Pathological Mechanisms in Oral Lichen Planus

A Study of Apoptosis - Regulatory Proteins and Risk Markers for Malignant Transformation

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PhD Thesis

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Research gives you ups and downs
Research makes you go around
Thinking of peculiar things

Research is finding something new
Even though you start with a clue
That may be a wrong path

Research can make you feel quite lonely
Just think that you are not the only
Person with that feeling

Researchers have to trust each other
Although if your experience is another
This can cause a lot of bother

Even so we must remain open-minded
For new collaborations

Evelyn Neppelberg
2007

CONTENTS

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2. LIST OF PAPERS

This thesis is based on the following papers, hereafter cited by their roman numerals:

I. Neppelberg E, Johannessen AC, Jonsson R. (2001) Apoptosis in oral lichen planus. Eur J Oral Sci 109: 361-4.

II. Neppelberg E, Loro LL, Øijordsbakken G, Johannessen AC. Altered CD40 and E-cadherin expression – putative role in oral lichen planus. J Oral Pathol Med, *in press*

III. Neppelberg E and Johannessen AC. DNA content and Cyclooxygenase-2 as risk markers of cancer development in oral lichen planus. In manuscript.

Approval to reproduce the papers was obtained from the publishers.

3. ABBREVIATIONS

Ab	Antibody
AEC	3-amino-9-etylcarbazole
ABC	Avidin-biotin-enzyme complex
BSA	Bovine serum albumin
Cox-2	Cyclooxygenase-2
DAB	Diaminobenzidine
DEPC	Dietylpyrocarbonate
E-cadherin	Epithelial-cadherin
EDTA	Ethylene-diamine-tetraacetic-acid
FasL	Fas ligand
FasR	Fas receptor
ISH	*In situ* hybridization
LP	Lichen planus
NF-κB	Nuclear factor kappa B
OLP	Oral lichen planus
OM	Oral mucosa
OSCC	Oral squamous cell carcinoma
PBS	Phosphate-buffered saline
mAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
pAb	Polyclonal antibody
TBS	Tris-buffered saline
TNF	Tumor necrosis factor
TNFR-1	Tumor necrosis factor receptor-1
TRAF	TNF receptor associated factor
TUNEL	Terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridine-5´- triphosphate (dUTP)-biotin nick end-labelling

4. ENGLISH SUMMARY

Oral lichen planus (OLP) is a chronic mucocutaneous disease characterized by basal cell destruction and a subepithelial band-like mononuclear inflammatory cell infiltrate predominated by T cells. Molecular biological changes in the basal cell compartment and keratinocyte cell death have been a matter of particular interest in later OLP research. The majority of OLP lesions run a benign course. However, OLP have been associated with an increased risk of malignant transformation.

The aims of this study were to investigate apoptotic cell death and putative regulatory proteins in keratinocytes (Papers I-III), as well as potential risk markers for malignant transformation in OLP (Paper III).

Biopsies from clinically and histologically verified OLP were investigated (Papers I-II), as well as a biopsy material from OLP patients where a certain amount of them had developed epithelial dysplasia and oral squamous cell carcinoma (OSCC) (Paper III).

Tissues were evaluated by histomorphometry, imunnohistochemistry (Papers I-III), TUNEL method (Paper I), mRNA *in situ* hybridization (Paper II) and image cytometry for measurement of DNA content (Paper III).

Our data show that apoptosis is increased within the epithelium of OLP compared with normal oral mucosa (OM), and that both Fas receptor (FasR) and Fas-ligand (FasL) apoptosis regulatory proteins are expressed within the epithelium and subepithelial cell infiltrate of OLP (Paper I). In actively diseased OLP lesions, basal keratinocytes are CD40 negative and epithelial (E)-cadherin negative in focal areas (Papers II-III). Cyclooxygenase-2 (Cox-2) is up-regulated within the epithelium of OLP lesions, compared with normal OM (Paper III). According to DNA content measurements, all biopsies were classified as diploid including OLP, epithelial dysplasia and OSCC, except for one biopsy from an OLP lesion with epithelial dysplasia which was tetraploid (Paper III).

Based on these findings we conclude that basal keratinocytes in OLP die by apoptosis and regulation of apoptosis appears to involve following mechanisms; (1) dysfunction in FasR/FasL system; (2) by down-regulating CD40 in diseased areas, basal keratinocytes may escape CD40-CD40L mediated apoptosis; (3) an up-regulation of Cox-2 , which may inhibit apoptosis; (4) loss of E-cadherin in basal keratinocytes may promote apoptosis and contribute to reduced basal cell structural integrity in OLP, allowing T cells to enter the epithelial compartment. Our biopsy material indicates that neither DNA content, nor expression of Cox-

2 and E-cadherin are reliable as prognostic markers to select the OLP patients at risk for development of OSCC.

Key words: oral lichen planus, apoptosis**,** FasR, FasL, CD40, CD40L, E-cadherin, Cox-2, DNA content, epithelial dysplasia, oral squamous cell carcinoma.

5. NORSK SAMMENDRAG (Norwegian summary)

Oral lichen planus (OLP) er en kronisk slimhinnelidelse karakterisert av destruksjon av basalcellelaget og båndformet betennelsesinfiltrat under epitelet med hovedsakelig T-lymfocytter. Molekylærbiologiske forandringer i basalcelleområdet og celledød er spesielt aktuelle felt innen nyere forskning på OLP. De aller fleste OLP tilfellene forblir godartete, men i sjeldne tilfeller kan munnhulekreft utvikles.

Hovedmålene for studien var å undersøke celledød i form av apoptose og proteiner som kan regulere den, samt mulige markører for utvikling av kreft i lesjonene.

Vevsprøver fra klinisk og histologisk verifiserte OLP lesjoner ble undersøkt (Artikkel I-II), og i tillegg et arkivmateriale med vevsprøver fra OLP pasienter hvor noen av pasientene utviklet celleforandringer og kreft over tid (Artikkel III).

Vevet ble undersøkt ved mikroskopi, immunhistokjemi (Artikkel I-III), TUNEL-metoden (Artikkel I), mRNA *in situ* hybridisering (Artikkel II) og bilde cytometri for måling av DNA innhold.

Studien viste økt forekomst av apoptose i epitelet ved OLP sammenlignet med normal munnslimhinne, og at proteinene Fas receptor (FasR) og Fas ligand (FasL) som kan starte apoptose, var tilstede i epitelet og betennelsesinfiltratet (Artikkel I). De basale epitelcellene var negative for CD40 i aktive sykdomsområder og tapte også epitelialt (E)-cadherin noen steder (Artikkel II). Cyclooxygenase-2 (Cox-2) var økt i OLP manifestasjoner sammenlignet med normal munnslimhinne (Artikkel III). Normalt DNA innhold (diploid) ble funnet i alle vevsprøvene fra OLP og munnhulekreft, men en av vevsprøvene fra OLP med celleforandring viste økt og balansert DNA innhold (tetraploid) (Artikkel III).

Basert på disse funn konkluderes at de basale epitelcellene dør ved apoptose i OLP og synes å involvere mange mekanismer: (1) dysfunksjon i Fas/FasL systemet, (2) ved å regulere ned CD40 kan de basale epitelcellene unngå CD40 mediert apoptose, (3) økt Cox-2 kan hemme apoptose, (4) tap av E-cadherin i de basale epitelcellene kan gi dårligere integritet, øke apoptose, og dermed bidra til at T-lymfocytter vandrer inn i epitelet. Vevsprøvematerialet (Artikkel III) antyder at verken DNA innhold, påvisning av Cox-2 eller tap av E-cadherin er gode nok markører for å velge ut OLP pasienter med økt risiko for munnhulekreft.

6. INTRODUCTION

6.1. Oral lichen planus

Lichen planus (LP) is a chronic inflammatory mucocutaneous disorder. *Lichen* comes from the Greek word leichen, meaning flat, and possibly the striking clinical colour of the pimples on skin led to the designation leichen ruber (latin; red). *Planus* refers to the clinical appearance of the skin papulae; flattened, smooth and depressed on the summit, as first described by Wilson in 1869 (Wilson, 1869). Although first described almost 150 years ago, and more than 5200 papers were present in the database PubMed in January 2007, many aspects of the pathogenesis of LP are yet not fully understood.

The prevalence of oral LP (OLP) varies from 0.02-2.2% in adults according to studies of different ethnic populations (Pindborg *et al.*, 1972), (Bouquot and Gorlin, 1986), (Axell and Rundquist, 1987), (Ikeda *et al.*, 1995), (Reichart, 2000). Clinically, six types of OLP have been described: reticular, papular, plaque, atrophic, erosive or ulcerative and bullous, but the lesions are most often seen as white, erythematous and/or ulcers. The white reticular appearance is most common, and more than one type of oral lesion may occur at the same time (Fig. 1) (Andreasen, 1968).

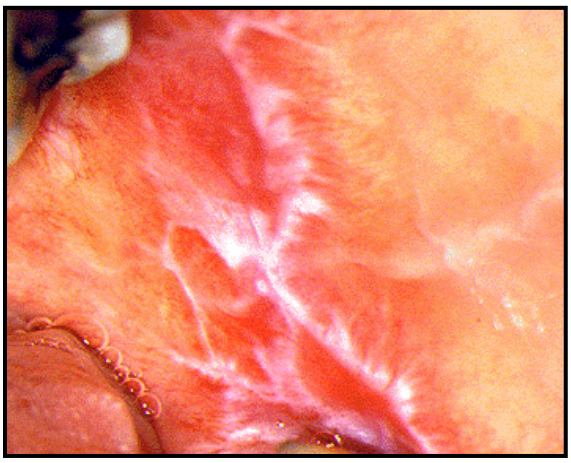

Figure1. Oral lichen planus lesion with reticular and erythematous manifestations.

The two most important histological features of OLP are the subepithelial band-like inflammatory cell infiltrate predominated by lymphocytes, and destruction of the epithelial basal cell layer (Fig. 2 A-C).

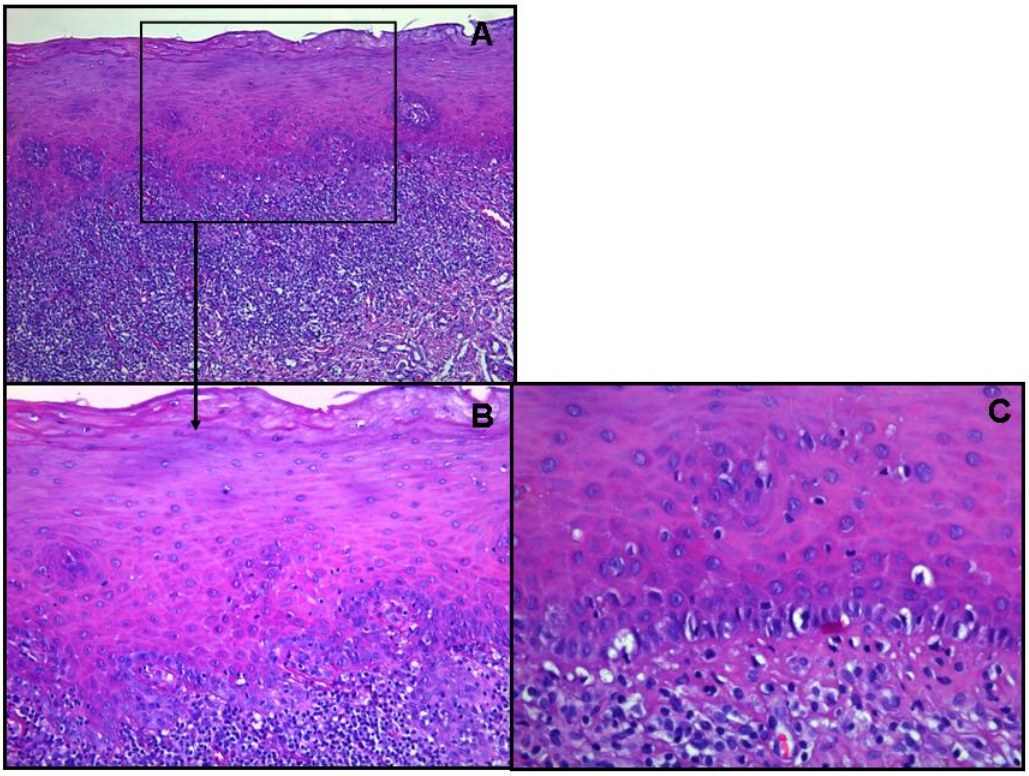

Figure 2. Oral lichen planus with subepithelial chronic inflammatory cell infiltrate (A), destruction of the basal cell layer (B) and cell death of basal keratinocytes (C). Hematoxylin & Eosin. Orig. magn: x100 (A), x200 (B), x400 (C).

Lichenoid phenotypes – oral lichenoid lesions

Oral lichenoid lesions may resemble idiopathic OLP both clinically and histopathologically. The oral lichenoid lesions may however have a defined or suspected cause in the patient history or clinical relation. The list of drugs that may cause drug-related lichenoid reactions in oral mucosa and skin is long (Thompson and Skaehill, 1994), (McCartan and McCreary, 1997), (Edwards and Kelsch, 2002). Lichenoid contact hypersensitivity lesions describe local lesions developed in the oral mucosal membrane with direct clinical contact with a dental restoration (Lind *et al.*, 1984), (Thornhill *et al.*, 2003). Also, Hepatitis C and graft-versus-host reactions may present with oral lichenoid reactions (Lodi *et al.*, 2005b), (Vargas-Diez *et al.*, 2005).

Etiology and pathogenesis

OLP is a T cell mediated inflammatory disease (Regezi *et al.*, 1978), (Gilhar *et al.*, 1989), (Porter *et al.*, 1997), (Sugerman *et al*., 2002). The antigen or antigens triggering OLP have not yet been identified, and there is no generally accepted pathogenesis theory.

Terminal differentiation and cell loss at the epithelial surface in oral mucosa (OM) requires to be balanced by basal cell proliferation (reviewed in Squier and Kremer, 2001). This homeostatic balance is disturbed in OLP, resulting in regions of epithelial acanthosis (increased thickness), atrophy (reduced thickness) or complete loss of epithelium (ulceration). Langerhans cells ($CD1^+$) play an important role in processing and presentation of antigens, and increased number of Langerhans cells have been described in OLP compared to oral leukoplakia and normal OM (Ragaz and Ackerman, 1981), (Rich and Reade, 1989), (Villarroel Dorrego *et al.*, 2002). T cells ($CD3^+$, Fig. 3A) including T helper cells ($CD4^+$, Fig. 3B) and suppressor/cytotoxic T cells ($CD8^+$, Fig. 3C) are present intraepithelially in OLP (Matthews *et al.*, 1984), (Kilpi, 1987), (Zhou *et al.*, 2002).

Basal keratinocytes appear to be the primary site of immunological injury in OLP, and molecular biological changes in the basal cell compartment have been a matter of particular interest in later research in OLP (reviewed in Sugerman *et al.*, 2002). Of current interest is the presence of cytotoxic T cells in OLP with the potential of targeting basal keratinocytes (Khan *et al.*, 2003), (Santoro *et al.*, 2004), based on that T cell lines cultured from LP skin lesions have proven to lyse autologous lesional keratinocytes *in vitro* (Sugerman *et al.*, 2000).

The basement membrane, delineating the border between the epithelium and connective tissue is a highly specialised structure that forms a dense meshwork built up by collagen type IV, laminin, heparin sulphate, entactin and fibronectin (Merker, 1994). The basal keratinocytes are responsible for constant renewal of several basement membrane components including laminin and collagen IV. In OLP, a variation in the intensity and thickness of collagen IV and laminin-5 expression is described, with breaks in some regions (Jungell, 1990), (Ramirez-Amador *et al.*, 1996), (Zhou *et al.*, 2001).

The subepithelial cell infiltrate consists mainly of T cells ($CD3^+$, Fig. 3A) including T helper cells ($CD4^+$, Fig. 3B) and suppressor/cytotoxic T cells ($CD8^+$, Fig. 3C). The CD4:CD8 ratio in the subepitelial infiltrate varies between 2:1 and 3:1 (Matthews *et al.*, 1984), (Ishii, 1987),

(Jungell *et al.*, 1989), (Sugerman *et al.*, 1993). During disease progression there may be a gradually increase of $CD8^+$ cells demonstrating an antigen-stimulated T cell population (De Panfilis *et al.*, 1983), (Scully and el-Kom, 1985).

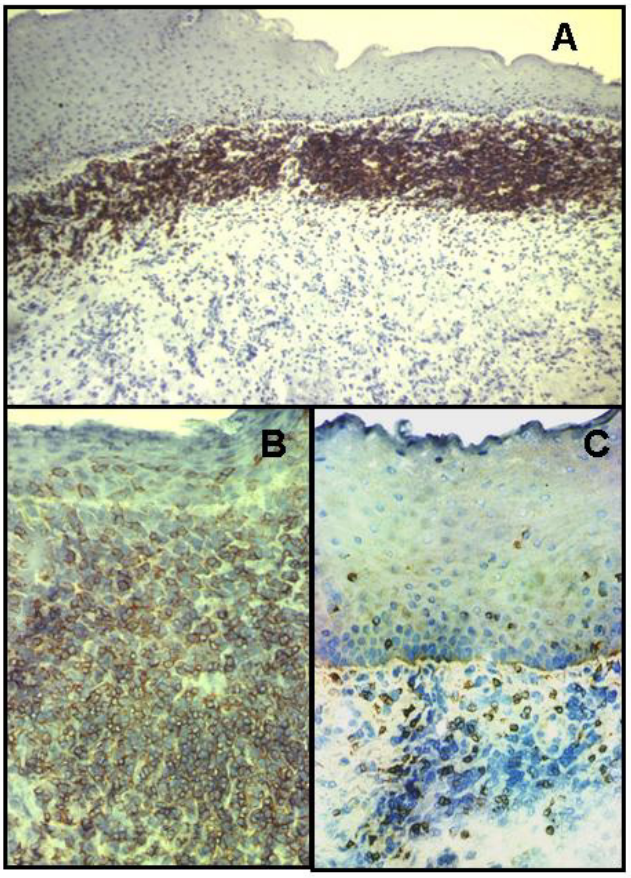

Figure 3. CD3+ (A), CD4+ (B) and CD8+ cells (C) in the epithelium and subepithelial cell infiltrate of oral lichen planus. Immunohistochemistry. Orig. magn: x100 (A), x250 (B, C). (B, C are also presented in Paper I: Fig. 1A, B).

Recruitment and retention of lymphocytes in OLP are mediated by release of cytokines from activated keratinocytes, T cells and endothelial cells (Fig. 4) (Yamamoto and Osaki, 1995), (Simark-Mattsson *et al.*, 1999), (Khan *et al.*, 2003).

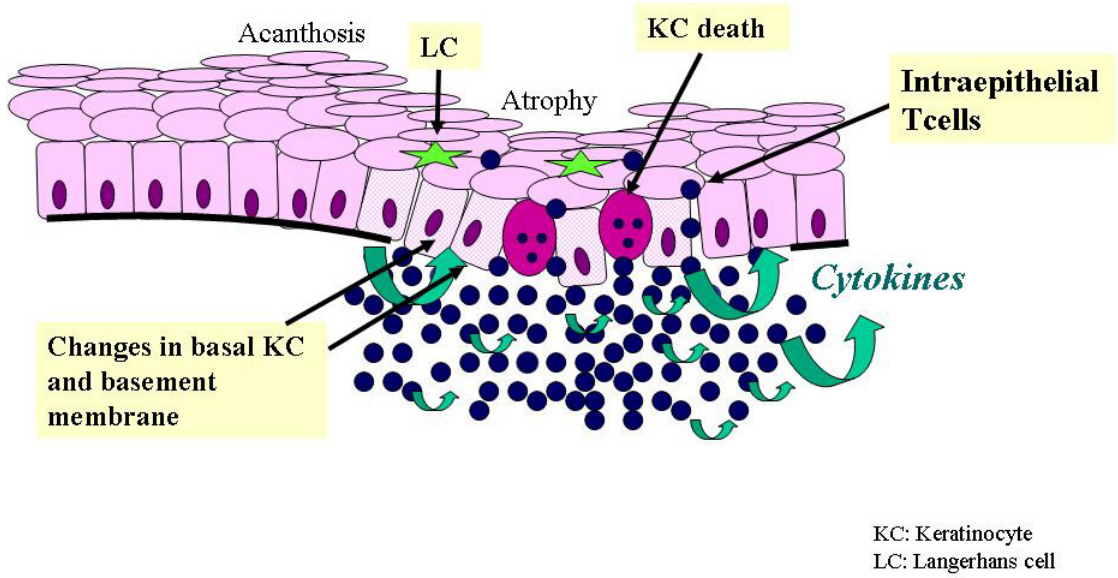

Figure 4. Pathogenetic aspects of oral lichen planus

6.2. Apoptosis

Apoptosis, from the Greek meaning falling (as in the falling of leaves from trees) was first described in 1971 (Kerr, 1971). Kerr and co-workers further described the morphology of apoptosis and its biological implications in tissue kinetics (Kerr *et al.*, 1972). Apoptosis is often described scientifically as programmed cell death, which refers to a series of morphological changes during cell death that are different from necrosis (Wyllie *et al.*, 1980). In contrast to necrosis, apoptosis is an active process of cellular self-destruction where single cells are usually affected within living tissues and with no provocation of inflammation (Cohen, 1993).

The apoptotic process and its regulation

The process of apoptosis may be summarised into four phases (Vaux and Strasser, 1996); (1) the initiation stimulus that may be internal or external, (2) the detection and signal transduction phase, (3) the effector phase that includes the caspases, and (4) the degradation of DNA phase. The initiation phase may be induced by a variety of signals including DNA damage, radiation, toxins, hypoxia, lack of nutrients and activation of death receptors. A central component of the apoptotic machinery is the caspase family of proteases, responsible for proteolytic cleavages (effectors) and upstream regulation (initiators) (Thornberry and Lazebnik, 1998). Several factors are involved in the regulation of apoptosis including the tumour necrosis factor (TNF) receptor family, tumor suppressor genes, oncogenes, mitochondrial factors and a number of enzymes (Dragovich *et al.*, 1998).

Fas and CD40 in regulation of apoptosis

Fas receptor (FasR, CD95, Apo-1) is a member of the tumor necrosis factor (TNF) family of proteins, expressed on the cell surface of rapidly proliferating cells including activated T and B cells (Nagata, 1994). Its ligand, FasL (CD95L, Apo-1L) is a cell surface molecule expressed predominantly by activated T cells (Nagata, 1999). FasL binds to FasR leading to a transduction signal that finally induces apoptosis via a caspase dependent pathway (Nagata, 1997), (Peter and Krammer, 1998), (Nagata, 1999). FasR and FasL are expressed in normal OM, where FasR has been reported to be restricted to the basal cell compartment (Leithauser *et al.*, 1993), (Loro *et al.*, 1999). In OLP, two immunohistochemical studies on FasR and FasL have shown different results, from no subjective difference to altered staining pattern

with more intense expression in epithelial spinous and surface layers, compared to normal OM (Dekker *et al.*, 1997), (Muraki *et al.*, 1997).

CD40 is another TNF family member that is constitutively expressed on cells with high proliferative potential such as keratinocytes and antigen presenting cells including T cells, Langerhans cells and macrophages (reviewed in Schönbeck and Libby, 2001, and Xu and Song, 2004). Its ligand, CD40L (CD154/ gp39) is transiently expressed on many cell types including mature $CD4^+$ and $CD8^+$ cells (Roy *et al.*, 1993), (reviewed in Xu and Song, 2004). CD40 and CD40L have shown to be involved in several biological processes such as regulation of normal squamous epithelial cell growth, cell survival, differentiation and inflammatory cell responses (Denfeld *et al.*, 1996), (Peguet-Navarro *et al.*, 1997), (Young *et al.*, 1998), (Schönbeck and Libby, 2001). CD40-CD40L interactions may either induce (via Fas and/or tumour necrosis factor receptor -1: TNFR-1) or inhibit apoptosis (via nuclear factor κappa B: NF-κB) in different cancer cell lines (Grell *et al.*, 1999), (Eliopoulos *et al.*, 2000), (Xu and Song, 2004). CD40 lacks intrinsic catalytic activity itself, so adapter proteins of the TNF-associated factor (TRAF) family mediate activation of the CD40 signalling cascades, converting the CD40-initiated signal into different functional outcomes (Chung *et al.*, 2002), (Harnett, 2004), (Xu and Song, 2004). Normal basal keratinocytes in OM express CD40, as well as CD40L in the basement membrane region (Peguet-Navarro *et al.*, 1997), (Loro *et al.*, 2001), (Villarroel Dorrego *et al.*, 2006). However, yet its specific biological role(s) have not been elucidated.

Cell death of basal keratinocytes in OLP

Many years ago, an apoptotic mechanism for keratinocyte cell death in OLP was proposed (Hashimoto, 1976), (Sumegi, 1979), (Weedon, 1980). Based on electron microscopic studies, the colloid or Civatte bodies were described to be typical of apoptotic cells (Kerr *et al.*, 1972), (Weedon, 1974).

In OLP, cytotoxic $CD8^+$ T cells are suspected to be involved in keratinocyte cell death. However the mechanisms by which the cytotoxic T cells interact with keratinocytes have not yet been clarified. T cells may induce apoptosis in keratinocytes via several different mechanisms including; (1) FasL on T cell surface binding FasR on keratinocyte cell membrane, (2) T cell secreted TNF-α binding TNFR-1 on keratinocyte cell surface, (3) T cell surface CD40L binding CD40 on keratinocytes (followed by apotosis via Fas and/or TNFR-

1), (4) T cell secreted granzyme-B entering the keratinocyte through membrane perforin induced pores (Fig. 5).

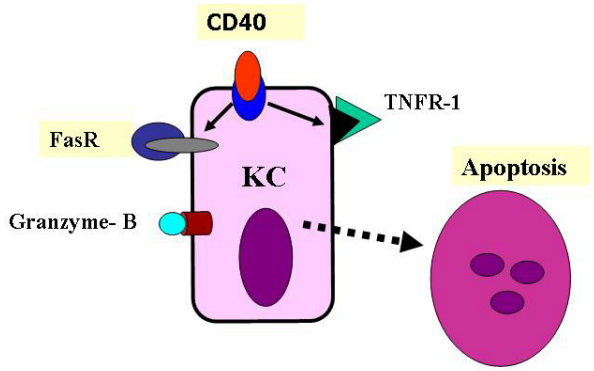

Figure 5. Possible mechanisms of keratinocyte apoptosis in oral lichen planus mediated by T cells

In OLP, apoptosis of keratinocytes may also be induced by other mechanisms than those initiated by T cells, as for instance loss of cell-to-cell or cell-to-matrix contacts (Fig. 6). This type of apoptosis, termed anoikis (loss of home) may be induced in the absence of focal cell contact (reviewed in Grossmann, 2002).

Epithelial (E)-cadherin, belonging to the cadherin family (classical type I) of proteins, is one type of cell-to-cell adhesion protein expressed in normal stratified squamous epithelium including normal OM (Downer and Speight, 1993), (Jensen *et al.*, 1997), (Thomas and Speight, 2001), (reviewed in Gooding *et al.*, 2004). Maintained cell contacts by E-cadherin have been shown to prevent apoptotic cell death in mouse keratinocyte cell lines by inhibiting caspase-3 (a major effector of apoptosis) (Galaz *et al.*, 2005).

CD44 is a transmembrane adhesion molecule belonging to a family with multiple isoforms that take part in different biological functions like cell-to-cell and cell-to-matrix interactions in stratified squamous epithelium (Thomas and Speight, 2001). The role of CD44 in apoptosis is not clear-cut. On the one hand, CD44 has been shown to inhibit apoptosis in human colonic cancer cell lines (Lakshman *et al.*, 2004) and normal mouse colonic epithelium (Lakshman *et al.*, 2005). On the other hand, it promotes apoptosis in human synovial cell lines from patients with rheumatoid arthritis (Fujii *et al.*, 2001). Normal OM keratinocytes express CD44 (Oliveira and Odell, 1997), and two immunohistochemical studies indicate that also keratinocytes in skin LP lesions express CD44 (Harris *et al.*, 1997), (Orteu *et al.*, 1997).

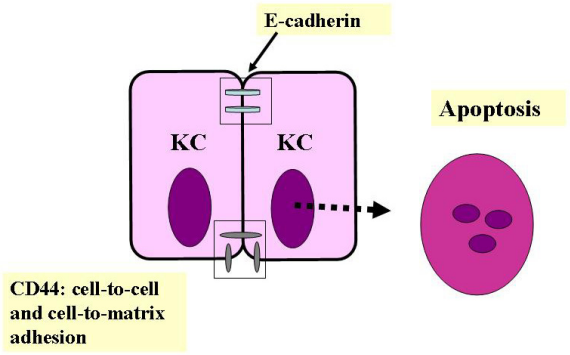

Figure 6. Loss of cell contacts may induce apoptosis in oral lichen planus.

To our knowledge, no reports regarding the expression of CD40, CD40L, E-cadherin or CD44 in OLP have been published in English literature.

Hypothesis

Basal keratinocytes die by an apoptotic mechanism, and a dysregulation of apoptosis and regulatory molecules is involved in basal cell destruction in OLP.

6.3. Malignant potential of OLP lesions and potential risk markers

Although OLP is defined as a precancerous condition by the World Health Organization (Pindborg *et al.*, 1997), the malignant potential of OLP lesions itself is still controversial and debated among researchers (Krutchkoff and Eisenberg, 1985), (Eisenberg, 2000), (Lozada-Nur, 2000), (Lodi *et al.*, 2005a). The potential risk for cancer development in these patients is estimated between 0.4-6.25% (van der Meij *et al.*, 1999), (Gandolfo *et al.*, 2004), (Lodi *et al.*, 2005a). In all lesions considered to be premalignant there is a great need for objective markers to select patients at risk, but so far no such definite markers are available. Candidate prognostic markers for risk of cancer development may include genetic and molecular biological changes arising during carcinogenesis.

Nuclear DNA content – DNA ploidy

Aneuplody is defined as an unbalanced representation of chromosomes or "loss or duplication of chromosomes or chromosomal segments" (Lewin, 1997). The normal situations of euploidy denote the situations with whole integer multiples of chromosomes in the nucleus like haploid (one), diploid (two), triploid (three), or tetraploid (four). Aneuploidy is claimed to be the primary cause of multilateral genomic instability of neoplastic and preneoplastic cells (Duesberg *et al.*, 2004), and is a frequent change observed in epithelial cancers and pre-cancers (Rasnick and Duesberg, 1999). However, it is not yet clarified whether aneuploidy alone is a suitable prognostic factor in oral epithelial premalignant lesions and oral squamous cell carcinoma (OSCC) (Franzen *et al.*, 1987), (Baretton *et al.*, 1995), (Reibel, 2003), (Diwakar *et al.*, 2005). Previous reports of measurements of DNA content in OLP biopsies and cytologic samples have presented highly variable results from none to 41% of the lesions classified as being aneuploid (Biesterfeld *et al.*, 1991), (Mattila *et al.*, 2004), (Femiano and Scully, 2005), (Maraki *et al.*, 2006), (Rode *et al.*, 2006).

P53- a tumour suppressor

The p53 protein is involved in the regulation of the cell cycle, and coded for by the *TP53* tumour suppressor gene. Activation of p53 protein may either; (1) induce cell cycle arrest to allow repair of damaged DNA, or (2) induce apoptosis via several pathways including Fas and/or inhibition of Cyclooxygenase-2 (Cox-2) (reviewed in Bertram, 2000). Mutations in *TP53* are a frequent genetic change in OSCC as well as in other human cancers (reviewed in Nylander *et al.*, 2000). Mutant p53 protein has a prolonged half-life, and may be detectable

due to accumulation in cells, in contrast to the normal protein. Previous immunohistochemical reports of p53 expression in OLP have been inconclusive, from not detectable to expression of p53 detected in 91% of the lesions (Crosthwaite *et al.*, 1996), (Taniguchi *et al.*, 2002), (Lee *et al.*, 2005), (Acay *et al.*, 2006).

Cyclooxygenase-2

Cyclooxygenase-2 (Cox-2), which is an inducible enzyme in most cell types including keratinocytes, fibroblasts and T cells, catalyzes the synthesis of prostaglandins (reviewed in Smith *et al.*, 2000), (Kainulainen *et al.*, 2002). Several processes in cancer development may be influenced by Cox-2, such as cell proliferation, apoptosis, and angiogensis (reviewed in Prescott and Fitzpatrick, 2000). Cox-2 may inhibit apoptosis via different pathways, like down-regulation of arachidonic acid, up-regulation of the protooncogene Bcl-2 and down-regulation of Bax, thus contributing to increased cell survival (Tsujii and DuBois, 1995), (Prescott and Fitzpatrick, 2000), (Lin *et al.*, 2002). In normal OM, Cox-2 is generally not expressed, but a variable expression has been reported in oral epithelial dysplasias and OSCC (Shibata *et al.*, 2005). To our knowledge, there have not been any reports in English literature on Cox-2 expression in OLP.

E-cadherin

E-cadherin has been proposed as one prognostic factor in development of OSCC, based on the fact that loss of E-cadherin mediated cell contact is associated with invasion and metastasis in OSCC (Yamada *et al.*, 1997), (Diniz-Freitas *et al.*, 2006).

Hypothesis

Aneuploidy, p53, Cox-2 and E-cadherin expression are either alone or together, prognostic markers for the risk of cancer development in OLP. In addition, p53, E-cadherin and Cox-2 are involved in the regulation of keratinocyte apoptosis in OLP.

7. AIMS

The general aims of this study were to investigate for apoptosis and apoptosis regulatory proteins in OLP, and to identify potential predictive risk markers for cancer development in OLP.

Specific aims:

- To quantify the rate of apoptosis in the epithelium and subepithelial cell infiltrate, and localize apoptotic cells within the epithelium in OLP (Paper I).
- To characterize the expression of two well known apoptotic regulatory systems; FasR and FasL (Paper I), CD40 and CD40L (Paper II).
- To characterize the expression of E-cadherin, important in secured structural integrity of basal keratinocytes and epithelium of OLP (Papers II, III).
- To characterize the expression of Cox-2 in OLP, since this enzyme may be involved in regulation of apoptosis (Paper III).
- To search for potential predictive risk markers for cancer development in OLP; thus DNA-content, expression of Cox-2, and E-cadherin was investigated in OLP lesions, epithelial dysplasias and OSCC developed in OLP patients (Paper III).

8. MATERIALS - Tissue specimens

8.1. Tissues in OLP series

Biopsies from 27 admitted patients (8 men and 19 women), clinically and histologically diagnosed with OLP, mean age 59 yrs (range 30-79), were included (Papers I, II). Clinically, all OLP patients had the reticular type of lesions, of whom 14 also had erythematous lesions. Another 2 patients had ulcerous lesions. All patients presented with bilateral buccal lesions, 11 had gingival lesions, 6 had tongue lesions and 5 had lip lesions in addition. Cutaneous LP was reported in the history of 13 patients. Biopsies were taken from reticular lesions of the buccal mucosa, not associated with dental restorations. None of the patients had been treated with topic or systemic steroids. Six patients used systemic medications including four patients on platelet aggregation inhibitors (acetyl-salisylic-acid: Albyl-E), and two patients on a serum cholesterol reducer (Lovastatin: Mevacor).

The biopsy specimens were cut into two halves: one was snap frozen in isopentane, precooled in liquid nitrogen and stored at -70^0C, the other one was fixed in 4% buffered formalin (pH 7.2) and embedded in paraffin. Normal OM samples from healthy voluntary individuals (n=8), human tonsils (n=8) and OSCC (n=5) served as control specimens.

The regional committee for medical ethics in research approved the project (code: 146/96-45.96), and all patients included in this study gave their informed consent.

8.2. Biopsies from OLP, epithelial dysplasias and OSCC developed in OLP patients

Archival biopsies (n=78) from 45 patients with clinically and histologically verified OLP were included (Paper III). The patients' biopsies were divided into two groups. Group 1: biopsies from 26 patients (18 females and 8 males, mean age 55 yrs, and range: 31-71) who had been followed up for 6-225 (mean: 91) months and had more than one biopsy taken from their lesions. Six of these patients (6/26, 23 %) developed epithelial dysplasias in their lesions, of which five (5/26, 19 %) progressed to OSCC. Group 2: biopsies from 19 patients from primary lesions taken for diagnostic purpose, and selected due to typical histologic picture of OLP (11 females and 8 males, mean age 64 yrs, and range: 36-83). The biopsies were taken (1978-2003) at the Department of Oral Surgery and Oral Medicine, Faculty of Dentistry,

University of Bergen, and the Department of Maxillofacial Surgery, Haukeland University Hospital, Bergen, Norway.

Normal buccal OM (n=7) from voluntary healthy individuals were included as controls.

9. METHODS AND METHODOLOGICAL CONSIDERATIONS

9.1. Immunohistochemical methods

Immunohistochemistry was performed to characterize the expression of different proteins in OLP (Table 1). Both cryostat and formalin fixed, paraffin embedded tissue sections were used in the series. Two different immunohistochemical methods were performed; (1) the DAKO avidin-biotin-peroxidase (ABC) complex technique (Hsu *et al.*, 1981) (Papers I-II) and (2) the EnVision$^+$ System (EnVision$^+$ System kit, according to the manufacturers protocols, DAKO) (Papers II-III). The main difference between these methods is that the EnVision$^+$ System utilizes labelled polymers (either peroxidase or alkaline phosphatase) for detection of the epitopes instead of avidin and biotin (Jordan *et al.*, 2002). The EnVision$^+$ System may be more sensitive compared with the ABC method due to the labelled polymer that includes several secondary Ab (Jordan *et al.*, 2002). Another advantage of the EnVision$^+$ System is that it is a less time consuming procedure with fewer steps compared to the DAKO ABC method. In addition, a higher dilution of primary Ab can often be used with the EnVision$^+$ System due to utilization of the labelled polymer. However, since the EnVision$^+$ kit is expensive, the cost-benefit may vary according to different Ab. We experienced that the monoclonal Ab (mAb) CD40 (Calbiochem) was titrated to a dilution of 1:80 with the traditional DAKO ABC method, and 1:1000 with the EnVision$^+$ System. Avoiding biotin with the EnVision$^+$ System may also be an advantage due to problems with background staining from endogenous biotin.

The DAKO autostainer – Universal Staining System (DAKO-USA, Caripteria, California USA) was used for the immunohistochemical procedures after antigen retrieval in the later series (Papers II-III). The autostainer gave similar immunohistochemical results compared to running the procedures manually, with the main advantage of a more standardized and less time consuming procedure. A disadvantage with the autostainer is that more reagents including Ab is required compared to running the procedures manually, thus the cost-benefit may be lesser with expensive Ab.

Immunohistochemical double labelling

Immunohistochemical double labelling was performed with CD40 and CD3 (T cell marker), in order to further characterize the CD40 expressing cells detected in OLP (Paper II). Titrations of both Ab were performed separately, and together (one Ab followed by the other) in two steps. In order to avoid cross reaction, one mAb and one polyclonal Ab (pAb) were chosen (DAKO, double staining kit protocol). Best results were obtained using a two steps procedure with the mAb CD40 1:1000 (Calbiochem) initially, followed by the pAb CD3 1:50 (Novocastra). In general, it is recommended to start with the mAb against the epitope that is most abundantly expressed in the tissue, followed by the pAb against the second epitope (S. Nielsen, DAKO Corp). Retrieval of the polymer by blocking solution (EnVision^{+} Doublestain kit, DAKO) is recommended between the two Ab, especially if both Ab are monoclonals. We obtained best results with 4% formalin fixation for 2 min, followed by boiling in TRIS/ETDA (pH: 9) for 1 min.

As positive controls, parallel cut sections were incubated with the two Ab separately in all series.

Table 1. Primary antibodies used in the immunohistochemical series.

Target	Immune expression	Antibody, Clone	Dilution	Source	Paper
CD3	T cells	mAb: NA, Rabbit pAb	1/20, 1/50	Vector, Novocastra	I, II
CD4	Mainly helper T cells, MHC class II	mAb: NA	1/20	Vector	I
CD8	Mainly cytotoxic T cells, MHC class I	mAb: NA	1/20	Vector	I
FasR	Activated T and B cells, rapid proliferating cells	UB2	1/100	Medical & Biological Laboratories	I
FasL	Mainly activated T cells	DX2	1/5	Calbiochem	I
CD40	A vide variety of cells; as basal keratinocytes, T cells, Langerhans cells	EA-5, HB-14, rabbit pAb	1/80, 1/100, 1/1000	Calbiochem, Santa Crruz	II
CD40L	Activated T cells, basement membrane region in oral mucosa	24-31, rabbit polyclonal	1/100	Calbiochem, Santa Cruz	II
Laminin-5	Basement membrane	D4B5	1/100	CEMICON	II
Collagen IV	Basement membrane	CIV-22	1/5	DAKO	II
E-cadherin	Keratinocytes, macrophages, salivary duct cells	HECD-1	1/25, 1/1500	R&D	II, III
CD44	A vide variety of cells as keratinocytes, fibroblasts, mature T cells	DF1485	1/1500	DAKO	II
Ki-67	Epithelial cells, marker of cells in interphase - nuclear antigen	MIB-1, rabbit mAb SP6	1/25, 1/100	DAKO, NeoMarkers	NP
Cox-2	In most tissues and cell types (inducible enzyme); T cells, macrophages, keratinocytes	COX 229, SP21	1/1500	Zymed, Neo Markers	III
P53	Different cell types; as keratinocytes, T cells, indicating a mutant p53 protein.	DO-7	1/100	DAKO	NP

mAb: monoclonal antibody
pAb: polyclonal antibody
NA: not avaliable information of clone
NP: not presented in the papers included in this thesis

Evaluation of the OLP tissue sections – immunohistochemistry (Papers I-III)

Tissue sections were evaluated morphologically under a light microscope (Leica DMLM, GmbH, Munster, Germany). Special attention was given to evaluate the epithelium, basal keratinocytes and subepithelial inflammation in diseased areas compared to non-diseased areas of OLP. Sections were compared with normal OM controls. Cell counts were performed manually in the epithelial basal cells and subepithelial cell infiltrate in OLP for FasR, FasL, CD3, CD4, CD8 (Paper I), CD40 and CD40L (Paper II) under a light microscope (Leica GMBH) with a fitted ocular grid. Up to 1000 cells were counted for each slide at x 400 magnification. Results are presented in percent positive cells of total (number of positive cells/ total cells).

For Ki-67 a semi-quantitative registration was performed, dividing the lesions into three groups according to expression; (+): few occasional cells (<10%) in the basal cell region, (++): 10-50% positive cells in the basal cell region, and (+++): >50% positive cells in the basal cell region. Positive cells in parabasal cell layers were also noted.

Semi-quantitative registration of Cox-2 expression in the epithelium of OLP was performed (Paper III), and the lesions were divided into three groups according to expression; (1) in the basal cells only with a perinuclear localization, (2) in both basal and parabasal cell layers with perinuclear and cytoplasmic localization, or (3) in focal areas with both perinuclear and cytoplasmic localization of expression also including more superficial cell layers. In addition, cell counts were performed for Cox-2 in the subepithelial cell infiltrate for each slide at x 400 magnification and presented as percent positive cells of total.

For p53 the lesions were evaluated as positive or negative, and if less than 5% of the cells expressed p53, the lesion was considered as negative.

9.2. TUNEL method

For visualization of DNA fragmentation, the Tdt-mediated dUTP-biotin nick end labeling (TUNEL) method was performed (Paper I) (Gavrieli *et al.*, 1992), (Loro *et al.*, 2000). DNA fragmentation occurs during other processes than apoptosis such as autolysis and necrosis, as well as during pre-treatment of tissue sections and processing (Stadelmann and Lassmann, 2000). The level of DNA fragmentation detected in the specimens is highly dependent on the technical procedures, such as retrieval techniques, treatment by enzymes, duration of exposure, and timing of colour reaction (Saraste, 1999), (Loro *et al.*, 2003). Standardization of the experiments and controls should therefore be conducted in order to evaluate apoptotic cell death by TUNEL (Potten, 2001), (Loro *et al.*, 2003).

Both cryostat and formalin fixed, paraffin embedded tissue sections were used in the TUNEL series. Pretreatment of freshly cut cryostat sections included fixation in 1% paraformaldehyde or 1% formalin for 30 min, without any obvious difference. For formalin fixed and paraffin embedded tissue sections, best results were obtained with a combination of microwave treatment (850 W for 5 min, followed by 500W for 5 min) in citrate buffer (10mM, pH: 3.0), and proteinase K (25 μg/ml in PBS) for 15 min at room temperature.

Biotin-labelled dUTP was utilized for detection of DNA fragmentation, since it may give more reproducible results, is a less time consuming detection process, and is less expensive compared to digoxigenin-labelled nucleotides (Loro, 2001). Endogenous peroxidase block was performed after the TdT reaction (Loro, 2001), since hydrogen peroxide is known to induce DNA breaks and reduces TdT activity (Migheli *et al.*, 1995). The signals were detected with Neutravidin-HRP, due to less non-specific staining compared to Streptavidin horse radish peroxidase (Loro, 2001), and developed with 3-amino-9-etylcarbazole (AEC).

For positive controls, specimens were treated with DNAse 0.5 mg/ml (Roche Diagnostics GmH. Mannheim, Germany) in Tris-buffered saline (TBS) buffer for 15 min at 37^0 C, prior to incubation with bovine serum albumin. The specificity of the TUNEL reaction was tested using unbiotinylated dUTP (Roche) in the TUNEL labelling mixture instead of biotinylated dUTP (negative control).

Evaluation TUNEL sections from OLP lesions (Paper I)

Cell counts of positive cells per mm epithelial length (apoptotic index) were performed in tissue sections of OLP under a light microscope (Leicha GMBH) with a fitted ocular grid at x250 original magnification (Paper I). At least two parallelly cut sections from each lesion were included, and cell counts of corresponding regions were performed.

9.3. *In situ* mRNA hybridization

In situ hybridization (ISH) is a molecular technique used to study DNA and RNA in tissue sections. This method was performed in order to further investigate the expression of CD40 and CD40L at the mRNA level on OLP tissue sections (Paper II).
Cryostat sections were used in the ISH series, and fixation of freshly cut sections was performed in 4% formalin (Heikinheimo and Happonen, 1991).

The set of the probes (Paper II: Table 1) were laboratory designed using National Biosciences oligo primer analysis software programme version 5.0 for Windows (Molecular Biology Insights, Cascade, Co, USA), and produced by Eurogentec (S.A. Prac Industr. Des Hauts-Sarts, Herstal, Belgium). Single stranded RNA probes were selected, since these are reported to be significantly more effective than DNA probes (Heikinheimo and Happonen, 1991). Small probes containing 21-27 bases were chosen (Paper II, Table 1) in order to facilitate penetration, so prehybridization with proteinase K could be avoided (Heikinheimo and Happonen, 1991). All probes were biotinylated at the 5'-end. One of the CD40 antisense probes selected (Paper II: Table 1; 1) has been previously described (Tone *et al.*, 2001). Best results were obtained with a cocktail of probes compared with incubation with individual probes alone. All probes were incubated over night. Incubations with probes at 50-52^{o}C gave best results. Probes that required higher incubation temperatures (<55 ^{o}C) gave problems with drying of the sections.

The signal amplification system using biotinylated tyramide is based on the peroxidase catalyzed deposition of biotinyl tyramide. Signal amplification cycles were performed one to three times using the catalyzed amplification system for ISH (Genpoint kit, DAKO), and best results were obtained with three amplifications (Table 2). Incubations with primary streptavidin horseradish peroxidase at different concentrations: 1:100, 1:200, 1:500, 1:1000 were performed from 15-30 min. Best results were obtained with primary streptavidin at a concentration of 1:500 for 15 min in each amplification cycle. Background staining was reduced by washing in 0.1% Triton-X-100 in TBS 3 min twice with stirring, distilled H_2O, and TBS for 3 min between each step in the amplification cycle.

Incubation with biotinylated sense probes complementary to the antisense probes, were performed to test the specificity of the hybridization reaction (negative control). Total mRNA

was detected using an 18-mer "poly-T" probe (positive control). Sections from normal OM and tonsillar tissue served as positive tissue controls for CD40 and CD40L mRNAs.

Table 2. Trouble-shooting guidelines for in situ mRNA hybridization.

NO	Pitfall	Trouble-shooting
1	Contamination	• Use gloves, especially during the prehybridization steps • Autoclave all glasses and instruments used for the prehybridization procedure • Use DEPC water for all prehybridization solutions • Immediately fix freshly cut cryostat sections in 1% formalin or paraformaldehyde
2	Too weak signal	• Use a cocktail of different probes • Preheat probes at 95^{o}C for 5 min and immediately immerse in ice, before placing on samples • Increase amplification cycles to 2 or 3 • Use higher concentrations of primary streptavidin • Increase the time of primary streptavdin in each cycle • Wash thoroughly in dH_2O and/or TBS after Triton-X-100 to remove remnants of Triton-X-100 which can dilute or interfere with the reagents applied in the next step of the procedure
3	Background staining	• Wash thoroughly with 0.1% Triton-X-100 between the different steps especially after the biotinyl tyramide step • Wash thoroughly in dH_2O and/or TBS after Triton-X-100 to remove remnants of Triton-X-100 which cause the sections to easily dry • Use lower concentrations of primary streptavidin • Decrease amplification cycles to 1 or 2 • Increase the temperature of the stringent wash (between 5-20 ^{o}C < melting point of the probe)
4	Detachment of tissue from the glass slides	• Use silane-coated slides • Let cryostat sections dry on the bench at room temperature for 5-7 min before starting the procedure
5	Drying of tissue sections	• Choose probes that do not require too high incubation temperature (<55-60^{o}C) • Use coverslips during incubation • Use a humidified chamber with a good seal

DEPC water: 0.1% dietylpyrocarbonate treated H_2O

9.4. DNA image cytometry - measurement of DNA content

We utilized a DNA image cytometry method to measure the DNA content in biopsies from OLP lesions, epithelial dysplasias and OSCC developed in OLP patients (Paper III). This method is based on quantitative DNA staining, and measurement of nuclear Integrated Optical Density as the cytometric equivalent of the DNA content. The DNA content is expressed in a "c" scale with 2c as the mean nuclear content of normal cells in G0/G1 cell cycle phase (diploid population) (Haroske *et al.*, 1998).

Formalin fixed, paraffin embedded tissue sections were enzymatically digested in 0.05% protease solution in PBS (5mg/ml, for 60 min) for the preparation of the isolated nuclei/ monolayers according to previous protocols (Hedley, 1994), (Pradhan *et al.*, 2006). The DNA content of Feulgen–Schiff stained nuclei was measured and analysed using the Fairfield DNA Ploidity System according to an established protocol (Haroske *et al.*, 1998), (Pradhan *et al.*, 2006). At least 500 cell nuclei were measured and stored in galleries for each case. The nuclei were picked manually based on morphologic appearance of epithelial cell nuclei, being aware of the high amount of lymphocytes in the samples. Lymphocytes were included as internal controls (calibration). Some samples were measured automatically for internal control as well (2000 cell nuclei). One advantage of this image cytometry method is that small pieces of tissue samples are required for the analysis, compared to methods based on flow cytometry. Other advantages are that cell nuclei may be carefully evaluated morphologically and can be compared with the parallel histological tissue sections when picked manually. Storing the galleries for each case allows later reevaluation of the samples also by others.

DNA Ploidy – classification

A lesion was classified as diploid if only one G0/G1 (2c) peak was present, if the number of nuclei in the G2 (4c) peak did not exceed 10% of the total, or if the number of nuclei with DNA content >5c did not exceed 1% of the total number of nuclei (Paper III: Fig. 2A).
A lesion was classified as tetraploid when one G0/G1 (2c) peak was present in the diploid position together with a G2 (8c) peak, or when the number of nuclei in 4c peak exceeded 10% of the total number of nuclei. (Paper III: Fig. 2B).
A lesion was defined as aneuploid if the peaks appeared outside the area of 2c, 4c or 8c, or if the number of nuclei with DNA content above 5c or 9c exceeded 1%.

A lesion was classified as polyploid when the number of nuclei at 8c exceeded 10% of the total, or if the number of nuclei at 9c exceeded 1%.

10. RESULTS

10.1. Apoptosis, Fas and FasL expression in OLP (Paper I)

Significantly increased apoptosis (mean: 1.4/0.25 mm epithelial length) was found within the epithelium of OLP (Fig. 7A-C), compared to normal OM (mean: 0.2/ 0.25 mm epithelial length). Most often apoptosis was confined to the basal cell region and areas of basal cell destruction. More apoptotic cells were observed in areas of atrophic epithelium. A low rate of apoptosis (mean: 1.11%) was observed in the subepithelial cell infiltrate in OLP (Fig. 7B, C), without significant difference in percentage compared to normal OM (0.93%).

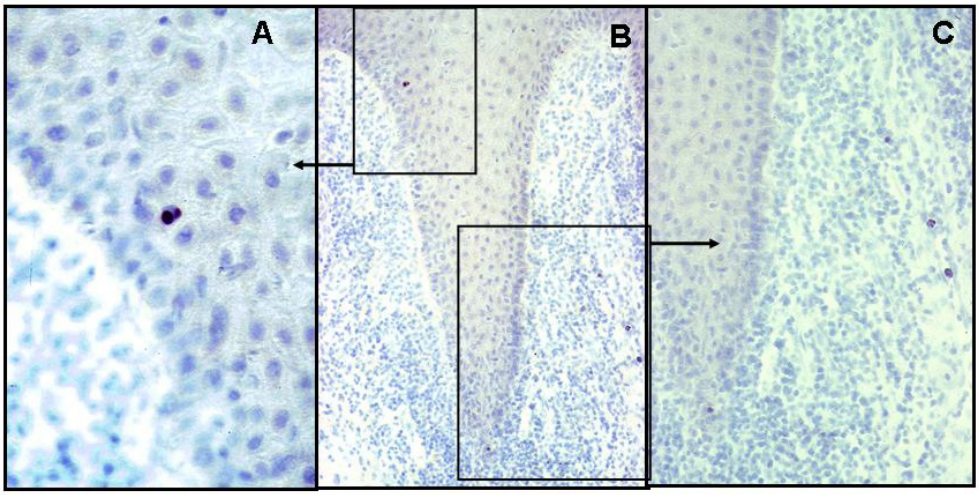

Figure 7. Apoptotic cells in the epithelium and subepithelial chronic inflammatory cell infiltrate in OLP. TUNEL. Orig. magn: x150 (B), x250 (C), x400 (A). Improved quality compared with Fig. 1E, F (Paper I) achieved by Adobe photoshop.

In the epithelium of OLP, FasR and FasL expression (Fig. 8) was more abundant in the basal cell area compared with suprabasal cell layers, and with no obvious difference in staining pattern compared to normal OM (Table 3). A high porportion of the mononuclear cells in the subepithelial cell infiltrate expressed FasR (mean: 65.0 %) and FasL (59.7 %).

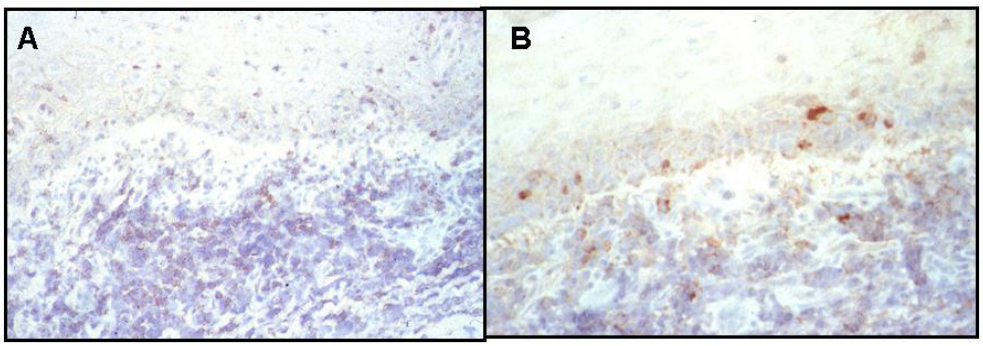

Figure 8. Immunohistochemical expression of FasR (A) and FasL (B) in oral lichen planus. Orig. magn: x150. Improved quality compared with Fig.1C, D (Paper I) achieved by Adobe photoshop.

10.2. Ki-67 expression in OLP

All OLP lesions (n=15) and normal OM (n=5) expressed Ki-67. A variable expression was observed between the OLP specimens. An increased expression of Ki-67 (+++) was observed mainly in basal and parabasal cell layers (Fig. 9, Table 3) in 7/15 (47%) of the OLP specimens compared to normal OM. More Ki-67 expressing cells were observed especially in parabasal cell layers in regions of atrophic epithelium, compared with regions with acathosis (Fig. 9). Moderate expression of Ki-67 (++) was observed in 4/15 (27 %) and less expression (+) in 4/15 (20%) of the OLP specimens. Ki-67 was also expressed in mononuclear cells (8-10%) of the subepithelial cell infiltrate of OLP specimens (Fig. 9). All normal OM investigated was in the (+) category.

These results are not presented in the papers included in this thesis.

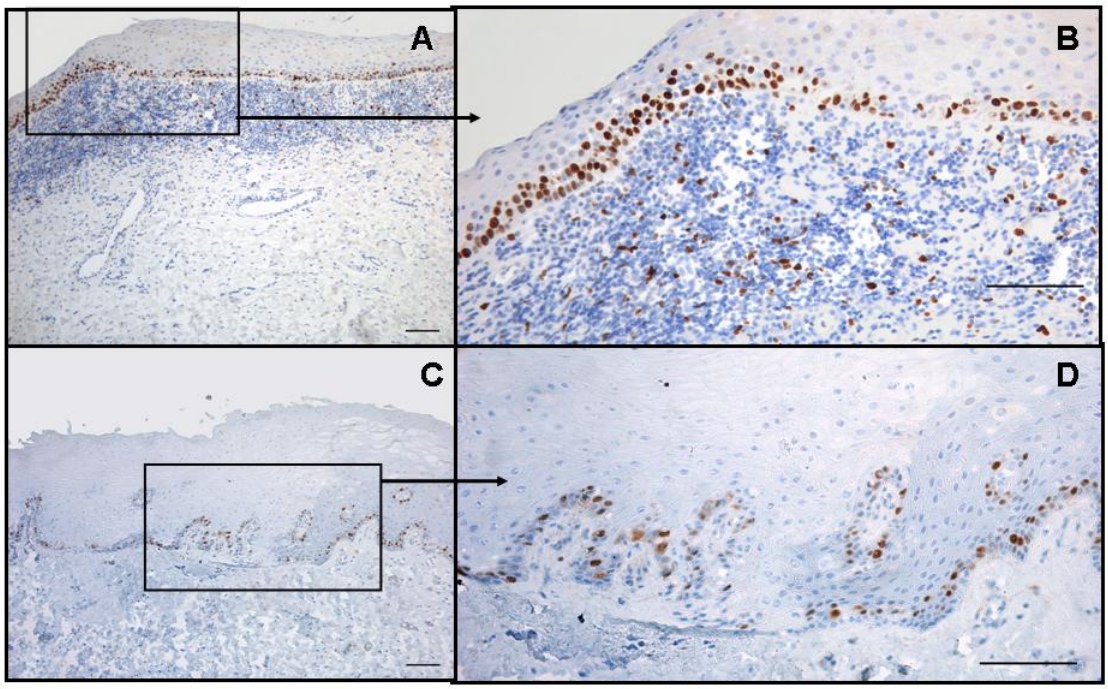

Figure 9. Ki-67 expression in epithelium and subepithelial mononuclear cell infiltrate in OLP (A, B) and normal OM (C, D). Immunohistochemistry. Scale bar: 100mμ.

10.3. CD40 and CD40L expression in OLP (Paper II)

Loss of CD40 and CD40L expression in OLP

Basal keratinocytes in non-diseased areas of OLP expressed CD40 (93-95%, Fig. 10A). At the borders between non-diseased and diseased epithelium of OLP, partial expression of CD40 (some cells expressed CD40 and some were negative) was observed in some regions with minor areas of focal inflammation (Fig. 10B). In areas exhibiting subepithelial inflammation and/or basal cell destruction few keratinocytes (10-15%) expressed CD40 (Table 3, Paper II: Fig.1A, B). CD40 expressing cells with the morphology of Langerhans cells were occasionally seen within the epithelium (Fig. 11A, B, Paper II: Fig. 1C, D). In the subepithelial cell infiltrate, CD40 was detected in more than half (55-60%) of the mononuclear cells (Paper II: Fig. 1A, B).

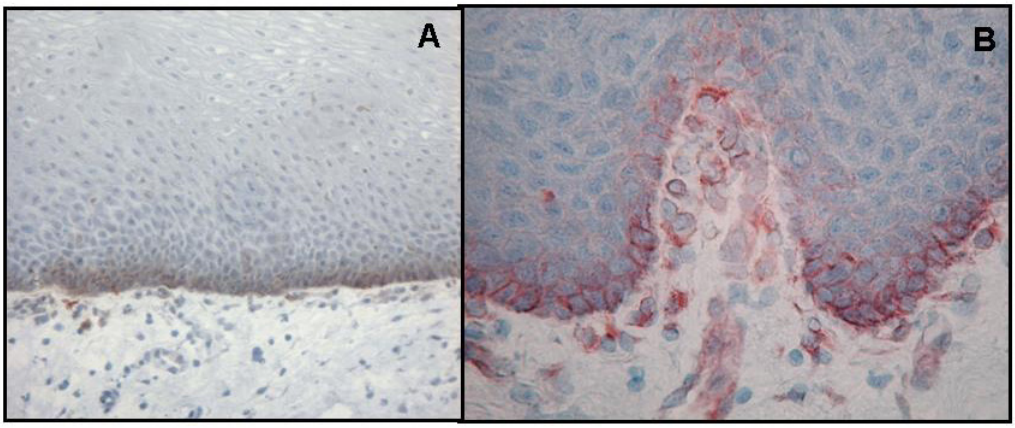

Figure 10. Immunohistochemical expression of CD40 in basal keratinocytes in non-diseased epithelium (A) and partial CD40 expression in areas with less inflammation at the borders between non-diseased and diseased epithelium of oral lichen planus (B). Orig. magn: x100 (A), x400 (B, C)

CD40L was lost in areas of OLP exhibiting basal cell destruction (Table 3), or the expression was weak and diffuse in areas where the basement membrane zone was not well defined (Paper II: Fig. 1E, F). Occasional CD40L expressing cells were observed in the epithelium. In the subepithelial cell infiltrate, <5% of the cells expressed CD40L.

Presence of CD40^{+} T cells within the epithelium of OLP

Scattered intraepithelial T cells (CD3^{+}) expressing CD40 were detected in OLP specimens (Fig. 11A-C, Paper II: Fig.1C, D). Some of the T cells were seen in close proximity CD40 expressing keratinocytes (Fig. 11A, B). Also, in the subepithelial cell infiltrate, some T cells (CD3^{+}) expressed CD40, while others did not (Fig. 11A, C, Paper II: Fig. 1C, D).

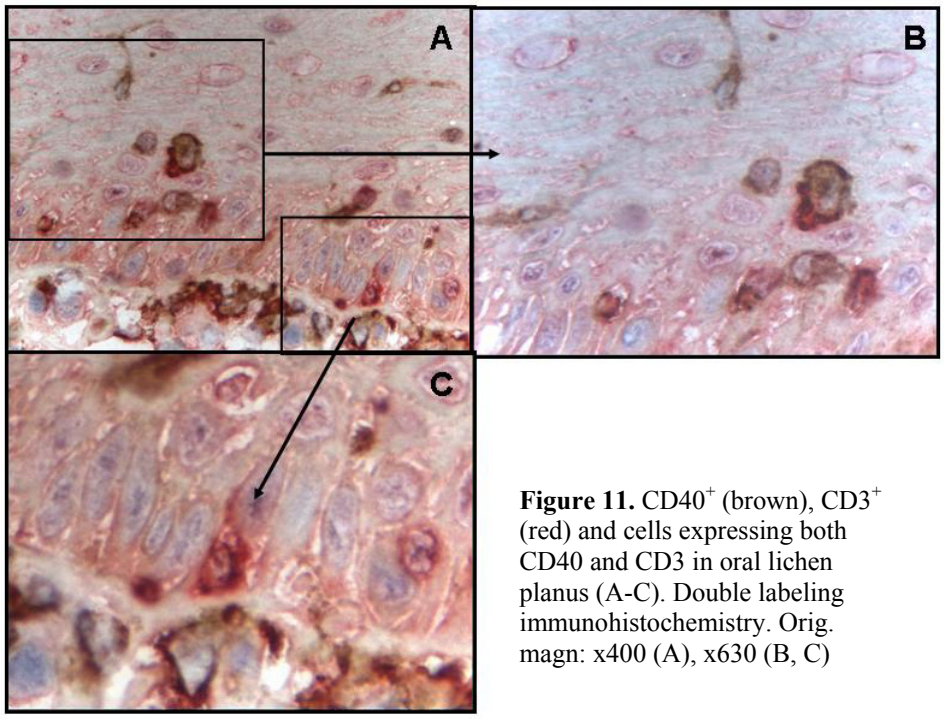

Figure 11. CD40^{+} (brown), CD3^{+} (red) and cells expressing both CD40 and CD3 in oral lichen planus (A-C). Double labeling immunohistochemistry. Orig. magn: x400 (A), x630 (B, C)

Expression of CD40 and CD40L mRNA in OLP

CD40 mRNA was detected in only a few keratinocytes (6-7%) of the basal cell layer in OLP (Paper II: Fig. 2B), compared with normal OM (85-90%) (Paper II: Fig. 2A). CD40 mRNA was widely detected in the subepithelial cell infiltrate (45-50%) (Paper II: Fig. 2B). Only a few (<5%) scattered cells demonstrated CD40L mRNA expression in the epithelium and in the mononuclear subepithelial inflammatory cell infiltrate in OLP (Paper II: Fig. 2D), compared with normal OM (Paper II: Fig. 2C). Total mRNA was demonstrated in sections from OLP lesions (Fig.12A), normal OM and tonsillar tissue (positive controls). Tonsillar tissue served as positive controls for expression of CD40 and CD40L mRNAs (Fig. 12C, D). Incubations with the sense probes were negative (Fig. 12B).

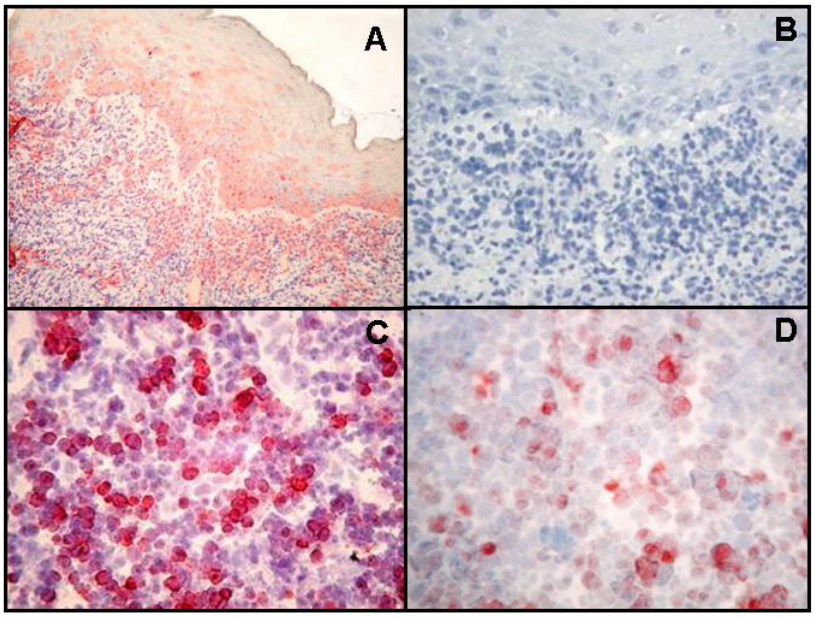

Figure 12. Total mRNA/ Poly-T (A), and negative sense probe control in oral lichen planus (B). Expression of CD40 mRNA (C) and CD40L mRNA (D) in tonsils. *In situ* mRNA hybridization. Orig. magn: x400 (A, B), x100 (C), x200 (D).

10.4. Loss of E-cadherin (Papers II-III) and maintained CD44 (Paper II) expression in OLP

In diseased areas of OLP, there was a focal reduction of E-cadherin expression in basal keratinocytes especially in areas with intense subepithelial cell infiltrate (Paper II: Fig. 3A, B, Paper III: Fig. 2C-D).

CD44 was detected in the epithelial basal cell region with a lower intensity in the spinous and superficial cell layers, with no difference in the expression observed in OLP compared to normal OM (Paper II: Fig. 3E, F). In the subepithelial infiltrates of OLP samples, CD44 was observed in approximately one third of the mononuclear cells.

10.5. Laminin-5 and collagen IV expression in OLP (Paper II)

Laminin-5 and collagen IV were detected along the basement membrane zone in all OLP and OM investigated (Fig. 13A-D). However, a variation in the intensity and thickness was observed between the OLP samples. Laminin-5 expression was observed to be more intense and labelled a thicker band along the basement membrane region in OLP (Fig. 13B) compared to OM (Fig. 13A). Regions with thickening (Fig. 13D) and minor breaks of polarized collagen IV expression were seen in focal areas of basal cell destruction in OLP compared with normal OM (Fig. 13C).

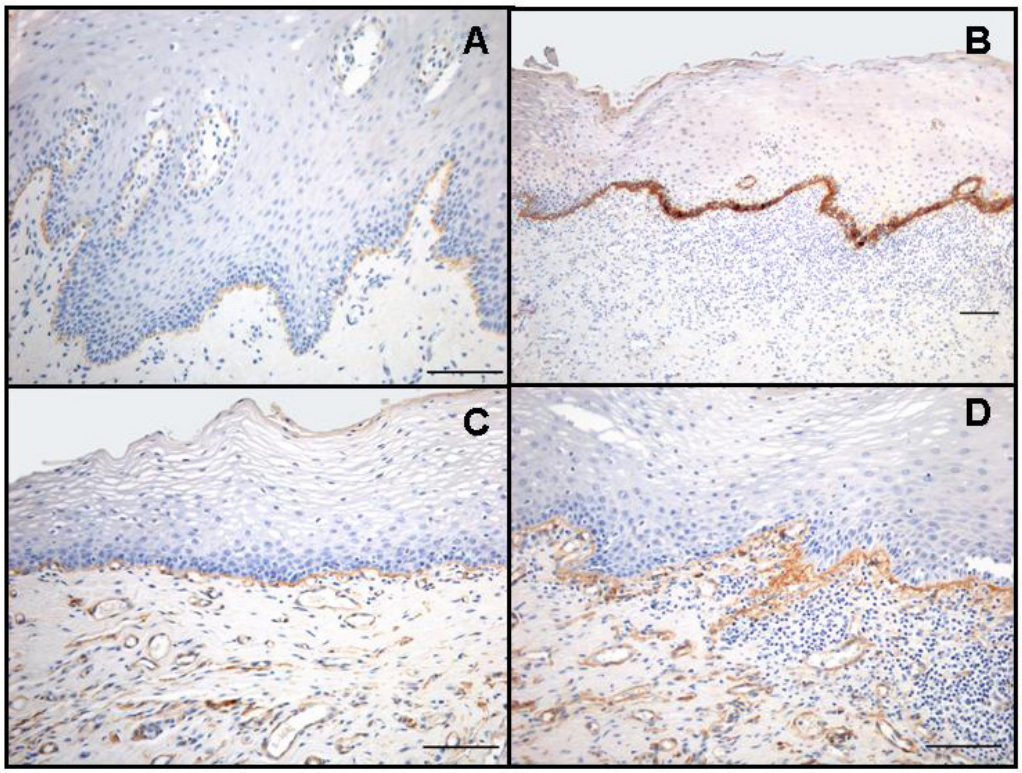

Figure 13. Laminin-5 expression in normal oral mucosa (A) and oral lichen planus (B), collagen IV expression in normal oral mucosa (C) and oral lichen planus (D). Immunohistochemistry. Scale bar: 100mμ.

10.6. Cox-2 and E-cadherin expression in OLP, epithelial dysplasia and OSCC developed in OLP patients (Paper III)

Cox-2 is expressed in the epithelium of OLP, epithelial dysplasia and OSCC

Cox-2 was expressed in all OLP specimens investigated (n=63). In the epithelium of OLP, Cox-2 was expressed in various regions; (1) in the basal cells only with a perinuclear localization (16 %), (2) in both basal and parabasal cell layers with perinuclear and cytoplasmic localization (44 %), or (3) in focal areas with both perinuclear and cytoplasmic localization of expression (40 %), also including more superficial cell layers (Paper III: Fig. 3A, B). In the subepithelial cell infiltrate of OLP, a varying degree of Cox-2 expression was observed, from between not detectable in some regions to expression of Cox-2 in 50 % of the mononuclear cells in other regions. In epithelial dysplasias (n=4), Cox-2 expression was observed in focal areas of the epithelium with both perinuclear and cytoplasmic localization in basal, parabasal and/or spinous cell layers. In OSCC (n=5) a focal Cox-2 expression was observed with both perinuclear and cytoplasmic localization in the tumour islands of infiltrating epithelial cells and in some infiltrating mononuclear inflammatory cells. The expression had no obvious relation to the tumour front.

Focal loss of E-cadherin expression in OLP, epithelial dysplasia and OSCC

E-cadherin was detected in all OLP specimens investigated (n=56), epithelial dysplasias (n=5), OSCC (n=7) and normal OM (n=7) investigated. In the epithelium of OLP, a focal reduction of E-cadherin expression was observed in the basal cells (Paper III: Fig. 3C, D) in areas with subepithelial mononuclear cell infiltrate, in 88 % of the biopsies. No obvious difference in E-cadherin expression was observed in 12 % of the OLP specimens, compared to normal OM. In the OLP lesions with epithelial dysplasia, E-cadherin was mostly maintained in the spinous cell layers and with a focal loss of expression in the basal cell region. In OSCC, E-cadherin expression was lost in some regions of the epithelial invasive tumour islands, but maintained in other regions.

10.7. P53 expression in OLP

P53 expression was detected focally in the basal cells (Fig. 14) in eight of the 15 OLP specimens investigated (53%). Neither of these patients developed epithelial dysplasia nor OSCC in their OLP lesions during the follow-up period. There was no obvious correlation between the areas of focal p53 expression and the thickness of the epithelium (atrophy or acanthosis). Few scattered p53 expressing cells were also observed in the subepithelial mononuclear cell infiltrate of OLP lesions. All normal OM (n=5) were negative. These results are not presented in the papers included in this thesis.

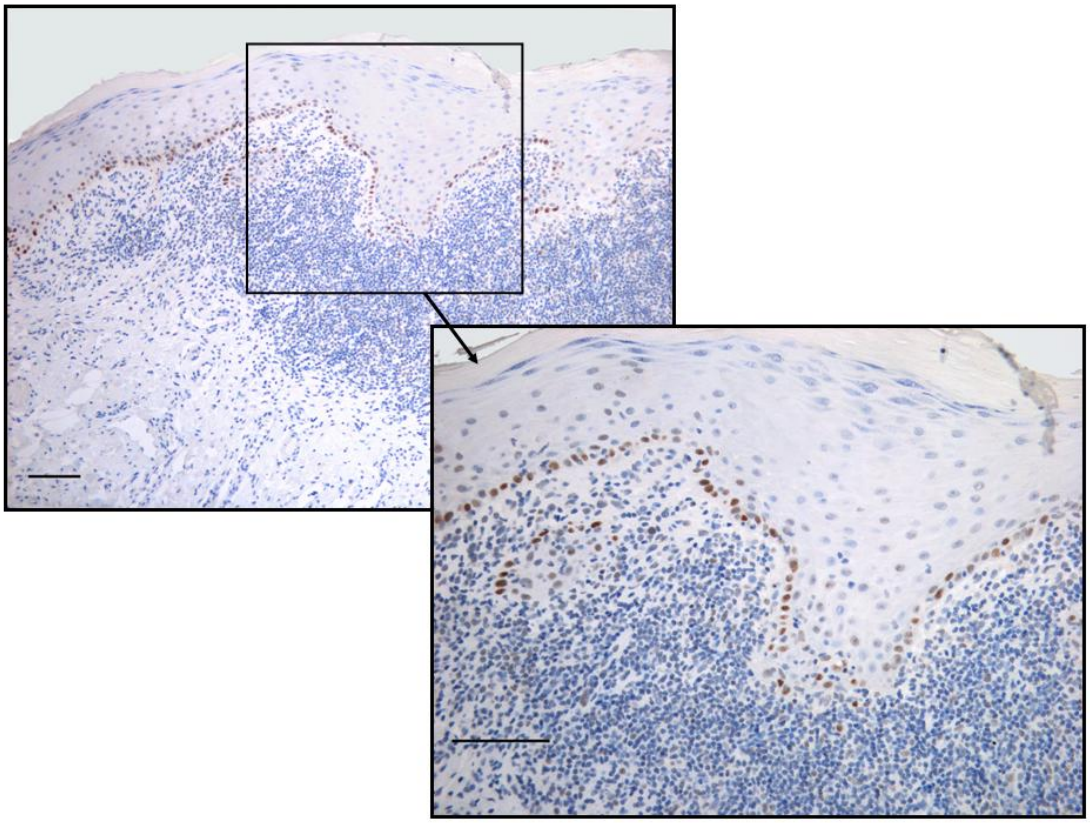

Figure 14. P53 expression in oral lichen planus. Immunohistochemistry. Scale bar: 100mμ.

10.8. Summary of immunohistochemical findings in OLP compared with normal OM

Table 3. Results of immune expression of different antibodies investigated in our series of oral lichen planus lesions compared with normal oral mucosa (Papers I-III).

Expression	Normal oral mucosa		Oral lichen planus	
	Epithelium	**Connective tissue**	**Epithelium**	**Connective tissue**
CD3	-	Scattered cells	Scattered T cells	Majority (66%) of the subepithelial cell infiltrate
CD4	-	Scattered cells	Scattered cells	Majority (60%) of the mononuclear cells,
CD8	-	Scattered	Scattered cells	Moderate (30%) of the mononuclear cells
FasR	Predominantly in basal cells	Scattered cells	Predominantly in basal cells	Majority (65%) of the cells in the mononuclear cell infiltrate
FasL	Predominantly in basal cells	Scattered cells	Predominantly in basal cells	Majority of the cells in the mononuclear cell infiltrate
CD40	Basal keratinocytes (95-97%)	Scattered cells	Loss in basal keratinocytes (85-90%) in areas with subepithelial inflammation. Scattered cells in the epithelium, some with the morphology of Langerhans cells	More than half of the mononuclear cells (55-60%)
CD40L	Expressed as a polarized zone in the basement membrane region	Few scattered cells in the underlying connective tissue	Scattered cells in the epithelium Polarized zone Lost in areas of basal cell destruction	<5% of the cells in the mononuclear cell infiltrate
E-cadherin	Marked expression in basal keratinocytes, and reduced intensity in superficial cell layers	Salivary glands deeper in the connective tissue	Focal loss in basal keratinocytes, but retained in other superficial cell layers with reduced intensity towards the surface	Scattered cells of the mononuclear cell infiltrate with a morphology suggestive of macrophages
CD44	Marked expression in basal keratinocytes, and reduced expression in superficial cell layers	Fibroblasts	No obvious difference compared to NOM	About one third of the mononuclear cells. Fibroblasts
Ki-67	Basal and parabasal cells	-	Increased in basal and parabasal cell layers, with a variation between the specimens. More expressing cells in atrophic regions	Expressed in mononuclear cells of the subepithelial cell infiltrate (8-10%)

- : not detected

Expression	Normal oral mucosa		Oral lichen planus	
	Epithelium	**Connective tissue**	**Epithelium**	**Connective tissue**
Laminin-5	Along the basement membrane zone	-	Variation in thickness of the band along the basement membrane zone, thicker in some regions	-
Collagen IV	Along the basement membrane zone	Basement membrane of vessels	Variation in expression along the basement membrane zone, minor breaks in areas of basal cell degeneration	Basement membrane of vessels
Cox-2	-	Muscle cells deeper down in the tissue	Expressed in focal regions of the epithelium, especially on the nuclear membrane of keratinocytes and in the cytoplasm	Varying expression from none in some regions to about half of the mononuclear cells in other regions Muscle cells deeper down in the tissue
P53	-	-	Increased expression in basal cells in some specimens	A few scattered cells in the subepithelial cell infiltrate

- : not detected

10.9. DNA-content in OLP, epithelial dysplasia and OSCC developed in OLP patients (Paper III)

In all biopsies of OLP investigated (n=58) the keratinocyte nuclei were classified as diploid (Paper III: Fig. 2A). One patient with epithelial dysplasia within the OLP lesion in the first biopsy was also classified as diploid, and later biopsies from this patient presented with OLP without signs of epithelial dysplasia (Paper III: Fig. 1, patient no: 6) Of the six epithelial dysplastic lesions which had developed in areas of OLP over time, one lesion was classified as tetraploid (Paper III: Fig. 2B), and this patient presented with OSCC 2 years after. All OSCC were classified as diploid (n=7). The OSCCs developed in patients with previous diploid lesions of epithelial dysplasia, except for one patient with tetraploid epithelial dysplasia. None of the biopsies investigated were classified as aneuploid.

11. DISCUSSION

11.1. Increased apoptosis in the epithelium of OLP

The keratinocytes in OLP lesions are under a constant cross fire from antigens, cytotoxic T cells and inflammatory mediators, the end result of which is basal cell destruction. The present study provides further evidence that basal keratinocytes die by apoptosis (Paper I). Later studies have confirmed this finding, using different techniques for detection of apoptosis, such as electron microscopy, anti-single-stranded DNA and cleaved-caspase-3 (Santoro *et al.*, 2004), (Tobon-Arroyave *et al.*, 2004), (Bascones *et al.*, 2005). Increased keratinocyte apoptosis in atrophic epithelium of OLP, has also been confirmed by one later immunohistochemical study using anti-single-stranded DNA (Santoro *et al.*, 2004). Not only keratinocytes, but also intraepithelial $CD4^+$ T cells may undergo apoptotic cell death in OLP, as shown by a combination of TUNEL and immunohistochemistry (Karatsaidis *et al.*, 2004). In order to investigate mechanisms involved in the balance between cell renewal (proliferation) and apoptotic keratinocyte cell loss, sections from OLP specimens were immunolabelled with an Ab against Ki-67 (a nuclear proliferation marker). We observed elevated Ki-67 expression within the epithelium of OLP, corresponding to what has been demonstrated in previous studies (Taniguchi *et al.*, 2002), (Hirota *et al.*, 2002), (Mattila *et al.*, 2007). Increased expression of other proliferation cell markers (proliferating cell nuclear antigen, topoisomerase IIα) has also been demonstrated in OLP, especially in atrophic lesions (Lee *et al.*, 2005), (Mattila *et al.*, 2007). Our findings and those of others indicate an imbalance between cell proliferation and cell death in OLP.

11.2. Regulation of keratinocyte apoptosis in OLP

The present study provides further information about biological changes in expression of apoptosis regulatory proteins in OLP (Papers I-III). There may be different complex pathways involved in apoptosis of basal keratinocytes, and these pathways may also have multiple interactions. Figure 15 illustrates some potential interactions in the apoptosis cascade between proteins investigated in the present study. These molecules take part in an array of complex biological processes. Accordingly, it is difficult to draw conclusions on each of the molecules' respective role in apoptosis regulation, based on its expression in keratinocytes or other intraepithelial cells. Nevertheless, alterations in protein expression of regulators of apoptosis presented in this study may play specific roles in the pathogenesis of apoptotic cell death in OLP.

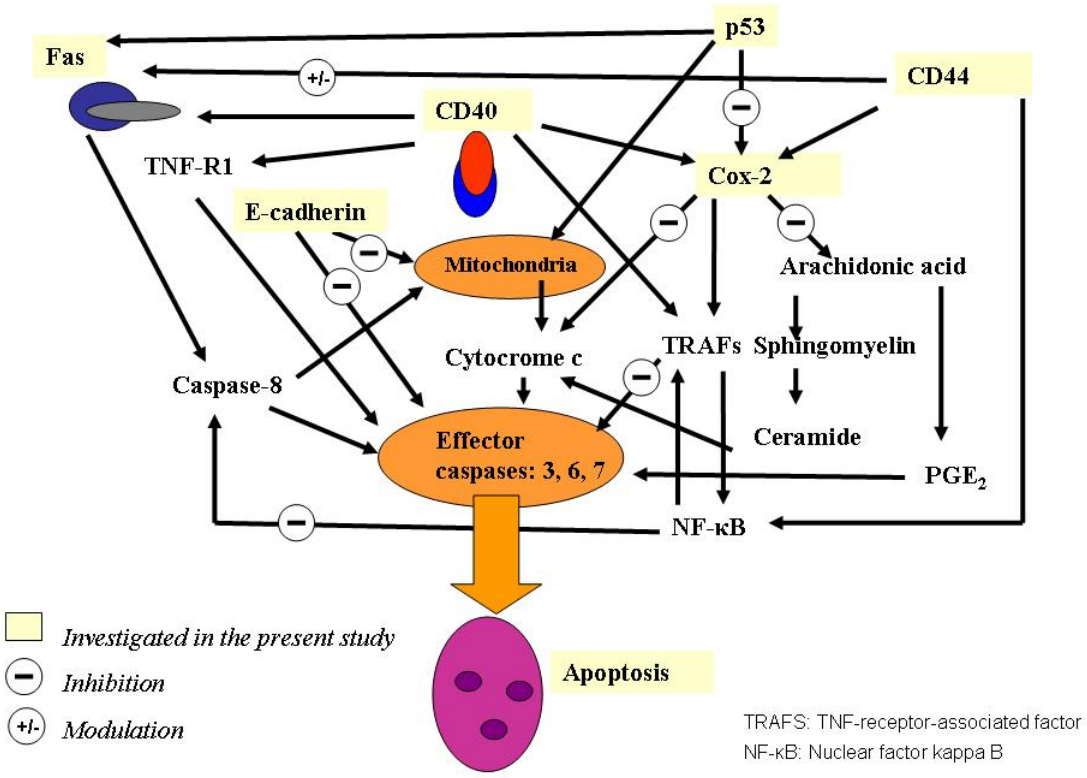

Figure 15. Summary of some potential interactions of mediators of apoptosis investigated in the present study of oral lichen planus.

FasR and FasL were highly expressed in OLP, both in the epithelium and in the subepithelial cell infiltrate (Paper I), as also found in a previous immunohistochemical study (Dekker *et al.*, 1997). Since the FasR-FasL system mediates apoptosis, it is surprising that the rate of apoptosis is rather low. This finding may indicate a functional defect in the FasR/FasL molecules or in the Fas mediated apoptotic pathway. Corresponding findings have been demonstrated in the chronic lymphocytic infiltrate of salivary glands of Sjögren's syndrome patients, suggesting that the lymphocytes are resistant to Fas induced apoptosis (Ohlsson *et al.*, 2001). In addition, genetic defects in the Fas gene have been associated with defective apoptosis in autoimmune disorders including systemic lupus erythematosus (Suda and Nagata, 1997), (Vaishnaw *et al.*, 1999), (Kühtreiber *et al.*, 2003).

The CD40-CD40L system may play several roles in the pathogenesis of OLP, since (1) CD40 and CD40L are expressed in different cell types of OLP (Paper II), and (2) multiple potential interactions of CD40/CD40L may occur with other molecules expressed in OLP (Fig. 15). The fact that CD40-CD40L ligation may induce or inhibit apoptosis dependent on the cell

type and context (Grell *et al.*, 1999), (Eliopoulos *et al.*, 2000), (Xu and Song, 2004), (Georgopoulos *et al.*, 2006), further complicates the picture of the putative role of the CD40-CD40 system in the pathogenesis of OLP.

The basal keratinocytes of diseased areas in OLP may escape CD40-CD40L mediated apoptosis via down-regulation of CD40 (Paper II). On the other hand, the CD40 down-regulation may together with the CD40L loss observed in the basement membrane zone, be a signal to promote keratinocyte renewal in areas of basal cell destruction of OLP. This suggestion is based on the fact that CD40 ligation inhibits proliferation of epidermal keratinocytes in monolayers by modulation of the cell cycle (Peguet-Navarro *et al.*, 1997), (Grousson *et al.*, 2000). In addition, an inverse relationship between CD40 and Ki-67, with decreased CD40 expression and increased Ki-67 expression, has been demonstrated in epidermal monolayers and normal skin biopsies (Concha *et al.*, 2003). Accordingly, elevated Ki-67 expression (Hirota *et al.*, 2002), (Taniguchi *et al.*, 2002) and other proliferation markers (Lee *et al.*, 2005), (Mattila *et al.*, 2007), may support the suggestion that CD40 down-regulation is a signal to promote proliferation in basal keratinocytes of OLP.

Down-regulation of CD40 may also affect other apoptosis regulatory proteins including Fas and Cox-2, by interaction mechanisms (Fig. 15). This suggestion is based on the fact that CD40 can up-regulate Fas in cancer cell lines (Eliopoulos *et al.*, 2000), as well as Cox-2 on human fibroblasts and endothelial cells *in vitro* (Zhang *et al.*, 1998), (Garlichs *et al.*, 2002), (Dongari-Bagtzoglou *et al.*, 2003). Cox-2 has the ability to inhibit apoptosis via several pathways (Fig. 15), as reducing both cytocrome-c and caspase activation (Sun *et al.*, 2002), and up-regulation of the protooncogene Bcl-2 (Tsujii and DuBois, 1995). Consequently, the reported up-regulation of Cox-2 expression in OLP (Paper III) may lead to inhibition of apoptosis and thus contribute to increased cell survival. Such an anti-apoptotic effect of Cox-2 may however be disturbed by p53, due to interaction with Cox-2 as well as the ability of p53 to induce apoptosis by its own (Fig. 15) (Bertram, 2000), (Tang *et al.*, 2006).

Loss of adhesive cell contacts such as E-cadherin (Papers II, III) may play specific roles in basal cell destruction of OLP. This suggestion is based on the fact that E-cadherin loss may; (1) promote apoptosis of basal keratinocytes in OLP, since reports have demonstrated that apoptosis may be prevented by E-cadherin mediated cell contact in immortalized cell lines (Peluso *et al.*, 2001), (Galaz *et al.*, 2005), (2) promote T cell migration into the epithelium, where they may easier target the keratinocytes and induce apoptosis (Paper II).

CD44 is most probably not directly involved in apoptosis of keratinocytes, since it is maintained in OLP (Paper II). However, CD44 may play an indirect role in regulation of keratinocyte apoptosis in OLP by modulating other apoptosis related molecules including Fas (Fujii *et al.*, 2001), (Hauptschein *et al.*, 2005) and Cox-2 (Murphy *et al.*, 2005).
Taken together, apoptosis regulation is complex in OLP involving several interrelated molecules including FasR/FasL, CD40/CD40L, Cox-2, p53, CD44 and E-cadherin. We have investigated some potential regulators that may be involved in apoptosis of basal keratinocytes. However, several other proteins expressed in OLP lesions that may play roles in apoptosis of keratinocytes needs further investigation, such as Topoisomerase IIα (Akimitsu *et al.*, 2003), (Mattila *et al.*, 2007), perforin-granzyme (Santoro *et al.*, 2004) and TNF-α-TNF-R1 systems (Khan *et al.*, 2003).

11.3. Candidate prognostic markers for malignant transformation of OLP

An ideal marker to predict the risk of cancer development in a premalignant lesion is an objective marker that indicates malignant transformation when present and exclude such transformation when absent. Numerous attempts have been performed to identify such a marker in different tissues, but so far no significant objective marker has fulfilled the premises. Accordingly, it is more likely that the combination of genetic and molecular biological markers together may strengthen the potential of prediction of malignant transformation. For this reason, several potential predictive markers were investigated on a unique biopsy material of patients with OLP where epithelial dysplasias and OSCC developed over time (Paper III).

Aneuploidy may be a good candidate as a predictive marker for malignant transformation in OLP, based on measurement of significant genetic changes at an early stage of cancer development. This is however a gross measurement of DNA aberrations.
In OSCC, several reports of ploidy status, mostly based on flow cytometry, have reported a wide variation in diploid cancers from 24-70% (reviewed in Diwakar *et al.*, 2005). A recent study indicates that this may be due to heterogeneity within the tumour itself, so several analysis from different parts of the tumour may be needed to confirm the ploidy status (Diwakar *et al.*, 2005). This may address concerns about the sensitivity and cost-benefit of ploidy as a routine method to select lesions at risk. Heterogeneity within OLP lesions may partly explain the wide variation in previous reports of ploidy status in OLP (Biesterfeld *et al.*,

1991), (Mattila *et al.*, 2004), (Femiano and Scully, 2005), (Maraki *et al.*, 2006), (Rode *et al.*, 2006). Other explanations may be sample variation and/or lack of calibration between laboratories. Accordingly, careful consideration must accomplish clinical interpretation of DNA ploidy status. The present study adds further evidence that aneuploidy is not sensitive enough alone as a screening test to select the OLP patients at risk for development of OSCC. On the other hand, our results indicate that OLP lesions with epithelial dysplasia may be selected due to polyploidy DNA pattern. However the patient material is limited with respect to further development of epithelial dysplasia and OSCC, so additional studies on larger patient materials is necessary to further evaluate the clinical value of this marker.

In order to investigate other potential biological objective predictive markers, the immunohistochemical expression of p53, Cox-2 and E-cadherin was investigated on biopsies from the same patient material (Paper III).
Immunohistochemical detection of p53 is controversial (Reibel, 2003). Previous investigations of p53 expression in OLP have been inconclusive, from reporting no expression to labelling in up to 91% of the lesions (Crosthwaite *et al.*, 1996), (Taniguchi *et al.*, 2002), (Lee *et al.*, 2005), (Acay *et al.*, 2006). Therefore we screened 15 of the OLP biopsies for p53 expression. About half of the OLP specimens (8/15) expressed p53, which is in agreement with one of the above mentioned studies (Taniguchi *et al.*, 2002). Since the patients that expressed p53 did not develop epithelial dysplasia or OSCC, our results confirm the view that detection of the protein alone is not reliable as a risk marker of cancer development (Warnakulasuriya, 2000). Therefore we abandoned a further investigation of the full panel of OLP biopsies.

As a suggested link between chronic inflammation and development of OSCC (Wang, 2005), Cox-2 may be one potential predictive factor for the risk of cancer development in OLP. However, the wide expression of Cox-2 in OLP lesions makes it unsuitable as a prognostic marker as such. Studies have confirmed elevated Cox-2 expression in squamous cell carcinomas of the head and neck (Chan *et al.*, 1999), (Boldrup *et al.*, 2005) as well as in cancers of other organs (reviewed in Herschman *et al.*, 2003). The effect of Cox inhibitors (most of these drugs affect both Cox-1 and Cox-2) in treatment of cancer and precancerous conditions seems promising. Several studies on cancers in stomach, colon, and rectum have shown that aspirin and other non steroid anti-inflammatory drugs (NSAIDs) result in lower death rates (reviewed in Herschman *et al.*, 2003). Three ongoing clinical studies have the aim

to assess the safety and efficacy of a Cox-2 inhibitor (celecoxib) in the treatment of patients with premalignant lesions in esophagus (Barret's esophagus) (Thun, 2003). Recently however, a clinical study on patients with oral leukoplakias and epithelial dysplasias also involving the Cox-2 inhibitor celecoxib has been stopped (Rikshospitalet – Radiumhospitalet Medical Center, Norway). This was due to an unreliable co-ordinator of the project (J. Sudbø), and withdrawal of his publications on up-regulated Cox-2 expression in oral leukoplakias and epithelial dysplasias, of which the project was partly based on. Although Cox-2 is up-regulated in OLP, and is suspected to play a role in a process of cancer development, it is not yet advisable to prescribe Cox-2 inhibitors (neither traditional NSAIDs nor more selective ones) as prophylaxis for these patients. These types of drugs may give serious side effects like gastrointestinal bleeding and heart complications, and the risk for malignant transformation in OLP is very low. Other complications may also arise in OLP patients, since NSAIDs may cause exacerbation of existing OLP lesions and/or contribute to drug-related lichenoid reactions in OM (Potts *et al.*, 1987), (Thompson and Skaehill, 1994), (Bagan *et al.*, 2004).

E-cadherin, an additional potential prognostic marker, was not either suitable to select OLP patients at risk in the present patient biopsy material, since the focal E-cadherin loss was observed in most OLP specimens (Paper III).

Also markers of proliferation including Ki-67 have been suggested to be potential markers of cancer development. Ki-67 expression is, however, often elevated in OLP compared to normal OM (Hirota *et al.*, 2002), (Taniguchi *et al.*, 2002), (Mattila *et al.*, 2007), indicating that its not a suitable marker to select patients at risk.

Accordingly, measurements of DNA content and/or the biological changes in expression of p53, Cox-2, E-cadherin and Ki-67 in the present study on OLP are not suitable as objective predictive prognostic markers to select the OLP patients at risk.
Loss of heterozygosity (LOH) may be a more sensitive marker for risk of cancer development in OLP, compared with DNA aneuploidy. Studies have reported a low frequency (6%) of allelic loss of chromosomal regions that contain putative tumour suppressor genes (3p, 9p, 17p) in OLP compared to epithelial hyperplastic lesions (14%) and lesions with mild epithelial dysplasia (40-54%) (Zhang *et al.*, 1997), (Zhang *et al.*, 2000). The usefulness of LOH as a marker in OLP could, however not be evaluated from these two above studies, since

it is not known if those patients with LOH loss underwent malignant transformation. Thus, the clinical value of this marker must be evaluated in additional studies.

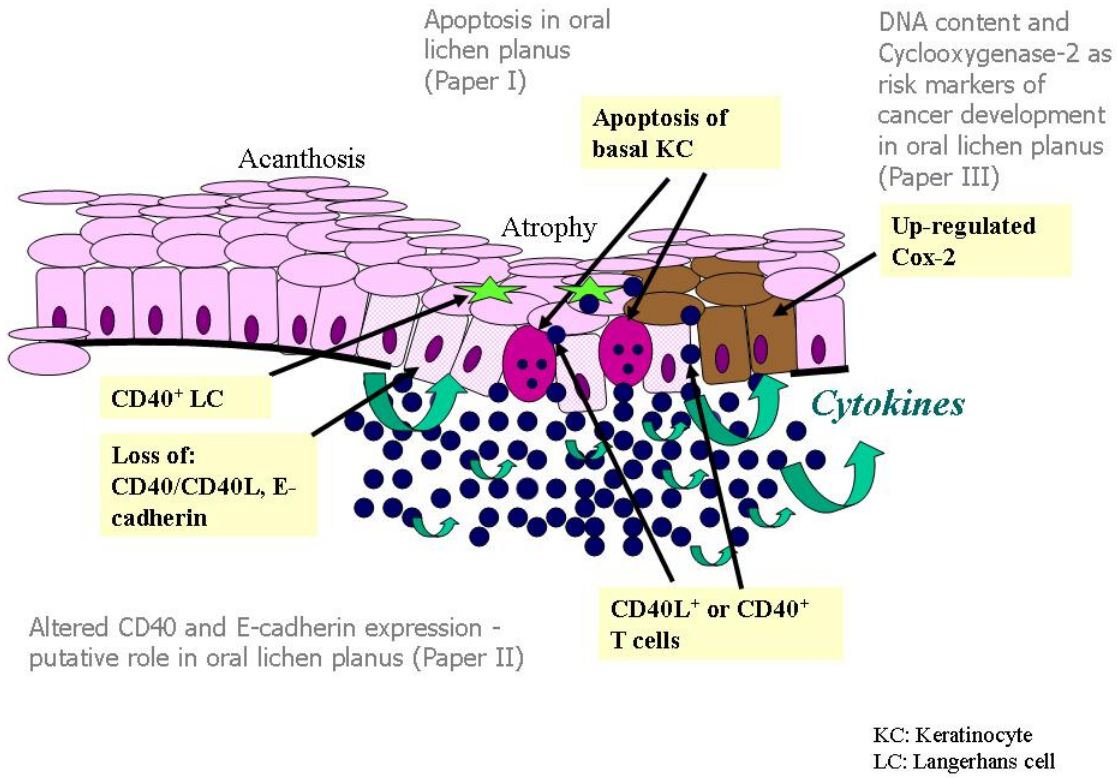

Figure 16. Summary of the pathogenesis of oral lichen planus including results and papers of the present study.

12. CONCLUSIONS

The following conclusions are drawn in accordance with the aims of this study:

Apoptotic cell death of epithelial keratinocytes is complex, and a number of biological changes that may induce or inhibit apoptosis are found in the basal cell compartment of OLP compared to normal OM.

- Basal keratinocytes die by apoptosis in OLP. Apoptosis is increased in areas with basal cell destruction and regions with atrophic epithelium in OLP. Apoptosis may be dysregulated in the subepithelial mononuclear cell infiltrate, contributing to maintenance of the massive inflammatory cell infiltrates in OLP.
- There may be a dysfunction in the FasR-FasL mediated apoptosis in keratinocytes and T cells of OLP.
- Basal keratinocytes may escape a CD40-CD40L mediated apoptotic mechanism in OLP by down-regulation of CD40 in diseased areas.
- Cox-2 is widely expressed and up-regulated in OLP, and may inhibit apoptosis of keratinocytes.
- E-cadherin loss in basal keratinocytes may promote apoptosis and contribute to basal cell destruction, allowing T cell migration into the epithelial compartment in OLP.
- DNA-content, Cox-2 and E-cadherin expression were not suitable as prognostic markers for risk of OSCC development in OLP in our patient material.

13. FUTURE PERSPECTIVES

Based on the findings of the present study, further studies may contribute to a better understanding of epithelial basal cell destruction and pathogenesis of OLP:

The trigger mechanisms and pathways of apoptosis need to be better clarified to understand the process of epithelial basal cell destruction in OLP, including targeting mechanisms by cytotoxic T cells and molecular biological changes that may promote apotosis in keratinocytes.

The present study indicates that expressions of several proteins in epithelial keratinocytes of OLP are changed, however further functional studies of these proteins, including FasR/FasL, CD40/CD40L, E-cadherin and Cox-2, are necessary.

Studies on larger patient materials are necessary to further evaluate the clinical value of aneuploidy as a predictive objective prognostic marker to select OLP patients at risk for development of OSCC.

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15. PAPERS (I-III)