1996, 32B: 394-400

Juhl M, Reibel J, Stoltze K. Immunohistochemical distribution of keratin proteins in clinically healthy human gingival epithelia. Scand J Dent Res. 1989, 97: 159-70

Kaelin WG Jr. The emerging p53 gene family. J Natl Cancer Inst. 1999, 91: 594-8

Kamer AR, Krebs L, Hoghooghi SA, Liebow C. Proliferative and apoptotic responses in cancers with special reference to oral cancer. Crit Rev Oral Biol Med. 1999, 10: 58-78

Kang MK, Bibb C, Baluda MA, Rey O, Park NH. In vitro replication and differentiation of normal human oral keratinocytes. Exp Cell Res. 2000, 258: 288-97

Kasperbauer JL, Neel HB, Scott RE Proliferation and differentiation characteristics of normal human squamous mucosal cells of the upper aerodigestive tract. Ann Otol Rhinol Laryngol. 1990, 99: 29-37

Kivela T, Uusitalo M. Structure, development and function of cytoskeletal elements in non-neuronal cells of the human eye. Prog Retin Eye Res. 1998, 17: 385-428

Kulkarni PS, Sundqvist K, Betsholtz C, Hoglund P, Wiman KG, Zhivotovsky B, Bertolero F, Liu Y, Grafström RC. Characterization of human buccal epithelial cells transfected with the simian virus 40 T-antigen gene. Carcinogenesis. 1995, 16: 2515-21

Langbein L, Rogers MA, Winter H, Praetzel S, Schweizer J. The catalog of human hair keratins II. Expression of the six type II members in the hair follicle and the combined catalog of human type I and II keratins. J Biol Chem. 2001, 276: 35123-35132

La Vecchia C, Tavani A, Franceschi S, Levi F, Corrao G, Negri E. Epidemiology and prevention of oral cancer. Oral Oncol. 1997, 33: 302-12

Leigh IM, Navsaria H, Purkis PE. Keratins (K16 and K17) as markers of keratinocyte hyperproliferation in psoriasis in vivo and in vitro. Br J Dermatol. 1995, 133: 501-11

Liu Y, Arvidson K, Atzori L, Sundqvist K, Silva B, Cotgreave I, Grafstrom RC. Development of low- and high-serum culture conditions for use of human oral fibroblasts in toxicity testing of dental materials. J Dent Res. 1991, 70: 1068-73

Liu Y, Egyhazi S, Hansson J, Bhide SV, Kulkarni PS, Grafstrom RC. O6-methylguanine- DNA methyltransferase activity in human buccal mucosal tissue and cell cultures. Complex mixtures related to habitual use of tobacco and betel quid inhibit the activity in vitro. Carcinogenesis. 1997, 18: 1889-95

Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature. 1991, 351: 453-6

Levrero M, De Laurenzi V, Costanzo A, Gong J, Wang JY, Melino G. The p53/p63/p73 family of transcription factors: overlapping and distinct functions. J Cell Sci. 2000, 113: 1661-1670

Lindberg K, Rheinwald JG. Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts. Differentiation. 1990, 45: 230-241

Loro LL, Vintermyr OK, Liavaag PG, Jonsson R, Johannessen AC. Oral squamous cell carcinoma is associated with decreased bcl-2/bax expression ratio and increased apoptosis. Hum Pathol. 1999, 30: 1097-105

Loro LL, Vintermyr OK, Ibrahim SO, Idris AM, Johannessen AC. Apoptosis and expression of Bax and Bcl-2 in snuff- and non-snuff associated oral squamous cell carcinomas. Anticancer Res. 2000, 20: 2855-60

Ludlow JW. Interactions between SV40 large-tumor antigen and the growth suppressor proteins pRB and p53. FASEB J. 1993, 10: 866-71

Maas-Szabowski N, Szabowski A, Stark HJ, Andrecht S, Kolbus A, Schorpp-Kistner M, Angel P, Fusenig NE. Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. J Invest Dermatol. 2001, 116: 816-20

Mackenzie IC, Binnie WH. Recent advances in oral mucosal research. J Oral Pathol. 1983, 12: 389-415

Mackenzie J, Ah-See K, Thakker N, Sloan P, Maran AG, Birch J, Macfarlane GJ. Increasing incidence of oral cancer amongst young persons: what is the aetiology? Oral Oncol. 2000, 36: 387-9

Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol. 1995, 146: 3-15

Maruoka Y, Harada H, Mitsuyasu T, Seta Y, Kurokawa H, Kajiyama M, Toyoshima K. Keratinocytes become terminally differentiated in a process involving programmed cell death. Biochem Biophys Res Commun. 1997, 238: 886-890

Matsuta M, Kon S, Sasaki K, Matsuta M. Immunohistochemical detection of p21WAF1/CIP1 and p53 proteins in formalin-fixed paraffin-embedded tissue sections of squamous cell carcinoma of the skin. J Dermatol Sci. 1997, 14: 233-9

Merrick DT, Blanton RA, Gown AM, McDougall JK: Altered expression of proliferation and differentiation markers in human papillomavirus 16 and 18 immortalized epithelial cells grown in organotypic culture. Am J Pathol. 1992, 140: 167-177

Metcalfe A, Streuli C. Epithelial apoptosis. Bioessays. 1997, 19: 711-20

Mitra RS, Wrone-Smith T, Simonian P, Foreman KE, Nunez G, Nickoloff BJ: Apoptosis in keratinocytes is not dependent on induction of differentiation. Lab Invest. 1997, 76: 99-107

Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell. 1982, 31: 11-24

Moore SR, Johnson NW, Pierce AM, Wilson DF. The epidemiology of mouth cancer: a review of global incidence. Oral Dis. 2000, 6: 65-74

Morgan P R, Leigh I M, Purkis P E. Site variation of keratin expression in human oral epithelia - an immunocytochemical study of individual keratins. Epithelia. 1987, 1: 31-43

Morgan PR, Economopoulou P, Leigh IM, Lane EB. 'Fast-turnover' keratin localisation in normal and neoplastic oral epithelia. J Dent Res. 1991, 70: 724-

Morgan PR, Su L. Intermediate filaments in oral neoplasia. Oral cancer and epithelial dysplasia. Eur J Cancer B Oral Oncol. 1994, 30B: 160-6

Mori H, Sugie S, Yoshimi N, Hara A, Tanaka T. Control of cell proliferation in cancer prevention. Mutat Res. 1999, 428: 291-8

Neugebauer P, Bonnekoh B, Wevers A, Michel O, Mahrle G, Krieg T, Stennert E. Human keratinocyte culture from the peritonsillar mucosa. Eur Arch Otorhinolaryngol. 1996, 253: 245-251

Nylander K, Dabelsteen E, Hall PA. The p53 molecule and its prognostic role in squamous cell carcinomas of the head and neck. J Oral Pathol Med. 2000, 9: 413-425

Oda D, Bigler L, Lee P, Blanton R. HPV immortalization of human oral epithelial cells: a model for carcinogenesis. Exp Cell Res. 1996, 226: 164-9.

Oda D, Savard CE, Eng L, Sekijima J, Haigh G, Lee SP. Reconstituted human oral and esophageal mucosa in culture. In Vitro Cell Dev Biol Anim 1998, 34: 46-52

O'Guin WM, Galvin S, Schermer A, Sun TT. Patterns of keratin expression define distinct pathways of epithelial development and differentiation. Curr Top Dev Biol. 1987, 22: 97-125

Pluquet O, Hainaut P. Genotoxic and non-genotoxic pathways of p53 induction. Cancer Lett. 2001, 1: 1-15

Polakowska RR, Haake AR. Apoptosis: the skin from a new perspective. Cell Death Differ. 1994, 1: 19-31

Polverini PJ, Nor JE. Apoptosis and predisposition to oral cancer. Crit Rev Oral Biol Med. 1999, 10: 139-52

Potten CS. Apoptosis in oral mucosa: lessons from the crypt. A commentary. Oral Dis. 2001, 7: 81-85

Poumay Y, Pittelkow MR. Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. J Invest Dermatol. 1995, 104: 271-6

Poumay Y, Herphelin F, Smits P, De Potter IY, Pittelkow MR. High-cell-density phorbol ester and retinoic acid upregulate involucrin and downregulate suprabasal keratin 10 in autocrine cultures of human epidermal keratinocytes. Mol Cell Biol Res Commum. 1999, 2: 138-144

Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity, function in health and disease. Crit Rev Oral Biol Med 2000, 11: 383-408

Presland RB, Kuechle MK, Lewis SP, Fleckman P, Dale BA. Regulated expression of human filaggrin in keratinocytes results in cytoskeletal disruption, loss of cell-cell adhesion, and cell cycle arrest. Exp Cell Res. 2001, 270:199-213

Prime SS, Nixon SV, Crane IJ, Stone A, Matthews JB, Maitland NJ, Remnant L, Powell SK, Game SM, Scully C. The behaviour of human oral squamous cell carcinoma in cell culture. J Pathol. 1990, 160: 259-69

Ralhan R, Agarwal S, Mathur M, Wasylyk B, Srivastava A. Association between polymorphism in p21(Waf1/Cip1) cyclin-dependent kinase inhibitor gene and human oral cancer. Clin Cancer Res. 2000, 6: 2440-7

Ravi D, Nalinakumari KR, Rajaram RS, Nair MK, Pillai MR. Expression of programmed cell death regulatory p53 and bcl-2 proteins in oral lesions. Cancer Lett. 1996, 105: 139-46

Ravi D, Ramadas K, Mathew BS, Nalinakumari KR, Nair MK, Pillai MR. De novo programmed cell death in oral cancer. Histopathology. 1999, 34: 241-9

Raybaud-Diogene H, Tetu B, Morency R, Fortin A, Monteil RA. p53 overexpression in head and neck squamous cell carcinoma: review of the literature. Eur J Cancer B Oral Oncol. 1996, 32B: 143-9

Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol. 1999, 17: 2941-53

Reiss M, Brash DE, Munoz-Antonia T, Simon JA, Ziegler A, Vellucci VF, Zhou ZL. Status of the p53 tumor suppressor gene in human squamous carcinoma cell lines. Oncol Res. 1992, 4: 349-57

Richard MH, Viac J, Reano A, Gaucherand M, Thivolet J. Vimentin expression in normal human keratinocytes grown in serum-free defined MCDB 153 medium. Arch Dermatol Res. 1990, 282: 512-515

Runembert I, Queffeulou G, Federici P, Vrtovsnik F, Colucci-Guyon E, Babinet C, Briand P, Trugnan G, Friedlander G, Terzi F. Vimentin affects localization and activity of sodium-glucose cotransporter SGLT1 in membrane rafts. J Cell Sci. 2002, 115(Pt 4): 713-24

Sand LP, Jalouli J, Larsson PA, Hirsch JM. Prevalence of Epstein-Barr virus in oral squamous cell carcinoma, oral lichen planus, and normal oral mucosa. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002, 93: 586-92

Sexton CJ, Proby CM, Banks L, Stables JN, Powell K, Navsaria H, Leigh IM. Characterization of factors involved in human papillomavirus type 16-mediated immortalization of oral keratinocytes. J Gen Virol. 1993, 74: 755-761

Shin KH, Tannyhill RJ, Liu X, Park NH. Oncogenic transformation of HPV-immortalized human oral keratinocytes is associated with the genetic instability of cells. Oncogene. 1996, 12: 1089-96

Schoop VM, Mirancea N, Fusenig NE. Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. J Invest Dermatol. 1999, 112: 343-53

Smack DP, Korge BP, James WD. Keratin and keratinization. J Am Acad Dermatol. 1994, 30: 85-102

Sonnenschein C, Soto AM. Somatic mutation theory of carcinogenesis: why it should be dropped and replaced. Mol Carcinog. 2000, 29: 205-211

Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. J Natl Cancer Inst Monogr. 2001, 29: 7-15

Stark HJ, Baur M, Breitkreutz D, Mirancea N, Fusenig NE. Organotypic keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. J Invest Dermatol. 1999, 112: 681-91

Su L, Morgan PR, Lane EB. Protein and mRNA expression of simple epithelial keratins in normal, dysplastic, and malignant oral epithelia. Am J Pathol. 1994, 145: 1349-57

Su L, Morgan PR, Lane EB Keratin 14 and 19 expression in normal, dysplastic and malignant oral epithelia. A study using in situ hybridization and immunohistochemistry. J Oral Pathol Med. 1996, 25: 293-301

Su TT. The regulation of cell growth and proliferation during organogenesis. In Vivo. 2000, 14: 141-8

Sugerman PB, Joseph BK, Savage NW. Review article: The role on oncogenes, tumour suppressor genes and growth factors in oral squamous cell carcinoma: a case of apoptosis versus proliferation. Oral Dis. 1995, 1: 172-188

Sundqvist K, Kulkarni P, Hybbinette SS, Bertolero F, Liu Y, Grafstrom RC. Serum-free growth and karyotype analyses of cultured normal and tumorous (SqCC/Y1) human buccal epithelial cells. Cancer Commun. 1991, 3: 331-40

Tanigaki-Obana N, Ito M. Can fibroblast growth factors substitute for bovine pituitary extracts in culture systems for hair apparatus cells? Arch Dermatol Res. 1994, 8: 484-9

Teraki Y, Shiohara T. Apoptosis and the skin. Eur J Dermatol. 1999, 9: 413-25

Tomakidi P, Fusenig NE, Koha A, Komposch G. Histomorphological and biochemical differentiation capacity in organotypic co-cultures of primary gingival cells. J Periodont Res. 1997, 32: 388-400

Tomkova H, Fujimoto W, Arata J. Expression of the bcl-2 family genes in the course of keratinocyte differentiation. Eur J Dermatol. 1999, 9: 191-196

Tsunenaga M, Kohno Y, Horii I, Yasumoto S, Huh N, Tachikawa T, Yoshiki S, Kuroki T: Growth and differentiation properties of normal and transformed human kertinocytes in organotypic culture. Jpn J Cancer Res. 1994, 85: 238-244

Vaccariello M, Javaherian A, Wang Y, Fusenig NE, Garlick JA. Cell interactions control the fate of malignant keratinocytes in an organotypic model of early neoplasia. J Invest Dermatol. 1999, 113: 384-91

Vaux DL, Strasser A. The molecular biology of apoptosis. Proc Natl Acad Sci U S A. 1996, 93: 2239-44

Vaidya M M, Sawant SS, Borge, AM, Ogale SB, Bhisey AN. Cytokeratin expression in precancerous lesions of the human oral cavity. Oral Oncol Eur J Cancer. 1998, 34B: 261-4

van der Velden LA, Manni JJ, Ramaekers FC, Kuijpers W. Expression of intermediate filament proteins in benign lesions of the oral mucosa. Eur Arch Otorhinolaryngol. 1999, 256: 514-519

Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000, 408: 307-310

Vondracek M, Xi Z, Larsson P, Baker V, Mace K, Pfeifer A, Tjalve H, Donato MT, Gomez-Lechon MJ, Grafstrom RC. Cytochrome P450 expression and related metabolism in human buccal mucosa. Carcinogenesis. 2001, 22: 481-8

Vondracek M, Weaver DA, Sarang Z, Hedberg JJ, Willey JC, Warngard L, Grafstrom RC. Transcript profiling of enzymes involved in detoxification of xenobiotics and reactive oxygen in human normal and simian virus 40 T antigen-immortalized oral keratinocytes. Int J Cancer. 2002, 99: 776-82

Wang N, Stamenovic D. Contribution of intermediate filaments to cell stiffness, stiffening, and growth. Am J Physiol Cell Physiol. 2000, 279: C188-94

Waseem A, Dogan B, Tidman N, Alam Y, Purkis P, Jackson S, Lalli A, Machesney M, Leigh IM. Keratin 15 expression in stratified epithelia: downregulation in activated keratinocytes. J Invest Dermatol. 1999, 112: 362-9

Watt FM. Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans R Soc Lond B Biol Sci. 1998, 353: 831-7

Weinberg RA. Oncogenes and tumor suppressor genes. CA Cancer J Clin. 1994, 44: 160-70

Weinberg WC, Azzoli CG, Chapman K, Levine AJ, Yuspa SH. p53-mediated transcriptional activity increases in differentiating epidermal keratinocytes in association with decreased p53 protein. Oncogene. 1995, 10: 2271-2279

Werner S, Smola H. Paracrine regulation of keratinocyte proliferation and differentiation. Trends Cell Biol. 2001, 11: 143-6

Winning TA, Townsend GC. Oral mucosal embryology and histology. Clin Dermatol. 2000, 18: 499-511

Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol. 1980, 68: 251-306

Xie X, Clausen OP, De Angelis P, Boysen M. The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue. Cancer. 1999, 86: 913-20

Yao L, Iwai M, Furuta I. Correlations of bcl-2 and p53 expression with the clinicopathological features in tongue squamous cell carcinomas. Oral Oncol. 1999, 35: 56-62

Zou CP, Hong WK, Lotan R. Expression of retinoic acid receptor beta is associated with inhibition of keratinization in human head and neck squamous carcinoma cells. Differentiation. 1999, 64: 123-32