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Mast Cells and Epidermis in Skin Culture

Doctoral dissertation

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ABSTRACT

Mast cells are numerous in the uppermost dermis of the normal skin, which is
normally exposed to external air. The mechanism why mast cells accumulate and persist
in the upper dermis is still obscure. Since there is a continuous cross-talk between the
epidermis and the dermis, it could be possible that the air exposure may be important to
the maturation or survival of mast cells in the dermis. This interaction was examined by
using air-liquid interface, submerged skin organ culture, proliferating keratinocytes, and
keratinocyte epithelium models. Also, release of soluble tryptase and chymase was
studied. Furthermore, the effect of air-exposure, retinoic acid, stem cell factor, TNF-α
and histamine on survival of dermal mast cells was studied, and the sequence of events
leading to mast cell death were described. Multiple histochemical techniques were used
and a new chymase-apoptosis double staining-method and objective measuring tool for
measuring histological staining intensity was developed.

The present results show that histamine and TNF-α can have strong inhibitory effects
on keratinocytes when functioning together, either simultaneously or sequentially. Mast
cells can be inhibitory, and even cytotoxic, to keratinocytes in their microenvironment.
These results show that tryptase is solubilized and can reach distant skin sites. The
chymase activity is partially inactivated and the rest of the activity, as well as most of
the protein, remain close to the site of mast cell degranulation. Chymase is rapidly
inactivated when released in physiological conditions but in pathological conditions,
e.g., in pemphigoid, the control mechanisms may fail and chymase can freely destruct
or affect surrounding structures. The present findings may also explain why an urticarial
wheal does not lead to blister formation every time the mast cells are activated and
degranulated. These results also showed that chymase-positive, tryptase-negative mast
cell may represent a dead mast cell in tissues. Despite its evident action on mast cells
during culture, stem cell factor could not prevent or accelerate the decay in tryptase- and
chymase-positive cells in skin organ culture. Possibly, the apoptotic process in mast
cells began rapidly in submerged conditions and therefore exogenous stem cell factor
had no chance to prevent it. Also, all-trans retinoic acid had no effect on mast cell
survival in skin organ culture. These results indicate, that air exposure to the epidermis
is essential for the homeostasis and survival of mast cells in the dermis.

These findings may provide an experimental background for future studies and
perhaps for therapeutic use of histamine and TNF-α in combination e.g. in cancer
biology and immunotherapy. In chronic inflammatory skin diseases tryptase is an
interesting target for developing enzyme inhibitors for possible therapeutic use.

Paul Ehrlich, 1854-1915 [1]

"Much testing; accuracy and precision is experiment;
no guesswork or self-deception."

The Collected Papers of Paul Ehrlich,
London-New York, 1856-1960

To my deepest love, Satu

My mother- and father-in-law, Sinikka and Pauli Kärkkäinen, have also supported me all the way making this research work possible, and only thanks are not enough to emphasize their role. It is also more than just love. Also, Sirpa and Matti have lived together with me while doing this work and their support is greatly appreciated.

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Kuopio, January 2004

Petri Kivinen

ABBREVIATIONS

AIOD	area integrated optical density
ALI	air-liquid interface
α_1-AC	α_1-antichymotrypsin
α_1-PI	α_1-proteinase inhibitor
AtRA	all-trans-retinoic acid
BPE	bovine pituitary extract
c-kit	proto-oncogene encoding Kit-receptor
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinoid binding protein
DMEM	Dulbecco's modified Eagle's medium
D-PBS	Dulbecco's phosphate buffered saline
EGF	epidermal growth factor
FcεRI	high-affinity receptor for IgE
FcεRII	low-affinity receptor for IgE
FcγRI, II, III	high-affinity receptor for IgG
FCS	fetal calf serum
GM-CSF	granulocyte macrophage-colony stimulating factor
H1, H2, H3, H4	histamine receptor 1, 2, 3 and 4, respectively
HB-EGF	heparin binding epidermal growth factor
ICAM-1	intercellular adhesion molecule 1, CD54
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INV	involucrin, a cornified cell envelope precursor protein
KC	keratinocyte
Kit	stem cell factor receptor, Kit-receptor, CD117
KSFM	Keratinocyte-SFM® serum free medium
MC	mast cell
MC$_C$	C type of mast cell containing chymase and carboxypeptidase
MC$_T$	T type of mast cell containing only tryptase
MC$_{TC}$	TC type of mast cell containing tryptase, chymase, carboxypeptidase and a cathepsin G-like protease
MNA	4-methoxy-2-naphthylamine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PSGL-1	P-selectin ligand glycoprotein 1, CD162
RAR	retinoic acid receptor
rh	recombinant human
ROI	region of interest
RXR	retinoid X receptor
SCF	stem cell factor (a ligand for Kit-receptor)
SCFR	stem cell factor receptor, Kit-receptor, CD117
sSCF	soluble stem cell factor, SCF-1
SM	submerged
TdT	terminal nucleotide transferase
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
UVB	ultraviolet B radiation (280-320 nm)
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals:

I Kivinen PK, Hyttinen M, Harvima RJ, Naukkarinen A, Horsmanheimo M, Harvima IT. Quantitative digital image analysis applied to demonstrate the stratified distribution of involucrin in organ cultured human skin. Arch Dermatol Res 1999: 291: 217-223.

II Kivinen PK, Hyttinen M, Lappalainen K, Harvima IT. Increased growth inhibition and cytotoxicity by simultaneous or sequential action of histamine and tumor necrosis factor-α on cultured human keratinocytes. submitted

III Kivinen PK, Kaminska R, Naukkarinen A, Harvima RJ, Horsmanheimo M, Harvima IT. Release of soluble tryptase but only minor amounts of chymase activity from cutaneous mast cells. Exp Dermatol 2001: 10: 246-255.

IV Kivinen PK, Hyttinen M, Harvima R, Naukkarinen A, Horsmanheimo M, Harvima IT. Mast cell survival and apoptosis in organ cultured human skin. Exp Dermatol 2003: 12: 53–60.

V Hjertson M, Kivinen PK, Dimberg L, Nilsson K, Harvima IT, Nilsson G. Retinoic acid inhibits *in vitro* development of mast cells but has no marked effect on mature human skin tryptase- and chymase-positive mast cells. J Invest Dermatol 2003: 120: 239-245.

ABSTRACT

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ABBREVIATIONS

LIST OF ORIGINAL PUBLICATIONS

1. INTRODUCTION

Mast cells are especially numerous in the uppermost dermis of the normal skin, but only occasionally can mast cells be found in close morphological contact with the epidermis. The mechanism why mast cells accumulate and persist in the upper dermis is obcure. The skin is normally exposed to external air, which is essential to maturation of epidermis but possibly also dermis and mast cells. Stem cell factor is important in the development of human mast cells, but a number of other factors can regulate the growth, differentiation, and proliferation of mast cells. Retinoids are commonly known modulators of hematopoietic differentiation but their importance to mast cells is poorly known. Mast cells take part in many pathophysiological conditions in human skin but their exact role in many skin diseases is still unclear. Mast cells and their proteolytic enzymes have been suggested to play a marked role e.g. in blistering skin diseases characterized by dermal-epidermal separation (1-3). Tryptase and chymase are the major serine proteinases of the skin mast cells and the biological significance of these two enzymes depends on their activity. Mast cells accumulate and persist in the upper dermis of the skin, and they can be found in contact with the epidermis or even inside the epidermis in chronic inflammatory skin diseases like psoriasis and chronic leg ulcers (4-7). Since mast cell mediators, e.g. histamine, heparin and TNF-α, have been shown to inhibit keratinocyte proliferation and epithelium growth *in vitro* (8-11), mast cells have been assumed to be involved in controlling the growth of the epidermis. To highlight the importance of these cells in the human body, mast cells have also been viewed to have crucial roles in several other diseases e.g. arthritis (12, 13), fibrosing diseases (14), atherosclerosis (15), myocardial infarction (16) and even dilated cardiomyopathy (17).

This study examined the possible interaction of dermal mast cells and epidermis by using skin organ cultures, proliferating keratinocytes and maturing keratinocyte epithelium together with powerful mast cell mediators, TNF-α and histamine. Also, the effect of retinoic acid on the mast cells growing in skin organ cultures and *in vivo* mast cells was studied. Furthermore, the effect of air exposure to survival of dermal mast cells was studied, and the sequence of events leading to mast cell death was evaluated. The survival and effects of mast cells and their mediators on growth of human keratinocytes and epidermis will be also discussed in later chapters. The roles of mast cells and in normal skin physiology and pathophysiology will be discussed.

2. REVIEW OF THE LITERATURE

2.1 The origin of mast cells and about the nomenclature

Mast cells were originally discovered from the frog mesentery by German pathologist von Recklinghausen (1833-1910) already in 1863 (18). Later the German physics and biochemistry student, nowadays known as Nobel-laureate, Paul Ehrlich (1854-1915) described large, distinctively stained cells containing basophilic granules, for which Ehrlich coined the term "mast cells" (german: *mastzellen,* well-fed cells), suggesting that these cells, rich in granules, could help in the maintenance of the nutrition of connective tissues (19).

Since the time of Ehrlich, scientific knowledge of the molecular and cell structures and their function has increased to form data chaos e.g. many new cell structures and gene products are described every year. To avoid confusions in the names of new structures, the cluster of differentiation (CD) was introduced as a standardized, numerical classification method for cells and their antigens based on monoclonal technology allowing for the specification of cells and their antigens according to their origin, differentiation stage, lineage, and activated state. Also, + or − signs are used to indicate whether the antigen is presented or not in that cell. B-lymphocytes produce immunoglobulins (latin: *immunis,* free + *globules,* a small ball) which are composed of Fc-fragment and the Fab-fragment, which in turn is subdivided into heavy and light chains (Figure 1). The five major classes of immunoglobulins (Ig) are IgA, IgD, IgE, and IgM antibodies. Each immunoglobulin (antibody) class is distinguished by certain effector functions and structural features including a unique heavy chain isotype, designated α (IgA), δ (IgD), ϵ (IgE), γ (IgG), or μ (IgM). Fc receptors (FcR) are glycoproteins that bind specific Fc-fragments of these immunoglobulins (20).

CD34 antigen (gp105-120) is expressed in early lympho-hematopoietic stem and progenitor cells, small-vessel endothelial cells, embryonic fibroblasts, and some cells in the fetal and adult nervous tissue (21). Also, mast cell (MC) precursors are now known to be hematopoietic CD34$^+$ cells in origin (22), being derived from bone marrow stem cells, which enter the circulation as mononuclear cells. The circulating pluripotent mast cell progenitors, CD34$^+$ cells, express the Kit (CD117) receptor for stem cell factor (SCF), IgG receptor FcγRII (CD32) (23), and FcϵRI (24). Human hematopoietic CD34$^+$ stem cells can proliferate and differentiate into mature mast cells even in the mouse skin (25).
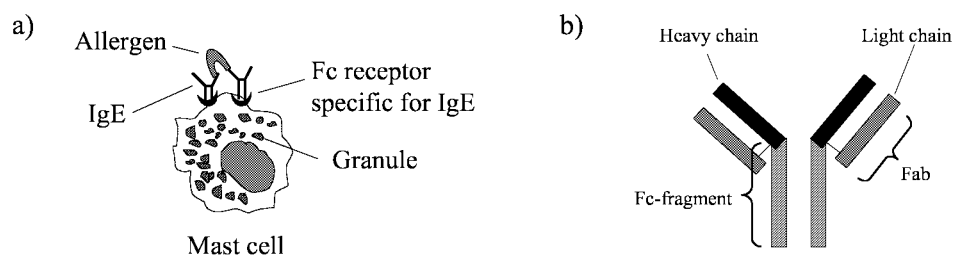
Figure 1. The schematic depiction of a) a mast cell and b) an immunoglobulin.

2.1.1 Homing of the circulatory mast cell progenitors to skin

The mast cell precursors leave the blood and enter the tissues, for example skin, where they undergo the final phase of their differentiation in tissue microenvironments rich in fibroblasts (26). The guiding mechanism for these progenitory mast cells out of vessels to mature in skin, the phenomenon known as the homing effect, is poorly understood. It is known that vessel endothelium is rich in various antigens that cause passing leucocytes to slow down or enable them to extravasate in response to chemokines and cytokines. Keratinocytes are also known to express these mediators, indicating their role in chemotaxis.

The selectin family of adhesion molecules consists of lectins, leukocyte (L-selectin) or endothelium (P- and E-selectin). Human skin-homing T lymphocyte (27) and hematopoietic progenitor cell entry are dependent on the cell-adhesive interactions between the cells and vascular E-selectin (known also as CD26E, ELAM-1, LECAM-2), which is constitutively expressed on postcapillary venules in the skin (28). E-selectin glycoprotein ligand identified on human progenitor cells is the predominant 220-kD sialomucin-like protein, though P-selectin ligand glycoprotein 1 (PSGL-1, CD162) (29, 30) is also expressed in human $CD34^+$ progenitor cells (31). PSGL-1 possibly may act as a skin homing receptor or cutaneous lymphocyte-antigen (CLA) (32). Normal MCs in skin express also $\beta 1$ integrin (CD29), which when activated cause crosslinking of the with specific integrins (CD49c, known also as $\alpha 3$-integrin chain or VLA-3; CD49d, $\alpha 4$-integrin chain, VLA-4; CD49e, $\alpha 5$-integrin chain, VLA-5) (33, 34). Via these interactions MCs are spontaneously associated with laminin and fibronectin *in vivo* and pericellular laminin complexes *in vitro* suggesting that the MC and laminin interactions may be important determinants of mast cell localization in the perivascular regions (33, 34). The possible supplementary linear pathway of human mast cell development from tryptase single positive mast cells into tryptase and chymase double positive mast cells as the cells mature suggests that this maturation process is promoted by interleukin 4 (IL-4) (35), though there is no strong evidence in support of this claim.

2.1.2 Heterogeneity, growth and differentiation of human mast cells

Histochemical studies have revealed the presence of two mast cell phenotypes distinguished by their content, the two different neutral proteases tryptase and chymase – MC_T contains only tryptase whereas MC_{TC} has both tryptase and chymase (36, 37). MC_{TC} are found mainly in the connective tissues and do not appear to be dependent on lymphocyte-growth factors. In normal human skin, the majority of the mast cells are MC_{TC} type (36, 37). Also, chymase-positive and tryptase-negative MC_C mast cell type has been identified (38, 39). The MC_C are rich in chymase and carboxypeptidase and are present in the skin only in low numbers (39, 40).

Mast cells have been considered to be stationary cells in tissues, just waiting to be activated and then releasing their mediators. The primary role of MC_{TC} cell is more likely to be the involvement in angiogenesis and tissue reconstruction but these cells also participate fully in IgE dependent allergic reactions (41). Mast cells have been shown to be present in normal skin in the superficial dermal zone (42, 43). In the recent study, it was also shown that there is no difference between males and females or young

or old individuals in the mast cell distribution (42). The number of mast cells in normal skin has been reported to be 7.3±3.0 x $10^3/mm^3$ of skin (44) responding approximately 384 mast cells per mm^2, though there are other estimates due to the different calculation methods, e.g., values of 48 mast cells per mm^2 in skin from the upper arm (45) or in normal skin 180 mast cells per mm^2 (46) have been reported. In the most recent studies, the density of mast cells in peripheral skin was even over 20 fold compared to central (e.g. abdominal skin) (42). Interestingly, mast cell number was highest in peripheral skin sites e.g. chin and nose, accounting 80 mast cells per mm^2 in upper dermis (42). In the other study, the variation was only minimal, e.g., scalp 7.3 $/mm^2$, neck 7.5 $/mm^2$, flexure of the elbow 8.4 $/mm^2$ but the calculation was done from thicker sections (47). Also, the density gradient, i.e. highest density at the dermo-epidermal junction (10 fold amount compared to amount in upper subcutis) and the lowest in the deeper regions, does not vary in different locations of the body (42, 43). Thus, healthy skin has a proximal/distal and a central/peripheral mast cell gradient (42).

Mast cells have been demonstrated to accumulate or decrease according to different skin conditions showing that they possess the capability to migrate in order to be redistributed within tissues. In mice, it is shown that dry environment may increase mast cell number and their histamine content (48) wheter this happens in humans is not known. There are slightly more mast cells in the atopic lesions as compared to healthy skin, but the increase has no correlation with the clinical severity of the disease (47). More specifically, an increase in the number of tryptase-positive MCs in the upper dermis of non-lesional and lesional atopic subjects is detected but the chymase has lost its activity (49). Mast cells are normally concentrated around blood vessels, nerves and appendages but the epidermis of healthy skin is normally devoid of mast cells (45). That led to hypothesis that mast cells may participate angiogenesis (50), a theory that was later confirmed several ways (51-54), thus mast cells may promote growth and progression of cancers.

2.1.3 Properties and degranulation

Originally mast cells being close to body surface were thought to defend the host against parasites (55). More recently, mast cells were viewed as being harmful due to their key role in allergic and anaphylactic reactions resulting from their degranulation. Degranulation is a sequence of events leading to release of various mast cell granules and their contents into the surrounding cell space. That can happen by immunological or non-immunological ways.

The immunological degranulation process involves binding of immunoglobulins to the specific Fc-receptors that human mast cells express on their surface: FcεRI, FcγRI, II and III. The signalling via Fc receptor may influence the activation of mast cells by either up- or down-regulating certain molecules. The specific nature of mast cell response is directly linked to motifs or subunits of the intracellular chains of these receptors termed immunoglobulin receptor tyrosine activation motifs (ITAMs) (56, 57) or immunoglobulin receptor tyrosine inhibitory motifs (ITIMs) (20, 57). The function of FcεRI receptor is type I hypersensitivity (anaphylaxis), parasite elimination and release of various cytokines (e.g., TNF-α, IL-4, IL-5, IL-6, IL-10). During activation, the tyrosine amino acid groups in the receptor motifs are phosphorylated by the src-kinase

(Figure 2) p56^{lyn} (58), resulting in activation of tyrosine kinase p72^{syk} followed by stimulation of a downstream signalling pathway (59). The activation leads to either the Ras-Raf-MAPK pathway for the cytokine gene expression (60) and arachidonic acid metabolite generation (61, 62) or the pathway for phospholipase C/ phospholipase D and calcium mobilization/ protein kinase C resulting in degranulation (63). FcγRI in turn binds IgG and is up-regulated by IFN-γ. Activation of this receptor leads to an increase in the production of LTC$_4$, PGD$_2$ but also various cytokines are released (e.g., TNF-α and IL-8). In contrast, the function of FcγRII is to inhibit FcεRI mediated mast cell activation by co-aggregating with FcεRI. FcγRIII mainly occurs in bone marrow.
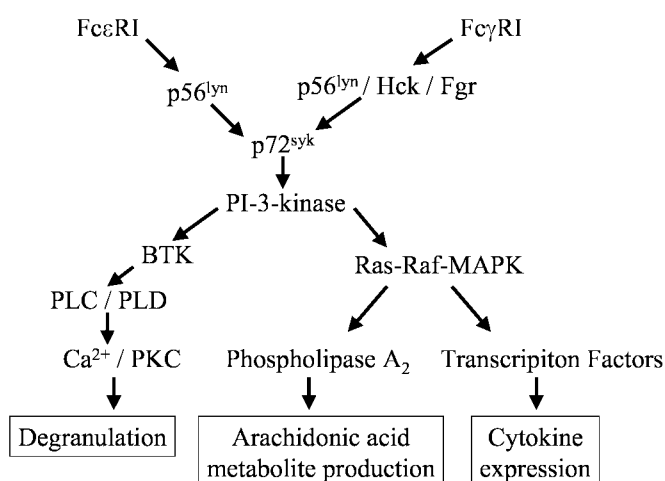
Figure 2. The signalling pathways of FcεRI and FcγRI in mast cells. PI-3 kinase = phosphatidylinositol-3 kinase; PL = phospholipase; BTK = Bruton's tyrosine kinase; PKC = protein kinase C. Other abbreviations are commonly approved nomenclature.

The non-immunological degranulation may occur in anaphylactoid reactions by the direct stimulus of various substances e.g. neuropeptides or by other basic secretagogues such as morphine, poly-L-lysine and compound 48/80 (64). Compound 48/80 is a mast cell degranulator that is widely used in mast cell research since it is a strong histamine liberator (65). Also, nitric oxide (NO) suppresses antigen-induced degranulation, mediator release, and cytokine expression. The action of NO on mast cells is time dependent, requiring several hours, and it is non-cGMP mediated (66). Other endogenous substances such as can induce mast cell degranulation like complement peptides C3a and C5a (67), neuropeptides Substance P and vasoactive intestinal peptide (VIP) (68), IL-4 (69) as well as tumor necrosis factor alpha (TNF-α) (69).

2.1.4 Mast cell mediators

Mast cells play a central role in allergic responses but also can be activated by immunological or non-immunological stimuli. In tissues, antigen-dependent mast cell activation is classically initiated by binding specific IgE to high-affinity FcεRI receptors on their cell surface leading to inflammatory mediator release (70). The presynthesized granule-associated and *De novo* synthesized lipid-derived mediators which are liberated in the immediate response are released rapidly, within 1-5 minutes. The release of cytokines and chemokines, instead, can require enhanced gene expression leading to delayed release of these mediators, it is maximal after 12 hours of antigen binding. The ever increasing list of mast cell mediators is shown in table 1. The supplementary source for the function of these mediators is available on the internet e.g. Cytokines Online Pathfinder Encyclopaedia (http://www.copewithcytokines.de/).

Table 1. A non-exhaustive list of the mediators stored, generated and secreted by human mast cells and their role in skin biology.

Mediator	Role e.g.	Ref
Presynthesized, granule-associated		
Tryptase α, βI, II, III	Potent mediator for inflammation, modulates cell growth. ↑ fibroblast proliferation and fibrosis, activates pro-stromelysin-3, cleaves fibronectin, fibrinogen. See also text.	(12, 71-74)
Chymase α, β	Various proteolytic actions, see also text. ↑ tumor cell cytotoxicity, ↓ keratinocyte and fibroblast growth. See also text.	(75-77)
Histamine	Via H1: contracts smooth muscle, ↑ prostaglandin production, PMN cell chemokinesis, eosinophil C3b receptors, fibroblast and endothelial cell growth, suppressor T lymphocytes, ↓ keratinocyte growth. H2: ↓ basophil histamine release, lymphokine release, polymorphonuclear migration, H1+H2: ↑ mucus production, vasopermeability. H1 and H3: ↑ neurons. See also text.	(11, 78-81)
Cathepsin G	Takes part in blood coagulation, ↓ ECM and BM	(40)
Heparin	↓ keratinocyte growth, Stabilizes granule contents	(11, 82, 83)
Urocanic acid	↑ neuropeptide release from peripheral sensory nerves, ↓ contact hypersensitivity induction via TNF-α	(84, 85)
Carboxypeptidase	↑ degradation of other proteins	(86)
Secretory leukocyte proteinase inhibitor	↓ chymase activity	(87)
De novo synthesized lipid-derived mediators		
Prostaglandin D$_2$	↑ Vasodilation, contracts smooth muscle, vasopermeability, neutrophil chemotaxis. Dominant prostanoid in mast cells. ↓ platelet aggregation	(68, 81, 88)
Leucotriene C$_4$, Leucotriene D$_4$	↑ Vasodilation, vasopermeability, DNA synthesis keratinocytes *in vitro* contracts smooth muscle, promotes prostaglandin and leucotriene E$_4$ generation	(64, 89, 90)
Platelet activating factor	↑ Vasoconstriction, vasopermeability, polymorphonuclear cell and monocyte chemotaxis	(91)
Cytokines and chemokines		
TNF-α	↑ adherence and migration of inflammatory cells, fibroblast growth and chemotaxis, mast cell histamine and tryptase release, adhesion molecule expression on endothelial cells, tumor cell cytotoxicity. See also text.	(92) (86)
IL-1α	↑ mast cell growth	(93)
IL-1β	↑ arachidonic acid metabolite formation	(94, 95)

IL-3	↑ mast cell survival	(96)
IL-4	↑ fibroblast proliferation, chemotaxis and matrix protein production, IgE production, B cell proliferation, B cell IL-6 production, T cell proliferation. ↓ macrophage killing activity and cytokine production	(25, 86, 97)
IL-5	↑ eosinophil chemotaxis, growth and survival	(86, 97)
IL-6	Contributes to allergic, other immunologically mediated and nonspecific inflammatory responses.	(86, 98))
IL-8	↑ IgE production, T cell growth and differentiation	(99)
IL-10	↑ neutrophil recruitment, ↑ polymorphonuclear cell chemotaxis ↑ proliferation of mast cells, ↓ cutaneous inflammation, ↓ cutaneous type 1 inflammation and normalization of keratinocyte maturation, regulation of tissue remodeling	(100, Reich, #754; 101: 2001)
IL-13	↑ IgE expression, e.g. in atopic dermatitis	(102, 103)
IFN-γ	Regulation of immune and inflammation processes	(104)
Monocyte chemotacting protein -1, 5	Potent activator of monocytes and mast cells, chemotactic for monocytes, regulates expression of cell surface antigens and IL-1, IL-6 ↑ degranulation, release of histamine, synthesis of leukotriene C4, type I collagen expression in cultured fibroblasts.	(105-107)
Monocyte inhibiting protein -1a, 1b	May initiate, augment, or inhibit histamine release	(108-110)
RANTES	May initiate, augment, or inhibit histamine release	(108, 109)
Transforming growth factor -β	↑ production of monocyte chemotacting protein-1 in fibroblasts	(107)
Stem cell factor	↑ mediator release from MC, MC survival. See also text.	(111, 112)
Nerve growth factor	Additional mast cell growth factor, may activate MC, ↑ survival of UVB induced keratinocytes	(113, 114)
Fibroblast growth factor -2, 7	Activates fibroblasts	(115)
Granulocyte-monocyte colony stimulating factor	↑ MC mediator release, keratinocyte proliferation and apoptosis	(108, 116)
Platelet derived growth factor	↑ MC growth	(117)
Vascular endothelial growth factor	↑ Vasopermeability, angiogenesis	(54, 118)
Heparin binding epidermal growth factor	Activates fibroblasts	(115)

↑ = increase, ↓ = decrease, BM = Bone marrow, CGRP = Calcitonin gene-related peptide, ECM = Extracellular matrix, HI-3 = histamine receptor type, IFN = interferon, IL = interleukin, MC = mast cell, RANTES = Regulated upon Activation, Normal T-cell Expressed, and presumably Secreted, VIP = Vasoactive intestinal peptide.

2.1.4.1 Histamine

Histamine (β-imidazolethylenamine) was the first identified mast cell mediator (119). It is a biogenic amine synthesized in mast cells by the action of histidine decarboxylase (120) from the amino acid L-histidine (121, 122). The molecular weight of histamine is 111 Da and it is stored in mast cell granules bound to the carboxylic residues of the heparin proteoglycan (82, 123). The concentration of histamine within mast cell secretory granules approximates 100 mM (1-2 pg/cell, 40% cell volume in granules, 10 μm cell diameter) (77). There are other estimates of the concentration of histamine in the dermal skin, i.e. about 50 μM based on an assumption of 3100 mast cells/mm^3 (124) or 5-8 μg histamine/g wet weight of skin and assuming that 1 g skin equals to 1 ml (125, 126).

Four G-protein-coupled histamine receptors have been characterized, H1 (78), H2 (127), H3 (128) and recently H4 (80). H1 functions through activation of phospholipase C with the generation of inositol 1,4,5-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) and increased cytosolic Ca^{2+} (129). H2 receptors are coupled both to the adenylate cyclase and phosphoinositide systems though the stimulation of the H2-receptor results primarily in cAMP formation (130). H3 receptor is expressed on histaminergic neurons in the brain, with low expression in peripherial tissues (130). The novel H4 receptor and H3 receptor both activate cells by inhibiting cAMP accumulation and increasing intracellular Ca^{2+} (130). Allergic reactions evoked by histamine are mediated mainly via the H1 receptor. H1 receptor antagonists are commercially available e.g. cetiritzine, hydroxyzine, loratadine, which all are referred to "antihistamines". Also, specific H2 receptor antagonists are avalaible, e.g., cimetidine, famotidine and ranitidine, which are known as "H2 receptor blockers" used for hyperacidicity of gastric mucosa. Purified histamine is commercially available.

Histamine can be detected classically from human plasma or other fluids by using radio enzyme assay (REA), high performance liquid chromatography (HPLC) with post-column derivatization system or radio immuno-assay (RIA) (131), gas chromatography and mass spectroscopic detection (132) or also by immunohistochemistry (133). Also, a histamine-free, histidine decarboxylase knockout mouse model has been developed (134). Mouse models represent interesting tools because recently it was found that over 90% of the mouse and human genomes can be partitioned into corresponding regions of conserved synteny, reflecting segments in which the gene order in the most recent common ancestor has been conserved in both species (135, 136).

Histamine is a potent mediator for vasodilation and increased capillary permeability. It can be released from mast cells during degranulation via slow IgE-dependent (more than 5 minutes) and rapid (less than a minute) non-immunological stimulation (137). After degranulation, histamine is catabolized along two main routes, deamination by diamine oxidase (138) and methylation of the imidazole ring by methyltransferase (121, 126, 139). The various metabolites have no significant physiological activity and are excreted in the urine (140), the main metabolite is tele-methylimidazoleacetic acid (141).

Histamine has been found to inhibit epidermal cell outgrowth and mitosis (79, 142). Inhibition was maximal at a histamine concentration of 10^{-4} M (142). In contrast,

imidazole acetate, a histamine breakdown product, was found to be a striking mitotic stimulator in organ culture (143). Histamine also modulates the proliferation of keratinocytes - by binding to the H2 receptor on the keratinocyte membrane it induces activation of adenylate cyclase and phospholipase C through a GTP binding protein and evokes a transient increase in intracellular Ca^{2+} (144), thus inhibiting the growth. In skin organ cultures, histamine significantly stimulates the proliferation of keratinocytes as compared to controls, reaching maxima within a range from nano- to micromolar concentrations (145). These findings support the proposal that mast cell products can modulate keratinocyte proliferation, and point to a role for mast cells in the regulation of epidermal tissue turnover under physiological conditions. The suppression of TNF-α synthesis by histamine is likely to be a transcriptional event, since histamine caused a four-fold reduction in TNF-α mRNA levels this effect being reversed by cimetidine or ranitidine, i.e., H2 receptor antagonists (146).

High histamine levels have been measured in blister fluids of bullous pemphigoid (147). Also, acute ultraviolet light B (UVB) exposure decreases the treshold for histamine stimulation in skin cells but also induces histamine release from mast cells (81). Elevated histamine levels are also found in suction blister fluids collected from UVB-exposed skin (148, 149).

2.1.4.2 Tryptase

Tryptase (EC 3.4.21.59) is a 135 kDa tetrameric serine proteinase (150) found inside the granules of MC_{TC} and MC_T cells. Tryptase is stored in the secretory granules bound to heparin proteoglycan which maintains the tryptase in a stable state in physiological conditions (151). Tryptase is released during mast cell degranulation as an active heparin complex. The adult foreskin contains 35 pg tryptase per mast cell (77). The human mast cell tryptase gene is located to chromosome 16p13.3 (73, 152) in the protease gene cluster encoding tryptases α, βI, βII, βIII, transmembrane tryptase (TMT) (153), and eosinophil serine protease-1 (Esp-1, testisin, PRSS21) (154). Tryptase βII and βIII probably are allelic variants of the same gene (152). Recently, tryptase δ (74) and tryptase ε (PRSS22) have been characterized (73). The mast cells that reside in various human connective tissues express many combinations of tryptases α, βI, βII, βIII, and TMT (155). The tryptase α is rather inactive, though it is also secreted and stored in skin mast cells. Tryptase β is the predominant form of tryptase in the skin and it consists of monomeric subunits (156). The human β-tryptase, after mast cell degranulation and exposure to neutral pH in the tissue, may become dissociated into active monomers and possibly, some of the biological activities of human tryptase may be attributable to active tryptase in its monomeric form (157).

Tryptase can be inhibited by synthetic inhibitors e.g. N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), leupeptin (158), and by the recently developed nonelectrophilic MOL 6131 based upon a β-strand template (159).

Tryptase is capable of cleaving synthetic substrates which have the basic amino acids lysine and arginine, e.g., 1,2-benzisothiazol-3-one 1,1-dioxide (160), Z-Gly-Pro-Arg-p-nitroanilide (pNA), Z-Gly-Pro-Arg-AMC, benzoyl-L-arginine ethyl ester (BAEE) and

tosyl-L-arginine methyl ester (TAME) all can function as substrates (158, 161, 162). These synthetic substrates have widely been used for the detection of tryptase from biologically active samples (71, 126, 150), commonly by micro-ELISA reader.

The effects of tryptase on surrounding cells and matrix proteins are largely dependent on its enzyme activity. Tryptase is stable when bound to heparin, and no physiologic inhibitors for tryptase have been found. This makes sense while mast cells predominantly are present in tissues, e.g., mucosa and skin where they can come into contact with exogenous bacteria, and other biologically active material and have important protective functions. Agents which displace tryptase from heparin can inactivate this enzyme since the tryptase tetramer dissociates into inactive monomers (163). Interestingly, tryptase may be a potent stimulus of microvascular leakage (164) leading to inflammatory conditions. The anatomical association of mast cells with nerve tissue and blood vessels may have role in dermal neurogenic inflammation (165-167). Tryptase is able to modulate the biologic effects of neuropeptides, e.g. by cleaving the VIP. Protease activated receptors (PAR-1 and PAR-2), are members of the G protein signal transduction receptors that are activated by proteolysis (168). Tryptase can also hydrolyse PAR-2 receptors present in the surrounding keratinocytes and fibroblasts (168) during inflammation in atopic dermatitis and psoriasis (168). Tryptase has also an important role in normal regulation of extracellular matrix turnover, wound healing and tumor metastasis (115, 169). Furthermore, tryptase has been found to stimulate histamine release from synovial cells (13). Tryptase can bind efficiently to heparin and can degrade the basement membrane and induce dermal-epidermal separation. Tryptase can degrade the pericellular matrix of fibroblasts, as well as cleaving fibronectin *in vitro* and in the basement membrane *ex vivo* (170, 171) contributing focal dermal-epidermal separation and blister formation. Tryptase can also stimulate fibroblast proliferation (115, 172) and collagen synthesis (173, 174) as well as vascular tube formation and hence angiogenesis (51). Thus, there is evidence in favour of concept of a synergistic effect of the mast cell mediators.

Recombinant human β-tryptase has already been produced (175). Furthermore, a mouse model for studying the effect of tryptase in asthma has been developed, in this model MOL 6131 reduced airway inflammation (159).

2.1.4.3 Chymase

Chymase (EC 3.4.21.39) is a glycosylated chymotrypsin-like serine proteinase stored as a heparin bound complex in the granules of MC_{TC} cells (176, 177). The name chymase was proposed by David Lagunoff and Earl P. Benditt in 1963 (178) to denote an enzyme similar to pancreatic chymotrypsin that had been first detected in mast cells 10 years previously (179). Human chymase is a monomer of 27 kDa the activity of which is regulated within the mast cell granule by pH and heparin (180). Chymase is encoded by a gene located in 14q11.2 (181). It was initially purified from rat skin (75) though it had earlier been purified from mast cells of rat thyroid (182). Subsequently, it has been cloned and produced as recombinant human chymase (183). In the adult skin, the majority of the mast cells are MC_{TC} cells (36) and the chymase content per single mast cell has been found to be 4.5 pg (77). Chymase has been classified by structural analysis and reclassification into two groups α and β (184). There are 5 known mouse

chymases of which all but chymase 5 are β chymases (185) similar to rat chymases 1 and 2 (186). The mouse chymase 5, rat chymase 5, dog chymase and human mast cell chymase all are referred to α chymases (186).

Chymase activity can be measured by using commercially available substrates like N-benzoyl-L-tyrosine ethyl ester, N-acetyl-L-tyrosine ethyl ester, and Suc-Ala-Ala-Pro-Phe-pNA (75, 187-189). Mast cell chymase can be inhibited by diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) (190), lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), α_2-macroglobulin (191), α_1-proteinase inhibitor (α_1-PI), α_1-antichymotrypsin (α_1-AC) (192). Recently, it was found that secretory leukocyte proteinase inhibitor (SLPI) can also inhibit chymase, especially if chymase and heparin are released from mast cell granules simultaneously (87). SLPI is also located and produced in mast cells (193, 194). Chymase-induced mast cell accumulation may occur via the ability of chymase to process membrane-bound SCF on the epidermal keratinocytes (195).

Chymase is a major constituent of the secretory granules of human mast cells, but little is known of the contribution of this serine proteinase to acute allergic reactions. It has recently been found to induce microvascular leakage in vivo (196). Little evidence was found for synergistic interactions between chymase and either histamine or tryptase (196). Thus, chymase could contribute to the increases in microvascular permeability seen following mast cell degranulation in allergic diseases (196). Chymase cleaves a peptide bond within the SCF protein between Phe[158] and Met[159] resulting in a soluble bioactive SCF which in turn may stimulate mast cell proliferation and differentiation (197). Mast cell chymase, like leukocyte elastase can efficiently release matrix-bound latent TGF-β1 complexes from cultured epithelial cells, possibly contributing to the accumulation of connective tissue in inflammation (198), i.e., promoting the formation of fibrosis. The rat chymase can activate specifically latent TGF-β1, indicating that mast cells can act as potent paracrine effector cells both by secreting active and by enhancing the TGF-β1 response in target cells (199). Chymase-released TGF-β1 inhibits the growth of smooth muscle cells and induces their apoptosis (200, 201). Also, the MC density and chymase activity has been found to increase in dermal fibrotic processes, e.g., in burn scars in mice (202).

PAR-1 (thrombin receptor) can be hydrolysed by the chymase (168). Chymase can also cause degradation of the epidermal-dermal junction leaving the bullous pemphigoid antigen on the epidermal side and the laminin on the dermal side of the split (1). Chymase has been reported to induce the mitogenicity of fibroblasts (203). Chymase can release fibronectin and soluble CD44 from the pericellular matrix of airway smooth muscle cells in vitro (204). Both tryptase and chymase can also promote matrix degradation indirectly via activation of collagenolytic metalloproteinases and urinary-type plasminogen activator (75, 170, 198, 200, 204). In addition to causing matrix degradation, tryptase and chymase are thought to be involved in repair processes and matrix deposition. Chymase, like tryptase, can bind efficiently to heparin. Chymase seems to be more effective, since purified chymase separated fully the epidermis from the dermis at the level of lamina lucida whereas purified tryptase induced only a focal separation (1, 171).

There are also many knock-out mouse models developed for investigating the role of chymase in skin pathophysiology, e.g., models of atopic dermatitis (205), fibrosis in scleroderma (206) and angiogenesis (52).

2.1.4.4 TNF-α

The tumor necrosis factor superfamily has been characterized in humans as at least three members: cachectin alias tumor necrosis factor alpha (TNF-α, TNF2), lymphotoxin alpha (LTA, TNF1, TNF-β) and lymphotoxin beta (LTB, TNF3). TNF-α is a 17-kDa proinflammatory cytokine (207) that is stored and secreted by human dermal mast cells (92). Elsewhere in the body, it is mainly secreted by macrophages and it can induce cell death of certain tumor cell lines. It is a potent pyrogen causing fever by direct action or by stimulation of IL-1 secretion and it is implicated in the induction of cachexia. Under certain conditions, it can stimulate cell proliferation and induce cell differentiation. The gene for human TNF-α is located on human chromosome 6p21.3 and it encodes a 233-amino-acid-long 26 kDa polypeptide. That polypeptide is further cleaved by TNF-alpha-converting enzyme (TACE) releasing the soluble TNF-α (208). TACE is a member of "A Disintegrin And Metalloprotease-" (ADAM) family of zinc metalloproteases (208). Also, epidermal keratinocytes secrete c which exerts its biological activities via binding to specific cell surface receptors present in almost all cells including keratinocytes (209). Two distinct TNF-α cell surface receptors (TNF-R, 55 and 75 kDa) have been identified and cloned (210, 211). In normal epidermal keratinocytes, the effects of TNF-α are apparently mediated via 55-kDa TNF-R (CD120a, TNFRI, p55) (209). In the skin mast cells are the primary source of TNF-α but also activated keratinocytes may produce it (212).

The TNF-α can be detected from human samples, e.g. plasma, by using highly sensitive ELISA-methods or from tissues by means of specific antibodies. The synthesis of TNF-α is induced by many different stimuli including interferons, IL-2, GM-CSF, substance P, bradykinin, immune complexes, inhibitors of cyclooxygenase and platelet activating factor (PAF). The production of TNF-α is in turn inhibited by IL-6, TGF-β, vitamin D3, prostaglandin E2, dexamethasone, cyclosporin A, and antagonists of platelet activating factor (PAF). The specific inhibitors e.g. etanercept and infliximab for TNF-α are already in clinical use. Etanercept is a recombinant dimeric form of the soluble TNF p75 receptor that binds tightly to TNF and to lymphotoxin, keeping them in biologically inactive form (213). Infliximab is a chimeric (mouse-human) monoclonal antibody that binds and neutralises the TNF-α (213). Both of these agents are already in clinical use in the treatment of severe psoriasis and psoriatic arthritis.

TNF-α also acts on a variety of cells including mast cells (92), thus, enhancing inflammatory and immune processes. Unregulated increases in the levels of TNF-α could be pathogenic also in inflammatory diseases. TNF-α is cytostatic for normal keratinocytes and may thus play a role in inhibiting epidermal proliferation (9). The production and secretion of TNF-α depends on the state of cellular stimulation. TNF-α can induce local proliferation of fibroblasts, capillaries, and epidermal cells (214) and is thought to play a major role in many inflammatory skin diseases. TNF-α expression has been shown to be markedly increased after UVB-exposure in the epidermis (215). This

cytokine has been thought to be responsible for the formation of sunburn cells that do not express differentiation markers like involucrin (216). TNF-α can induce ICAM-1 expression on keratinocytes and dermal endothelial cells providing adhesion sites for leukocytes (8, 217, 218). Stimulation of mast cells by rh-TNF-α evokes a concentration dependent release of histamine and tryptase (219). TNF-α appears to be a direct stimulus for causing mast cells to degranulate and to release both histamine and tryptase (219). Also in contrary, histamine has found to inhibit TNF-α release by mast cells through H2 and H3 receptors (220). TNF-α has been found to inhibit cell proliferation in human keratinocytes cultured in serum-free medium in a time- and dose-dependent manner with a minimum effective dose of 10 U/ml and a 50% inhibitory dose of 100 U/ml (217). Even at subnanomolar concentration, TNF-α significantly inhibits the incorporation of labelled thymidine by epidermal keratinocytes in murine skin organ culture (145) but its growth inhibition was completely reversible. Furthermore, TNF-α altered the morphology of the growing keratinocytes, inducing the appearance of a fusiform, fibroblast-like population (217, 221). It is claimed that TNF-α activity is determined by the balance of apoptosis-inducing and apoptosis-preventing factors that also may be exogenous mediators (222). The cytotoxic effect of TNF-α occurs primarily in the G_0-G_1 phase of the cell cycle (223, 224).

2.1.4.5 Stem cell factor

Stem cell factor (SCF, MGF, Kit ligand), the ligand for the receptor encoded by c-kit gene (225), is known to be produced by fibroblasts, keratinocytes and endothelial cells but also by human skin mast cells (226). The gene encoding SCF is located in chromosome 12q22 which produces a 31 kDa polypeptide. Two different SCF-specific mRNA splice variants encode for either soluble (SCF-1) or membrane-bound (SCF-2) forms. Their differential expression in immature and mature human mast cells, e.g., the secretion of SCF-1 or -2 by these cells, may play a role in autocrine stimulation, maintenance of survival and the differentiation of tissue mast cells (227). A possible feedback loop has also been described in which chymase released from mast cell secretory granules may solubilize SCF bound to the membrane of surrounding stromal cells (197). The liberated soluble SCF may in turn stimulate mast cell proliferation and differentiated functions; this loop could contribute to the abnormal accumulations of mast cells in the skin and hyperpigmentation, at the sites of chronic cutaneous inflammation. SCF is normally expressed in blood progenitory cells, melanocytes, germ cells, basal keratinocytes and mast cells ad it is considered to be important in their growth and development.

SCF is the principle cytokine that has been discovered to promote mast cell proliferation and/or differentiation (228). SCF acts to maintain MC viability and maturation (228), it is a growth factor that can act synergistically with other growth factors and matrix components (228). SCF has also been shown to be a chemoattractant cytokine for human mast cells (229).

Mast cells express also the receptor tyrosine kinase, the stem cell factor receptor (CD117, SCFR, Kit protein) encoded by the proto-oncogene c-kit (225). Ligation of SCFR induces its dimerization and activation of its intrinsic tyrosine kinase activity leading to activation of Raf-1, phospholipases, phosphatidylinositol 3-kinase, and

extracellular signal-regulated kinases (230). SCF can augment FcεRI-mediated JNK activation and cytokine gene transcription but this occurs via pathways that are regulated differently than those activated through FcεRI (230). Imatinib (STI 571 or CGP 57148B) is a selective inhibitor for Kit receptor (231). This drug is in clinical use as an anticancer drug in hematological malignancies.

The capacity to expand subsets of antigen-specific lymphocytes that become activated by environmental antigens is termed "acquired" immunity. Immunologic memory, although a fundamental aspect of mammalian biology, is a relatively recent evolutionary event that permits organisms to live for years to decades. "Innate" immunity, mediated by genes that remain in the germ line conformation and encode for proteins that recognize conserved structural patterns on microorganisms, is a much more ancient system of host defense. The studies using genetically mast cell-deficient WBB6F1-Kit^{W}/Kit^{W-v} and congenic wild-type (WBB6F1-+/+) mice indicate that mast cells can also promote health, by participating in natural immune responses to bacterial infection (232). Repeated administration of SCF also can enhance survival in mice that genetically lack TNF-α, demonstrating that the ability of SCF treatment to improve survival does not solely reflect the effects of SCF on mast cell- dependent (or - independent) production of TNF-α (232). These findings point to c-kit and mast cells as potential therapeutic targets for enhancing innate immune responses.

The recombinant human SCF (rhSCF) has been developed and widely used for mast cell studies. RhSCF potentiates histamine release from MC through immediate and delayed mechanisms, but has no effect on TNF-α release (233). This regulation of MC by SCF may be important in allergic and other inflammatory diseases. After subcutaneous injection of rhSCF, a wheal and flare reaction develops at the injection site and electron microscopy reveals that most dermal mast cells at these sites exhibit extensive, anaphylactic-type degranulation (234). RhSCF significantly increases dermal mast cell density at sites distant to the injection with the cytokine and also increases both urinary levels of the major histamine metabolite, methyl-histamine, and serum levels of mast cell alpha-tryptase (234) thus indicating that rhSCF can promote the functional activation of human mast cells *in vivo*. It has also been shown, that though the numbers of resident mast cells are very low in human cutaneous scars, the tissue does not contain a mast cell subpopulation that is chymase -, avidin -, tryptase +, Kit + (235). This may suggest massive mediator release from these cells into fresh wounds and increased immigration and/or proliferation of immature mast cells and their precursors (235). Also, a mast cell deficient mouse model has been developed to study the effect of SCF (236).

2.2 The structure of human skin

The skin is the largest organ of the human body, consisting of epidermis (appr. 0.03-0.13 mm in thickness), dermis, and subcutis with fat tissue. The thickness of different skin layers varies in different locations of the body, e.g. subcutis is thin at the face and thick at the abdominal area, or stratum corneum and granulosum are thicker in the soles of the feet than at the face. Epidermis, as the outermost surface, mainly consists of keratinocytes, melanocytes and Langerhans cells. Keratinocytes form four different layers, named according to their histological appearance: one cell layer of stratum basale or germinativum, three to four layers of stratum spinosum, two to three layers of stratum granulosum, and multilayered stratum corneum (Figure 3) (237).
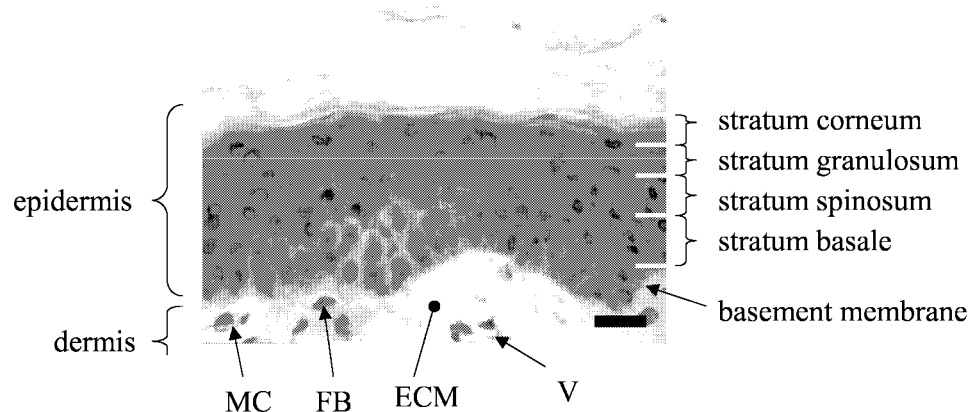
Figure 3. The histological structure in the cross-section of the human skin stained with hematoxylin and eosin. Hyperkeratotic appearance is due to one day cultivation prior tissue processing. Abbreviations: ECM = extracellular matrix, FB = fibroblast, MC = mast cell, V = blood vessel. Scale bar = 10 μm.

Melanocytes produce melanin pigment that darkens the skin and has an important protective function. Langerhans cells in turn take part in the immunological first line defence of the skin. The majority of epidermis consists of keratinocytes. Two types of the basally located keratinocytes have been identified: stem cells that have high proliferative capability, and transit amplifying keratinocytes that undergo actively terminal differentiation (238). The basal keratinocytes are connected to the basement membrane, which is the tight, separating border between dermis and epidermis. Basement membrane consists of three layers: the lamina lucida, the lamina densa, and the lamina fibroreticularis (237). When the keratinocytes receive the signal for the differentiation, multiple regulative events occur and the keratinocytes lose their contact to the basement membrane and migrate outwards forming the rest of the epidermal cell layers. Furthermore, the keratinocytes actively produce proteins and lipids needed to form the lipid bilayers at the squamous layer as a barrier against water loss and also provide defence against the microbial, mechanical and chemical attack (239). The simplest and most common way to remove the dead keratinocytes is to scale from the

outer surface of the skin. The dead keratinocytes can also be removed in some cases by leukocytes and adjacent cells in the opsonization or endocytosis after necrotic or programmed cell death. The time required for transition of basal cells to the surface of skin is 47-48 days (240), denoted as the epidermal turnover time. Dermis consists mainly of fibrillar collagen networks, elastin fibers, glycosaminoglycans, proteoglycans, fibronectin, that all support fibroblasts, nerves and sensory nerve endings, different sized vessels, hair bulbs but also mast cells (237).

Basal cells are in close contact with basement membrane, other basal cells and upper spinous keratinocytes, showing important polarity. They also contact with melanocytes and are also possibly affected e.g. by mast cells and their cytokines and growth factors. The spinous layer forms bundles of keratin filaments, together with the desmosomes and the adherens junctions (241), giving the spinous appearance. The cornified cell envelope precursor protein, involucrin, is synthesized already in upper stratum spinosum (242). In the granular layer, keratohyalin granules are synthesized and in the uppermost layers they form lamellae between first cornified cells. In the stratum corneum, the lipid and intermediate filament containing keratinocytes rapidly lose their nuclei and form a dense barrier, sheding the lower skin structures against attacks of outside world. Also, keratinocytes and mast cells are immunologically active (109), ready to secrete cytokines and chemokines when need be. Immunological and inflammatory reactions are mediated by complex network of soluble products but also by intercellular adhesion molecules.

Intercellular adhesion molecule-1 (ICAM-1, CD54) is coded by a gene that is located in chromosome 19p13.3-p13.2. It is typically expressed on non-hematopoietic cells such as vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, and fibroblasts, and on hematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal center dendritic cells in tonsils, lymph nodes, and Peyer's patches (243). ICAM-1 may be a ligand for an integrin leucocyte function associated antigen one (LFA-1, known also as integrin heterodimer CD11a/CD18 or $\alpha L \beta 2$) dependent reactions (244). Both histamine and TNF-α have been shown to induce ICAM-1 expression in cultured human keratinocytes and these mediators function synergistically leading to increased ICAM-1 expression (8, 245). Retinoic acid (246) and histamine (247) have been found to enhance TNF-α-induced ICAM-1 levels in human keratinocytes. However, ICAM-1 expression in keratinocytes does not only lead to T cell activation but can also cause increased lysis of keratinocytes by cytotoxic T cells (9, 248), thus showing the active participatory role of keratinocytes in cutaneous immunohomeostasis.

The keratinocytes as the principle epidermal cells produce and secrete multiple cytokines that are modulated by one or more other cytokines, including several agents that keratinocytes themselves secrete (Table 2). These effects appear to be mediated by high-affinity cytokine receptors on keratinocytes. However, the cytokines are also capable of interacting with dermal cells. Thus, the function of skin as an immune organ is extremely diverse.

Table 2. The non-exhaustive list of the mediators stored, generated and secreted by human epidermal keratinocytes and their role in skin biology. The role of mediators are partly supplemented from the database located http://www.copewithcytokines.de/.

Mediator	Role, e.g.	Ref
TNF-α	May cause apoptosis in KCs, ↓ proliferation of keratinocytes. Multiple immunological events. See also text.	(215, 221, 249)
Histamine	↑ UVB-induced IL-6 production by KCs, ICAM-1 expression , ↓ proliferation of keratinocytes	(11, 245, 250)
IL-α	↑ T-helper cells, B-cell activation and immunoglobulin production, proliferation and activation of fibroblasts, adhesion of leukocytes, IL-6 expression	(212, 251, 252)
IL-3	May participate in the regulation of hematopoietic cells and turn on early nonspecific host defense mechanisms	(212, 253)
IL-4	↑ proliferation and differentiation of activated B-cells, ↓ migration of Langerhans cells	(254)
IL-6	modulates keratinocyte differentiation,	(212, 252)
IL-7	↑ adhesion of the epidermal T cell to laminin-5	(255, 256)
IL-8	↑ metabolism of reactive O₂ species, chemotaxis, expression of adhesion molecules. Antagonizes IgE production.	(212, 249)
IL-10	↓ synthesis of a number of cytokines, ↑ as a costimulator (IL-3, 4) proliferation of mast cells	(257, 258)
IL-12	↑ Th1 activation	(259)
IL-15	↑ mast cell proliferation	(260)
IL-18	↑ IFN-γ, Fas-ligand (CD-95L)	(261, 262)
VEGF	↑ angiogenesis	(263)
VPF	↑ vascular permeability	(263)
IFN-γ	May amplify allergen-specific T-lymphocyte-triggered responses in contact dermatitis, ↑ ICAM-1 expression	(264, 265)
PDGF	↑ MC growth	(117, 212)
EGF	Strong mitogen, chemoattractant for fibroblasts and epithelial cells, differentiation factor. ↓ TGF-beta receptor	
FGFs	↑ angiogenesis, growth of fibroblasts, KCs	(54, 266)
TGF-α	↓ degeneration of epidermis	(212, 267)
TGF-β	most potent known growth inhibitor	(212)
GM-CSF	↑ induces keratinocyte proliferation, ↓ induces keratinocyte apoptosis	(116, 212)
Nerve growth factor	↑ promotes keratinocyte growth autocrinically, mediates the effects of retinoic acid	(268, 269)
HB-EGF	↑ basal KC growth	(270, 271)
Amphiregulin	autocrine growth factor of KC	(272), (271)
Epiregulin	autocrine growth factor of KC	(271), (273)

↑ = increase, ↓ = decrease, EGF = epidermal growth factor, FGF = fibroblast growth factor, GM-CSF = granulocyte monocyte colony stimulating factor, HB-EGF = heparin-binding growth factor, IFN = interferon, IL =interleukin, KC = keratinocyte, MC = mast cell, PDGF = platelet derived growth factor, TGF = transforming growth factor, VEGF = vascular endothelial growth factor, VPF = vascular permeability factor

2.3 General characteristics of cell and skin organ cultures

The long term cultivation of human epidermal cells was first achieved several years ago. Kitano (in 1970) firstly introduced a method so that trypsination could separate the epidermal cells and that the keratinocytes grew as cells attached to each other forming a sheet (274). This method was improved later by Prunieras (275) who demonstrated the regeneration ability of skin *in vitro*. Flaxman and Harper (1975) studied the effect of various agents on proliferation by using keratinocyte cultures (143). Later it was found that keratinocyte growth depends on endocrine stimulation by other organs, including the brain (276). At the beginning of the 80's, the efforts to improve culture of keratinocytes in vitro focused on two main aspects – ways to improve the proliferation by hormones or growth factors in culture medium but also to search for some way to achieve terminal differentiation of keratinocytes *in vitro* (277). By culturing the keratinocytes in delipidized serum, i.e., in the absence of vitamin A, the keratinocytes started to terminally differentiate (278), leaving the cell cycle (Figure 3).

The cell cycle (Figure 4) of keratinocytes consists of four phases: a DNA synthesis phase (S) and cell division phase (M) separated by two gap phases (G_1 and G_2). Non-dividing or non-cycling cells are resting in the G_0 phase, from where they can be driven into the cycle if required (279). Then the life cycle continues, alternatively the cells may reach the non-cycling state to either terminally differentiate or just stop growing and be dormant waiting for the appropriate stimulus for the start of cycling. The whole of the cycle is strictly controlled by various growth factors and molecules. The response of growth modulating molecules may vary depending on the cell cycle phase.
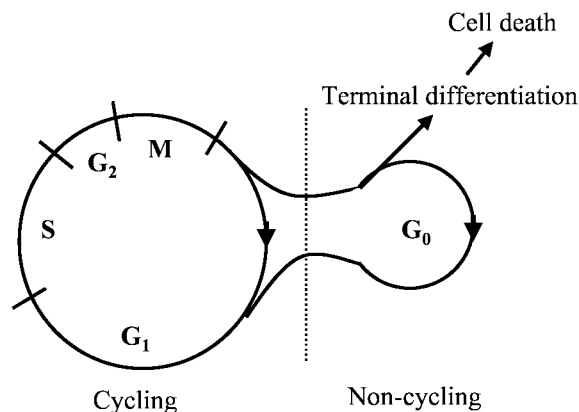
Figure 4. A schematic representation of the cell cycle for keratinocytes.

Human epidermal keratinocytes in culture retain many of the properties of the intact epidermis. One useful marker for an early stage of terminal differentiation is the presence of involucrin, a soluble protein precursor of the cross- linked envelope (280). Involucrin is synthesized after keratinocytes have left the basal layer and started to enlarge, but some time before the onset of envelope cross-linking, which occurs only in the outermost cell layers (280). If the level of calcium is raised in the keratinocyte

culture to induce stratification, involucrin- positive cells are selectively expelled from the basal layer (280-282).

The skin organ culture is a method for studying *ex vivo* skin biology. The modified method of Trowell (283) is widely used with slight modifications (275, 284, 285). The method of Trowell failed to become a popular experimental tool because organ cultured human skin grew poorly and lacked some of the structures of skin *in vivo* (286). It was also demonstrated that the temperature, oxygen tension, and pH of the medium could all affect the physiology of cultivated skin tissue (287). The cultivation was in most cases non-physiological due to immersion in medium but using the air-exposure achieved by growing the cultures at the air-liquid interface made the cultures more physiological (288). Nowadays, expensive living skin equivalents mimicking normal skin are commercially available but mast cells and the remaining physiological structures are commonly omitted. In organ cultured human skin, the dermal cells are found to survive up to 10 weeks (289) but the degradation of dermis and especially epidermis can be seen already at day 7 (285). Furthermore, high levels of both serine and matrix metalloproteinases are present in skin organ culture fluids suggesting that degeneration of the tissue is taking place (290). *Ex vivo* models were developed to mimic the *in vivo* conditions, due to the cultivation difficulties encountered with pure mast cells. Recently, a culture model has been developed where normal dermal mast cells and fibroblasts were enclosed in a collagen gel and normal keratinocytes were grown on top with exposure to an air interface (291).

2.3.1 Survival of mast cells

Apoptosis is a mechanism of physiological cell death that deletes cells during development and homeostasis (292). The cytoplasms of apoptotic cells become condensed and dyskeratotic, and the cells are then phagocytozed as fragments by surrounding cells (292). At the biochemical level, the fragmentation of cellular DNA into oligonucleosome-sized particles is characteristic of apoptosis (293). Fas antigen is a member of nerve growth factor and tumor necrosis factor receptor superfamily, acting as a membrane protein that induces apoptosis in human keratinocytes (294). Fas-ligand (CD95, APO-1L) induces apoptosis and it is regulated by metalloproteases (295).

The differential expression of the soluble (SCF-1, sSCF) or the membrane-bound form of SCF (SCF-2) in immature and mature human mast cells may play a role in autocrine stimulation, maintenance of survival and differentiation of tissue mast cells (227). A possible paracrine stimulation has also been described in which chymase released from mast cell secretory granules may solubilize SCF bound to the membrane of surrounding stromal cells (197), and the liberated bioactive sSCF may in turn stimulate mast cell proliferation and specialized functions.

Mast cells live in tissues for several months but they are dependent on exogenous factors for their survival. Mast cell numbers within tissues have been found to remain constant in the normal state, reflecting the balance between proliferation or migration and apoptotic cell death (296). It has been claimed that SCF may maintain mast cell survival by suppressing apoptosis (225). The regulation of tissue mast cell number depends both on the rate of production of mast cell precursors and the length of survival of mature mast cells within tissues. Human endothelial cells can also regulate survival

and proliferation of human mast cells (297), which may be a reason for perivascular location of mast cells. Once mast cell precursors target to tissues, their survival may largely be dependent upon the local production of SCF but also on the interactions between mast cells and their extracellular environment, e.g., collagen, laminin and fibronectin may be important for migration and reactivity (298). Furthermore, human skin mast cells have been shown to express receptors for laminin and fibronectin (229). SCF appears to stimulate adhesion to fibronectin by activating mast cells through its interaction with Kit/CD117. Thus, SCF stimulates mast cell adhesion, which is an evidence for claim that it may be a major factor responsible for the adhesion of mast cells to the connective tissue matrix under physiologic conditions (299).

SCF and interleukin-3 (IL-3) are the two principle cytokines that have been discovered to promote mast cell proliferation and/or differentiation. IL-3 seems to be important for early MC proliferation whereas SCF acts to maintain MC viability and maturation. *In vitro* (96) and *in vivo* (112), withdrawal of IL-3 results in mast cell apoptosis and a decrease in endogenous Bcl-X$_L$ and Bcl-2, which are down-regulated but the apoptotic changes are prevented by SCF (96, 225, 296, 300, 301). Thus, SCF may maintain mast cell survival by suppressing apoptosis. When overexpressed, bcl-2 prolongs survival of bcl-2-transfected mast cells following IL-3 deprivation (300, 302). IL-2 and IL-4 in combination enhance mast cell survival for up to 15 days (303). Moreover, IL-4 enhances the release of histamine, leukotriene C4, and IL-5 in MC activated by IgE receptor crosslinking. These facts indicate that also IL-4 is an important regulator of human MC function and is a support for the concept that mature MC retain the capacity to proliferate in a particular tissue environment (302).

IL-1α stimulates mast cell growth by a fibroblast-dependent mechanism, in which the SCF/Kit interaction may participate in a major way (93). TGF-β has been found to specifically prevent the SCF-mediated rescue from apoptosis, probably by down-regulating the expression of c-kit (300). Several other factors supporting mast cell development and survival have also been described, such as nerve growth factor (171), IL-6 (6) and thrombopoietin (77). Also, endothelial cells can be important for mast cell development *in vitro* (124). Thus, microenvironmental factors play an important role in regulating mast cell numbers by promoting survival in the periphery. However, there is still lack of proper cultivation method of pure mast cells, which has lead to use of organ cultures.

2.3.2 The effect of retinoic acid in human skin

Retinoic acid (atRA, all-trans retinoic acid, named also as retinoate, retinoic acid, tretinoin, 2,4,6,8-nonatetraenoic acid, vitamin A acid) is formed from β-carotene in the daily diet (Figure 5). It is converted to all-trans retinol which is delivered to the liver by retinol binding protein (RBP) and from there to skin vessels and capillaries close to the epidermis. In the human skin, retinyl esters are predominantly present in the storage form of retinol. The hydrolysis of retinyls produces free retinol that is oxidized to all-trans retinaldehyde and further to all-trans retinoic acid. The basal keratinocytes, which are capable of migrating and maturing, can be regulated also by retinoic acid and its derivatives. Normally, epidermis forms a gradient for atRA towards the surface of the skin.

All-trans retinoic acid has profound effects on epidermal homeostasis. However, the molecular mechanisms by which retinoids regulate keratinocyte cell proliferation and differentiation are not well understood. The knowledge of atRA as the major biologically active form of vitamin A (304), and nuclear retinoid receptors as the major mediators of all-trans retinoic acid actions, has provided exciting new insights into the molecular basis of vitamin A actions. Retinoids profoundly influence epidermal differentiation, and it has been presumed that antikeratinizing basis for retinoid activity is attributable to the dose-dependent alterations in transepidermal water loss and epidermal and stratum corneum loosening, which may, in turn, lead to loss of epidermal cohesion and abnormal barrier function. An undesirable side-effect of retinoid treatment is skin fragility due to compromised desmosomal adhesion.
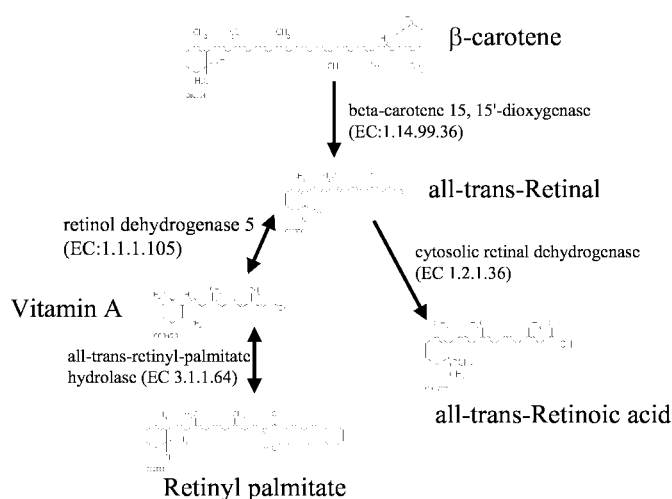
Figure 5. The metabolism of β-carotene to all-trans retinoic acid. Modified from the database "Retinol metabolism in humans" at the web site: http://www.genome.ad.jp/dbget-bin/www_bget?path:hsa00830.

2.3.2.1 Retinoic acid-binding proteins and receptors

Cellular retinoid-binding protein (CRBP) and cellular retinoic acid-binding proteins (CRABP) have been detected in normal human epidermis and dermis in the intracellular compartments (305). CRBP mainly binds (Figure 5) all-trans retinal and it is found in very high levels only in differentiating keratinocytes (306). CRABP-II is the protein, that transports the all-trans retinoic acid to nucleus, and CRABP-I proteins were found to be expressed in human skin (307). It is also known that the CRABP II mRNA is selectively induced in human skin *in vivo* and this process is regulated by exogenous all-trans retinoic acid and all-trans retinal (308, 309). In the nucleus, retinoic acid binds to its receptors in order to mediate its effects on the cell.
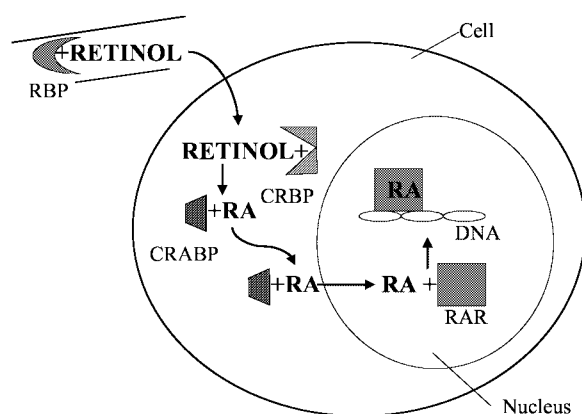
Figure 5. The schematic piture of the function of retinol binding proteins and retinoic acid receptor. RA = retinoic acid, RAR = retinoic acid receptor.

Three types of nuclear retinoic acid receptors (RAR) have been characterized, RAR-α, -β, -γ (310-314). In human skin, the effect of all-trans retinoic acid is mediated mainly by RAR-γ accounting for 87 % of receptors, the remainder consisting of RAR-α (311, 315). Also, three steroid hormone receptors (e.g. D_3-receptor, RARs) regulating receptors named retinoid-X-receptors (RXR-α, -β, -γ) have been characterized (311). RXR heterodimerizes with RARs forming a transcriptionally active regulator. The RXR-α is found in normal and psoriatic skin from keratinocytes, melanocytes, fibroblasts and Langerhans cells (311). It is found in the subcellular compartments of the basal keratinocytes, possibly participating in the change from proliferation into differentiation. RXR-α is upregulated in basal cell carcinomas and it is the target for potentially preventive or therapeutic treatment with RAR-α- or RAR-γ-selective retinoic acid metabolites (312). Transcription of the human CRABPII gene is inducible by retinoids in human skin keratinocytes but not in cultured cells, due to the insufficient levels of RARγ x RXRα (316).

2.3.2.2 The effect of atRA in normal skin and cultured human skin cells

In the mid 80's, atRA was found to cause cell death of epidermal cells in upper layers and to prevent the formation of stratum corneum (317). The expression of the terminal differentiation-specific keratins was completely suppressed by addition of retinoic acid to the culture medium, or the cultures in normal medium but in submerged cultures (318). However, the effect of growth medium, supplemented with fetal calf serum containing retinoids, was not fully understood. The removal of vitamin A by delipidization of the serum restored the keratinization process (318). In the organ cultures, maintained in serum-free, growth factor-free culture medium containing 0.15 mM Ca^{2+}, the ultrastructural appearance of skin degenerated rapidly (319), showing a

culture dependency on calcium and retinoic acid. Nowadays, the amount of calcium is reported to be critical for growth and differentiation of many cells (320). In the early 90's the retinoids were found to induce organ cultured skin samples to produce more vital layers *in vitro* (321-324). The addition of 1.4 mM Ca^{2+} in serum free growth medium inhibited the proliferation, and atRA could not alter the Ca^{2+} induced changes in adhesion (321, 325), leading to the hypothesis that the calcium concentration should be exactly controlled, together with atRA and all other regulative factors in all culture systems. The dermal connective tissue was found to be histologically well-preserved in atRA supplemented organ cultured human skin, which was an important finding of series of studies conducted by J. Varani and his collaborators (325). The other important finding of this era was that retinoids had often opposing effects *in vitro* and *in vivo*.

Topically applied atRA increases the thickness of the skin by increasing TGF-α secretion in an autocrine manner from keratinocytes (326) and by increasing the amount of cornifin (327), the cornified cell envelope (CCE) precursor, and via heparin-binding epidermal growth factor (HB-EGF) (328). Topical application of RA (0.1%) for 2 weeks *in vivo* resulted in suprabasal expression of α2, α3 and β1 integrin subunits (329), normally found in the hyperproliferative epidermis in wound repair and psoriasis. Integrins α2β1 and α3β1 (the connecting molecules for type IV collagen, laminin-5 and fibronectin) were detected in the epidermal layer in RA-treated skin (329). The atRA modulates also the synthesis of keratins, intermediate filaments that indicate the terminal differentiation of the epidermis and its barrier function (313, 330). The level of involucrin is virtually unaffected *in vitro* in skin equivalents (331, 332), but the fully grown confluent keratinocyte cultures up-regulate involucrin after treatment with atRA leading to the formation of a less adhesive stratum corneum (333). Furthermore, atRA has a strong antikeratinizing effect that leads to loosening of epidermal cohesion and fragility of the epidermis. This is also a common side effect of retinoid therapy.

Keratinocytes are an important source of vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF), and production of these factors is inhibited by retinoids (263). By RAR-β/RXR-α heterodimerization, atRA alone can stimulate (334) or potentiate TNF-α induced ICAM-1 expression (246). AtRA has been shown to reduce the production of nitrites by IgE-activated keratinocytes in a time- and concentration-dependent fashion (335). As a consequence, RA derivatives also can reduce the production of TNF-α (335). The level of inducible nitric oxide (NO) synthase (iNOS) activity in CD23 (FcεRII)-activated human keratinocytes was decreased after treatment of the cells with RA derivatives (335). Therefore, atRA derivatives are thought to downregulate TNF-α and the NO-transduction pathway, through the inhibition of iNOS transcription, thus possibly representing the mechanism of the anti-inflammatory activity of RA derivatives in skin diseases (335).

AtRA down-regulates also c-kit (336) which leads to an increase in the level of TGF-β which in turn potentiates the suppressive effect of atRA (326) on c-kit possibly affecting the growth of mast cells. The result of this regulative feedback is that the mast cell proliferation is decreased.

Proteoglycans participate in the assembly of the extracellular matrix, directly by interacting with other matrix components and indirectly by regulating cellular growth-factor responses. The dermal extracellular matrix is important in the structural integrity of the skin, forming a network for, e.g., nerves, vessels, fibroblasts, and mast cells. It is

regulated by stimuli entering into the dermis from outside of the body or from epidermal cells entering through the basement membrane. However, the dermal components and cells also regulate their own surroundings in the microenvironment. AtRA down-regulates human elastin gene expression which is elevated by a single exposure to ultraviolet B (UVB) at transcriptional and possibly protein levels in cultured human skin fibroblasts (337). All-trans retinoic acid decreases also the levels of type VII collagen (a major component of anchoring fibrils) (338) and reduces decorin production in cultured human skin fibroblasts (339). The anti-photoaging effect of atRA may be related, at least in part, to down-regulation of this elastin gene expression after its elevation by UVB (337). The platelet-derived growth factor-BB (PDGF-BB) and TGF-β can interact in a synergistic manner with retinoic acid to stimulate the production of tissue inhibitor of metalloproteinases (TIMP) in human skin and synovial fibroblasts (340). Retinoic acid also potentially inhibits basic-FGF (bFGF)- and EGF-stimulated collagenase protein production in skin fibroblasts (340). Table 3 summarizes previously described effects of atRA, TNF-α, histamine and stem cell factor (SCF) on atRA, TNF-α, histamine, SCF, VEGF/VPF, and ICAM-1 production.

Table 3. The effect of all-trans retinoic acid (atRA), TNF-α, histamine and stem cell factor (SCF) on atRA, TNF-α, histamine, SCF, VEGF/VPF and ICAM-1. ↑ = stimulates/increases, ↓ = inhibits/decreases, 0 = no effect.

Target	Stimulants			
	atRA	**TNF-α**	**Histamine**	**SCF**
atRA	—		↑ (341)	
TNF-α	↑ (342) ↓ (335)	—	↓ (146, 220)	0 (233)
Histamine	↓ (336, 344)	↑ (219, 345)	—	↓ (336) ↑, ↑↑ (233, 346)
SCF	↑ (347)		↑ (348)	—
VPF/VEGF	↓ (349, 350)			0 (118)
ICAM-1	0 (246) ↑ (334)	↑ (245, 246)	↑, ↑↑ (245, 247)	

3. AIMS OF THE STUDY

In normal human skin, mast cells are predominantly of the MC_{TC} type containing histamine, tryptase, chymase, carboxypeptidase and a cathepsin G-like protease. Mast cells can be found in high numbers in the dermis but only rarely in or in contact with the epidermis. In the dermis, however, the highest density of mast cells can be found in the upper dermis beneath the epidermis. The mechanisms for the growth and survival of mast cells as well as the accumulation of mast cells in the upper dermis are poorly understood. In chronic skin inflammation or inflammatory diseases, such as in psoriasis or leg ulcers, mast cells are increased in number in the uppermost dermis and mast cells can even be found in the epidermis. In addition, mast cells are assumed to participate in the separation of the epidermis from the dermis in bullous skin diseases, such as in pemphigoid. Thus, the mast cell and its mediators are supposed to interact with the epidermis, but this interaction is largely dependent on the diffusion of released mediators through the dermal matrix and on the availability of enzymatically active proteinase. Based on this condensed background, the aims of this study were

1) to cultivate normal human skin specimens submerged in the medium or placed at the air-liquid interface and to compare the epidermal expression of involucrin and tumor necrosis factor-α in these two culture models that form the basics for further experiments.

2) to cultivate normal human skin specimens submerged in the medium or placed at the air-liquid interface and to compare the survival and the sequence of events during death of mast cells in these two culture models.

3) to cultivate normal human skin specimens submerged in the medium in the presence of stem cell factor, histamine or tumor necrosis factor-α, and to study whether these essential mediators can affect mast cell survival or death.

4) to study the release of soluble histamine, tryptase activity and chymase activity from normal skin specimens during incubation with a mast cell degranulator, compound 48/80, and after rupturing mast cells by sonication.

5) to study the effect of histamine, tumor necrosis factor-α, or both in combination on the growth and survival of keratinocytes using cell and skin organ cultures.

6) to study the effect of retinoic acid on cutaneous mast cells during skin organ culture or during treatment of normal skin with a retinoic acid cream.

4. MATERIALS AND METHODS

4.1 Chemicals (I-V)

Histamine diphosphate monohydrate was purchased from Fluka (Buchs, Germany) and recombinant human TNF-α (1.0×10^7 units/mg) from Genzyme (Cambridge, MA, USA) or R&D Systems, Inc. (1.1×10^7 units/mg) (Minneapolis, MN, USA) (the concentrations of rhTNF-a purchased from the R&D Systems is given as ng/ml in figures). Keratinocyte-SFM (KSFM) serum-free medium, Dulbecco's modification of Eagle's medium (DMEM), fetal calf serum (FCS), Dulbecco's phosphate-buffered saline (D-PBS), penicillin and streptomycin were all purchased from Gibco BRL (Life Technologies, Inc., Grand Island, N.Y.). Recombinant human TNF-α and recombinant human SCF were purchased from R&D Systems Europe, Ltd (Oxon, U.K.) and histamine from Fluka (Buchs, Switzerland). Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Beidhaemek, Israel), and DMEM and penicillin-streptomycin solution from Gibco BRL (Life Technologies Ltd, Paisley, U.K). OCT compound for freezing the samples was purchased from Miles Scientific (Naperville, IL, USA). All-trans retinoic acid was purchased from Sigma (St. Louis, MO, USA). Ringersteril$^®$ and Novalan$^®$ base cream were obtained from Orion corporation (Helsinki, Finland).

For the thymidine incorporation assay, [^{3}H]-thymidine (1.0 mCi/ml, 15.5 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulphoxide, sodium hydroxide (NaOH), trichloroacetic acid and sodium dodecyl sulphate were all purchased from Sigma (Minneapolis, MN, USA). Ultima Gold liquid scintillation cocktail was purchased from Packard (Groningen, the Netherlands).

For enzyme- and immunohistochemistry, Z-Gly-Pro-Arg-4-methoxy-2-naphthylamide, Z-Gly-Pro-Arg-pNA and Suc-Val-Pro-Phe-4-methoxy-2-naphthylamide, were purchased from Bachem (Bubendorf, Switzerland). Sigma was also the source for aprotinin, α_1-proteinase inhibitor, compound 48/80, heparin sodium salt from porcine intestinal mucosa, bovine serum albumin (BSA), Tris(hydroxymethyl)aminomethane, Fast black K salt, Tween 20, and Fast Garnet GBC. N-Succinyl-Ala-Ala-Pro-Phe-pNA was purchased from Vega (Tucson, USA). α_1-Antichymotrypsin was purchased from Calbiochem (La Jolla, CA, USA). A mouse monoclonal anti-human involucrin antibody (1:400, clone SY5) was obtained from Novocastra Laboratories (Newcastle upon Tyne, U.K.), a mouse monoclonal antibody (20 µg/ml) against human stem cell factor (SCF) was obtained from Genzyme (Cambridge, MA, USA), a mouse anti-human CD117/Kit mAb (3 µg/ml) from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA), a rabbit polyclonal antihuman TNF-α from Sera-Laboratory (Crawley, Down, U.K.), and a mouse mAb against human mast cell chymase (0.1 µg/ml) from Biogenesis Ltd (Poole, U.K.). A rabbit polyclonal anti-tryptase antibody (0.19 µg/ml) was obtained from the source described earlier (162). The chemicals for immunohistochemical staining (Vectastain Elite ABC kit and alkaline phosphatase-conjugated secondary Ab) were from Vector Laboratories (Burlingame, CA, USA), and the TACS TdT *in situ* apoptosis detection kit and Red label substrate were from R&D Systems (Oxon, UK).

For the colour reaction in the immunohistochemistry, 3,3'-diaminobenzidine tetrahydrochloride (DAB) was purchased from Sigma (Minneapolis, MN, USA) and nickel chloride from Merck (Darmstadt, Germany). Aquamount and DePeX Mounting media were purchased from BDH Laboratory Supplies (Poole, UK)

4.2 Processing of skin samples (I-V)

4.2.1 Skin organ cultures (I-V)

The skin organ cultures were derived from fresh healthy-looking skin specimens obtained from 11 female donors undergoing mastectomy due to breast cancer or breast reduction surgery. The subcutaneous fat was removed with a knife and scissors and several 6-mm adjacent punch biopsies were taken from the healthy looking skin areas. The residual blood was washed away with phosphate-buffered saline, pH 7.4. For each well of the 6 well-plate, punch samples were chosen randomly from the skin specimen to minimize coincidental differences in the samples. The skin biopsies were cultured 0, 1-2, 7 or 14 days either at the air liquid interface or submerged (I) or in the other works (II-V) for 0, 1 or 3 days in the presence of histamine and/or TNF-α, all trans-retinoic acid at a final concentration of 0, 0.1, 1.0 or 10 μM or recombinant human stem cell factor (rhSCF) at a final concentration of 20 or 200 ng/ml in submerged conditions at 37 °C and 5% CO_2 by using 10% FCS and DMEM as the medium. The amount of atRA in used, fully supplemented medium prior to addition of atRA was detected by HPLC as previously reported (351) in the Department of Clinical Chemistry in Kuopio University Hospital: DMEM 0.29 μM, KSFM 1.05 μM.

At the end of each culture period, two skin biopsies were randomly taken from each well and were embedded in OCT compound and frozen in isopentane (-70°C) and kept frozen (-70°C) until further processing. The methods were approved by the Ethics Committee of Kuopio University Hospital, Kuopio, Finland.

4.2.2 Keratinocyte cultures (II)

Keratinocytes were cultured from human foreskin specimens using KSFM medium supplemented with 5 ng/ml epidermal growth factor, 50 μg/ml bovine pituitary extract, 100 U/ml penicillin and 100 μg/ml streptomycin (complete KSFM) at 37 °C and 5 % CO_2 as described in detail previously (352). The cells were used at third to fifth passage and cultured to 70-80% confluence before use. The viability of the cells was over 95% in all experiments. All incubations were made at 37 °C and 5% CO_2.

4.2.2.1 The *in vitro*-epithelialization model (II)

An *in vitro*-epithelialization model was developed by placing 4 adjacent and thick-walled stainless steel cylinders (inner diameter 6 mm) on the bottom of each well of a 6-well plate (Falcon). The cylinders were heavy enough to ensure sufficient sealing onto the plastic surface and thereby prevent the escape of keratinocytes. The wells and cylinders were equilibrated with 5 ml of complete KSFM. Then, 30,000 keratinocytes were added cautiously into each cylinder, and the cells were allowed to adhere onto the

plastic surface overnight. Next day, practically complete confluence of keratinocytes was reached and complete KSFM was changed to 5 ml of 10% FCS, DMEM, 100 U/ml penicillin and 100 µg/ml streptomycin. The cylinders were removed and after 1-2 h, modulating agents were added in varying concentrations and combinations into the wells as described in Results. The medium and modulating agents were changed every 1-3 days until the epithelium border was near the wall of the well. The cultures were terminated by removing the medium and after two washes with D-PBS, 4% formaldehyde was added into the wells for 24 h. After the fixation, the epithelium was stained with Mayer's hematoxylin for 24 h. On the next day, hematoxylin was removed, the epithelium was washed with deionized water, and then the epithelial cells were allowed to dry.

4.2.2.2 Proliferating keratinocyte cultures (II)

Proliferating keratinocytes were seeded at a density of about 5,000 cells/cm² into each well of the chamber slide, 6-well-plate and 24-well-plate by using complete KSFM in the 4-well chamber slides (Nunc Lab-Tek™, Nunc, Roskilde, Denmark). Keratinocytes were incubated for 24h to allow proper attachment to the base of the well. On the following day, histamine and/or TNF-α were added into the wells and the cells were cultured for 1 or 3 days. After the culture, the medium was removed and the wells were washed twice with D-PBS. Then, the cells were fixed in 4% paraformaldehyde for 10 min and the cells were air-dried and stored at -20°C prior to staining.

4.2.2.3 [^{3}H]-Thymidine-incorporation by cultured keratinocytes (II)

Proliferating keratinocytes were seeded at a density of 10,000-14,000 cells/cm² into the wells of a 24-well plate (Falcon, Becton Dickinson, Plymouth, UK) and the cells were cultured in 1 ml of complete KSFM for 1 day. On the following day, the complete KSFM was changed to basal KSFM (without EGF and BPE) and the cells were cultured for 1-2 days. Thereafter, the medium was replaced with fresh basal KSFM, varying concentrations and combinations of histamine and/or TNF-α were added into the wells, and the proliferative response of subconfluent keratinocytes was measured by adding 1 µCi [3H]-thymidine for about 20 h as described previously (11, 353). In this assay the exogenous thymidine is transported into the cells by enzyme thymidine kinase and incorporated in DNA during S-phase, which normally lasts 6-14 hours (354, 355). Finally, the wells were washed 3 times with ice-cold D-PBS to get away the non-incorporated thymidine and twice with 5% trichloroacetic acid. The radioactivity was solubilized with 0.1 M NaOH containing 1% sodium dodecyl sulfate. Ultima Gold liquid scintillation cocktail was mixed with the samples and radioactivity was counted in a scintillation counter (LKB-Wallac 1215 Rackbeta, Wallac, Turku, Finland). The experiments were performed in quadruplicate wells and the obtained results are expressed as the percentage of radioactivity compared to control wells.

4.2.2.4 MTT-analysis for the detection of cytotoxicity and cell viability (II)

The modified MTT-analysis was used (356) based on the ability of viable cells to pump the yellow coloured salt out of the cell – the more intense yellow colour in the cells, the less viable the cell. Keratinocytes were seeded into the wells of a 96-microwell plate the day before the experiment at the density of 4000 cells/cm2 using complete KSFM. The adhered keratinocytes were treated with modulating agents for 24 h and then 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in incomplete KSFM was added into the wells for 2 h. Thereafter, the MTT-solution was removed and the formed intracellular dye was solubilized by incubating with dimethyl sulfoxide for 15 min. The absorbance of the solution was measured at 550 nm using a micro-ELISA reader. The cultures and analyses were performed using 6 parallel wells and each experiment was repeated at least twice.

4.2.3 Treatment of normal human skin with retinoic acid cream (V)

Three healthy subjects (2 males, aged 37 and 41, and 1 female, aged 35) volunteered to participate in the study. Three treatment sites (each about 3 x 3 cm) on the non-sun exposed healthy skin of left medial arm or forearm were chosen and marked. The first area was treated with Novalan® base cream once a day for 6 weeks and used as the control skin area. The second skin area was treated first with Novalan® base cream for 4 weeks and subsequently with 0.1 % all-trans retinoic acid (atRA) dissolved in Novalan cream for 2 weeks. The third skin area was treated with 0.1 % atRA cream once a day for 6 weeks. After the treatment, one 4-mm punch biopsy was taken from each skin area following local anaesthesia (1 % lidocaine with epinephrine). Each specimen was immediately embedded in OCT compound and frozen in isopentane cooled with a mixture of absolute ethanol and dry ice. The methods were approved by the Ethics Committee of Kuopio University Hospital, Kuopio, Finland.

4.2.4 Induction of skin blisters by liquid nitrogen spray (III)

Two healthy males and four females (mean 40 years, range 25-61) volunteered to participate in the study. The liquid nitrogen was sprayed onto two adjacent, healthy looking skin areas of medial forearm until the skin was clearly frozen. One day later, clear freezing blister was seen and the fluid samples were collected with sterile handling from both blisters into Eppendorf® tubes on day 1 and 2. Then the fluid was centrifuged at 2.500 rpm for 10 minutes to remove possible cells. The collected supernatant was stored at –20°C for further processing.

4.3 Histochemical staining and analysis methods (I-V)

All samples were kept frozen (-20°C) until further processing. For the staining of mast cells, cryosections of 5 μm thickness were cut on poly-L-lysine coated slides which were stored at –20°C. The reagents used in these analysis are listed in detail Chemicals section.

4.3.1 Enzyme-histochemical staining methods (II-V)

Prior to staining, 5-µm cryosections were fixed in 0.6 % formaldehyde and 0.5 % acetic acid solution, pH adjusted to 7.4, for 10 min. The activity of tryptase in mast cells was demonstrated with 1 mM Z-Gly-Pro-Arg-4-methoxy-2-naphthylamide as the substrate and 0.5 mg/ml Fast black K salt (resulting black colour) or Fast Garnet GBC (resulting purple or reddish colour) as the chromogen at pH 7.5 (6). This substrate solution contained 0.5 mg/ml α_1-proteinase inhibitor to confirm the staining specificity (162). The activity of mast cell chymase was demonstrated with 1 mM Suc-Val-Pro-Phe-4-methoxy-2-naphthylamide as the substrate and 0.5 mg/ml Fast black K salt as the chromogen, at pH 7.5 (6), containing 0.5 mg/ml aprotinin to inhibit cathepsin G activity and to confirm the staining specificity (6, 40).

4.3.2 Immunohistochemical staining methods (I-V)

For immunohistochemistry, the skin cryosections were first fixed in ice cold acetone for 10-15 min followed by blocking with 100 µg/ml purifed goat IgG dissolved in 1% BSA in PBS. SCF, Kit protein, tryptase protein, and chymase protein were stained with specific antibodies dissolved in 1 % BSA-PBS. The bound antibodies on skin sections were visualized with the avidin-biotin-peroxidase (ABC) technique using the Vectastain Elite ABC kit (Vector) together with 0.05 % 3,3'-diaminobenzidine tetrahydrochloride, 0.04 % nickel chloride and 0.03 % hydrogen peroxide. The specificity of the staining was controlled by using the same skin samples with unrelated mouse monoclonals or rabbit polyclonal immunoglobulins (purified IgG) at the same concentration as the primary antibodies.

4.3.3 Apoptosis in chymase-positive mast cells (IV)

To identify apoptosis in mast cells, the immunohistochemical staining of chymase and the TACS TdT *in situ* apoptosis detection kit were used (in detail, see IV) in sequential order following the guidelines of the manufacturer (R&D Systems). Briefly, the cryosection or chamber culture slides were first fixed in cold acetone and permeabilized in Cytonin™ solution. Thereafter, chymase was stained by adding the primary anti-chymase mAb and the alkaline phosphatase-conjugated secondary Ab and finally Red Label Substrate. Next, endogenous peroxidase activity was quenched followed by incubation in the TdT labeling buffer. After the labeling reaction the samples were washed and then incubated in streptavidin-HRP. Finally, the samples were incubated in TACS Blue Label to visualize the apoptotic nuclei. DNase-free deionized water was used throughout the procedure. The slides were processed through increasing concentrations of ethanol, then xylene and finally covered with Depex mounting media. A TACS-nuclease-treated sample was used as a control for the labeling reaction and a slide stained without TdT enzyme was used as a negative control. Positive tissue control slides for apoptosis were provided by R&D Systems, and experimental nuclease-treated control was also used.

The mast cell was judged to be apoptotic when the red colour for cytoplasmic chymase and bluish colour for nuclear apoptosis were seen in close contact and the

morphologic criteria for apoptosis, such as cell shrinkage, membrane blebbing, and chromatin condensation, were fulfilled. The cells were counted as explained above and the apoptosis index (%) was defined as the number of apoptotic mast cells in relation to all chymase-positive cells. The proportion of apoptotic cells (given as the apoptotic index) (357, 358) was counted as positively stained and morphologically apoptotic considered cells divided by the total amount of counted positively stained cells. The positive cells were counted in a Olympus BH-2 microscope using 40x objective with 12 fields resulting in at least 130 cells/sample from 3 independent experiments and the results were given as percentage ± SD.

4.3.4 Sequential double staining methods (II, III, V)

The localization of the Kit receptor or TNF-α in tryptase-positive mast cells was stained by identifying first tryptase enzyme-histochemically (162) followed by photographing. Then, the red azo dye was dissolved away with Tween 20, followed by immunohistochemical staining with a mouse antihuman CD117/Kit monoclonal antibody (II) or a rabbit antihuman TNF-α polyclonal antibody (V), and finally photographing again at exactly the same site as the previous picture with the same (120x) magnification. By comparing the micrographs, the percentage of tryptase-positive mast cells expressing Kit or TNF-α was calculated as previously described (359).

For the staining of first chymase activity and then chymase immunoreactivity (III) in the same mast cells the sequential double-staining method was employed as previously described (189). The sections were first stained enzyme-histochemically for chymase activity and photographed. Thereafter, the blue dye was dissolved by overnight incubation in 15 % Tween 20 and the same sections were stained immunohistochemically with mouse monoclonal anti-chymase antibody. Finally, the restained sections were photographed again at exactly the same sites as the previous pictures. The sequential double-staining of first tryptase activity and then tryptase immunoreactivity (III) in the same mast cells was performed similarly using rabbit polyclonal anti-tryptase antibody as described (189).

4.3.5 Counting of mast cells (II, V)

Tryptase- and chymase-positive mast cells were counted separately in skin sections under high magnification (x400) with the Olympus BH-2 microscope equipped with an 0.2x0.2 mm ocular grid (Ella Graticules, Tonbridge, Kent, UK). Only the clearly identifiable mast cells were counted immediately beneath the epidermis. The number and the percentage of mast cells were counted by comparing the photographs from sequential double staining. The long lasting azo dye Fast black K salt was used as the chromogen when counting the number of tryptase and chymase positive mast cells.

4.4 Detection of histamine and enzyme activity in fluid samples (III)

Histamine was analyzed by radio enzyme assay using rat kidney histamine-N-methyltransferase and (^{3}H-methyl)-S-adenosyl-L-methionine as the methyl donor as described earlier (125).

The tryptase activity in different fluid samples was detected in duplicate with 0.2 mM Z-Gly-Pro-Arg-pNA substrate, 50 µg/ml porcine heparin and 1 mg/ml bovine serum albumin as the stabilizing agents, 1 mg/ml α_1-proteinase inhibitor to prevent possible background activity, and 100 mM Tris-HCl buffer, pH adjusted to 7.6. The chymase activity was analyzed with 0.2 mM Suc-Ala-Ala-Pro-Phe-pNA containing 50 µg/ml porcine heparin, 1 mg/ml aprotinin, 2 M KCl and 100 mM Tris-HCl, pH 7.6. The total reaction volume in each 96-well plate was 200 µl. The initial reaction velocity was measured as the increase in absorbance with a micro-ELISA reader at 405 nm. Spontaneous hydrolysis of the substrates was controlled by replacing the sample solution with the appropriate buffer. The results are expressed as U (U=µmol/min) per ml of sample.

4.5 Digital image analysis (I)

The skin specimens were examined using a Leitz Ortholux POL microscope with PL Fluotar 40/0.70 P objective (Leitz, Wetzlar, Germany) under monochromatic light (543 ± 10 nm) with an interference filter (Schott, Mainz, Germany). A 12-bit, Peltier-cooled digital camera (Photometrics CH 250, Tuscon, Ariz., USA) and a KAF-1400 (Kodak, Rochester, Minn., USA) CCD detector was used for digitizing the images. Prior to the image analysis, the greyscale response of the imaging system was calibrated. The logarithmic grey-optical density (OD) conversion constants and correlation coefficient were calculated for the pixel-grey value conversions, resulting in OD-converted pixels. The IP Lab software (Signal Analytics, Vienna, Va., USA) was used for conversions and measurements.

The spatial distribution of DAB staining was determined from 28.2 µm wide region of interest (ROI) with the height of the epidermal thickness within ROI. The width of the ROI was determined from the pixel-scaled width that was later converted to µm –scale. For each sample, five sections per slide were cut and stained. Within a section, the first technically acceptable section was selected systematically for digital image analysis. Within three sections, three similar separate ROIs were measured. The area-integrated optical density (AIOD) was determined from each ROIs by summing all the pixel values within each of the six subzones and dividing the OD sum by the sum of the individual subzone areas (Figure 8).

ROI = 6 x 1/6   Pixels
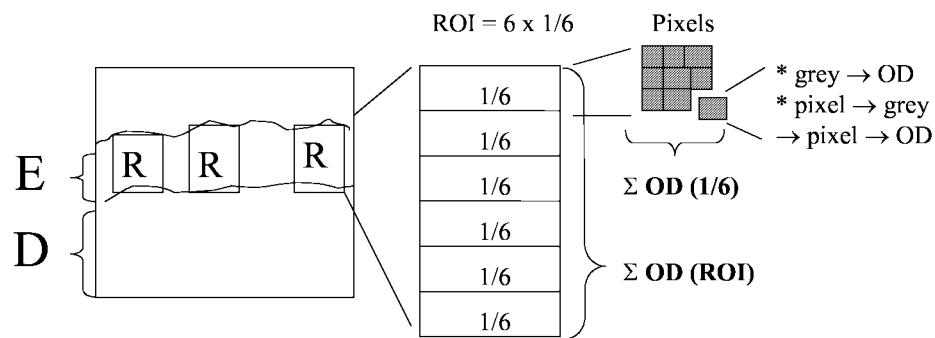
$$\text{AIOD} = \Sigma \text{ OD (1/6)} / \Sigma \text{ OD (ROI)}$$

Figure 8. The schematic presentation of how the area-integrated optical density (AIOD) is formed of a region of interest. E = epidermis, D = dermis, R = ROI = region of interest.

4.6 The measurement of epithelial outgrowth area (V)

The growth area of keratinocyte epithelium was measured using digital image analysis as previously described (11). Shortly, the initial area of seeded cells (4 cylinders) in the 6 well-plate was subtracted from the total area to yield the growth area by using calibrated measurements. The final results are given as the percentage of the growth area in the unstimulated control well.

4.7 Statistical methods (I-V)

Student's two tailed paired t-test (SPSS for Windows 9.0 and 10.0.5, SPSS Inc, USA) was used for statistical analysis when comparing the differences of the means in MTT-analysis and thymidine incorporation (II) or in the calculations of cells in histochemical samples (III, IV, V). The nonparametric Mann Whitney U-test (Statview 4.0 for MacIntosh, Abacus Concepts, Berkeley, Calif., USA) was used for the AIOD estimates because of the small sample size (the number of ROIs per section) (I).

4.8 Ethical aspects (I-V)

The Research Ethics Committee of the Kuopio University Hospital (Kuopio, Finland) approved the methods used in this study.

5. RESULTS

5.1 The suitability of digital image analysis for the detection of involucrin immunostaining (I)

The highest background OD in negative controls was markedly lower than the OD of specific immunopositivity in all cultured specimens after the corresponding cultivation periods, indicating that the measured values are real. The analysis method used revealed no positive immunoreactivity in negative controls. The lowest positive value in the subzones of the samples was determined with statistical tests between positive samples and negative controls. The means and variations within 3 and 5 measurements in one section were almost identical. Also the means and variations between different section measurements were almost identical between 3 and 5 measurements in any of other selected sections. The percentage of the coefficient of variations (CV%) were very small ranging from 0.45% to 7.11%. The variations within the same subzones in the same section were biologically low and acceptable. All grey-OD conversions were made against calibration (I, fig. 2).

5.2 Alterations in the epidermal expression of involucrin and TNF-α during skin organ culture (I, II)

The epidermal morphology in cultures became more loose during the cultivation but the air exposure seemed to keep the skin specimens vital for up to 7 days compared to submerged (SM) specimens. The involucrin immunostaining was highest in the uppermost zone of the epidermis on day 0. Involucrin immunoreactivity shifted downwards along with the progress of cultivation, a change which was significant when compared to the values on day 0 in both ALI and SM cultures. By culturing skin specimens in SM conditions, involucrin immunoreactivity slightly increased from the values on day 1 to those on day 3 (II, fig. 7) or from the values on day 0 to those on day 7 (I). The involucrin staining was diffuse and mainly cytoplasmic that made the staining suitable for semi-quantitative, but calibrated, digitized image analysis. When the change in area-integrated optical density (ΔAIOD) was measured, the results became more pronounced. Also, ΔAIOD was higher in the specimens in SM culture than in those in ALI culture. In the basal keratinocytes, the involucrin staining was more intense in advancing cultures. In the SM samples, the staining appeared to be somewhat more superficial than in the ALI samples and it peaked on day 7 in the mid epidermis. Thus, the selected division into six subzones was able to find differences between the samples and was found to be suitable for the detection of changes in staining (Figure 5 and 6, I).

The epidermal expression of TNF-α was also studied immunohistochemically in ALI and SM specimens. These results have not previously been published. In day-0 specimens (two of three samples, five sections per each patient), the immunoreactivity of TNF-α was found to be very faint, only single occasional dendritic cells were stained in the epidermis, a feature which prevented the appropriate use of image analysis (Figure 9). Already in day-1-2 specimens cultivated in ALI conditions, an increase in TNF-α immunoreactivity was observed and it increased during the cultivation. Instead, no such increase in TNF-α immunoreactivity was seen in SM samples (Figure 9). Thus,

the SM cultivation was associated with pronounced involucrin staining in the epidermis, but the ALI cultivation with increased TNF-α staining.

day 0

day 1 ALI   day 1 SM
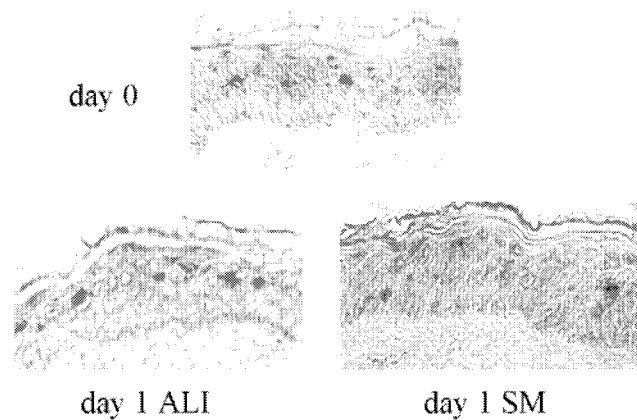
Figure 9. TNF-α immunoreactivity in organ cultured human skin. Positive immunoreactivity can be seen in the mid epidermis and also some immunoreactive cells can also be seen in basal cell layer.

In further experiments, skin specimens from 3 subjects were cultured in SM conditions in the presence of histamine and/or TNF-α for 1 or 3 days. As demonstrated in Fig. 7 (II), TNF-α inhibited the increase in involucrin immunostaining by only 0-5%, whereas histamine inhibited it by 15-17% when compared to control values. By combining histamine with TNF-α, the inhibition was 25%.

5.3 The effect of histamine and TNF-α on cultured keratinocytes (II)

Histamine decreased in a dose-dependent manner up to 40% of the thymidine-incorporation by keratinocytes, maximally at 0.5 mM histamine (II, fig. 1a). TNF-α could inhibit the thymidine-incorporation by about 20% (II, fig. 1b). By combining 1 μM histamine with increasing concentration of TNF-α up to 40% inhibition in the thymidine-incorporation was noted (II, fig. 2a). Although 0.5 mM histamine produced maximal inhibitory effect (II, fig. 1a), addition of increasing concentration of TNF-α together with 0.5 mM histamine to the keratinocyte culture gave rise to as high as 63% inhibition in the thymidine-incorporation (II, fig. 2b).

The growth of keratinocyte epithelium was inhibited by histamine in a dose-dependent manner by up to 46% at 1 mM histamine and the degree of inhibition by histamine was similar to that obtained in the thymidine-incorporation experiment (II). Addition of 10 ng/ml TNF-α together with 0.05 mM or 0.5 mM histamine to the epithelium culture resulted in clear potentiation in the growth inhibition when compared with the effect of

these mediators alone (II, fig. 3). Even more extensive growth inhibition was apparent when 50 ng/ml TNF-α was combined with 0.05 mM or 0.5 mM histamine (II, fig. 4).

When culturing keratinocytes for 1 or 3 days, up to 50 ng/ml TNF-α did not induce any changes in the apoptotic index compared to control values in one-day culture (II). Under experimental conditions, histamine at 0.5 mM produced the highest apoptotic index on day 1. However, when combining 0.5 mM histamine with TNF-α, a decrease in the apoptotic index was noted when compared to the value by histamine alone on day 1 and in day-3 cultures and the cells seemed to be dead in a cytotoxic way.

One-day treatment with histamine and/or TNF-α and MTT-assay were used to study possible cytotoxicity in keratinocytes using complete KSFM medium. Up to 0.5 mM histamine or up to 9 ng/ml TNF-α alone could not cause significant reduction in keratinocyte viability (II, fig. 5). When combining histamine and TNF-α together, however, a significant (up to 40%) reduction in cell viability was obtained, maximally at 0.5 mM histamine and 9 ng/ml TNF-α (II, fig. 5). In another experimental setting, keratinocytes were treated first with either 0.5 mM histamine or 9 ng/ml TNF-α for 1 day. On the following day, cells were treated with increasing concentration of TNF-α or histamine. The pretreatment of keratinocytes with either histamine or TNF-α for 1 day resulted in decreased viability by TNF-α or histamine, respectively, in a dose-dependent manner in both culture systems, maximally at 9 ng/ml TNF-α or 0.5 mM histamine (II, fig. 6a and 6b).

5.4 Release of soluble mast cell mediators from human skin (III)

5.4.1 Tryptase and chymase activity in the high-salt extract of human skin

Tryptase activity in the pooled high-salt extract (total protein 0.66 mg/ml) from the skin specimens of 20 donors was 242 U/l and 230 U/l in the absence and presence of 1 mg/ml α1-PI, respectively. Chymase activity in the same extract was 41.4 U/l and 39.8 U/l in the absence and presence of 1 mg/ml aprotinin, respectively. Thus, the ratio of tryptase to chymase activity was about 6:1 (III).

5.4.2 *In vivo*-release of tryptase and chymase activity to the blister fluid induced by freezing the skin with liquid nitrogen

Maximal tryptic activity in blister fluids collected from 6 subjects was detected after 1 day from freezing (III, table 1), and the majority of the tryptic activity can be attributed to tryptase that is known to resist the inhibitory action of α1-PI. On day 1, all 6 subjects showed chymotryptic activity to varying extent but only 2 of them revealed low aprotinin-resistant chymotryptic activity suggestive for chymase (III, table 1). The ratio of total tryptic to total chymotryptic activity ranged from 37:1 to 778:1. Thus, in contrast to the results of high-salt extract only very low levels of chymase activity could be recovered to the blister fluid.

5.4.3 *Ex vivo*-release of tryptase and chymase activity and histamine from skin specimens induced by compound 48/80 and subsequent sonication

Normal skin specimens were incubated in Ringersteril® in the presence or absence of 10 or 100 µg/ml compound 48/80 for up to 30 min, 240 min, 1 day or 2 days and thereafter the specimens were sonicated efficiently (III). However, no release of chymotryptic activity, measured in the presence and absence of 1 mg/ml aprotinin, could be detected at any time points, not even after the sonication, in 8 individual skin specimens. Only in 1 skin specimen out of 9, aprotinin-resistant chymotryptic activity was released markedly and even spontaneously during the 30-min incubation.

In contrast to chymase activity, a clear and parallel release of both tryptase activity and histamine was induced by compound 48/80 and subsequent sonication (III, fig. 1, table 2). In control solution after 4-h incubation, no release of tryptase activity and only slight spontaneous release of histamine was observed (III, fig. 1). However, on days 1 and 2 the spontaneous release of tryptase activity and histamine was markedly increased exceeding the release rate of both mediators by compound 48/80. Hence, these *ex vivo* results are similar with those obtained in freezing blisters *in vivo*.

5.5 Alterations in tryptase- and chymase-positive mast cells after incubation of skin specimens with compound 48/80 (III)

When skin specimens from 3 donors were incubated in diluent control (Ringersteril®), the number of mast cells showing chymase or tryptase activity remained apparently unchanged by day 1 (III, table 3). On day 2, the number of tryptase-positive cells tended to decrease in every case whereas the number of cells with chymase activity was unchanged, which resulted in an increase in the ratio of chymase to tryptase in all subjects although statistical significance was not reached.

In skin specimens incubated with 10 µg/ml compound 48/80, the number of cells showing chymase- or tryptase activity were sustained over 4h (III, table 3). On day 1, however, the number of cells with chymase activity and thereby also the ratio of chymase to tryptase were significantly reduced. This decrease is apparently due to chymase inactivation since the number of cells with chymase immunoreactivity did not change on day 1. On day 2, no further reduction in the number of cells with chymase activity was noted. Instead, the number of tryptase-positive cells decreased and simultaneously the ratio of chymase to tryptase increased significantly on day 2, a change which suggests solubilization of tryptase activity but not chymase activity.

5.6 Alterations in mast cells during skin organ culture (III, IV, V)

5.6.1 Alterations in the number of tryptase- and chymase-positive mast cells (III, IV, V)

Skin biopsies were cultured in ALI and SM conditions using DMEM and 10% FCS as the medium, and thus in the presence of serum protease inhibitors. In the ALI culture, the number of tryptase-positive cells in the upper dermis decreased steadily with time and the reduction was statistically significant on day 14 (III, table 4; IV, table 1)

meanwhile the slight decrease in the number of cells with chymase activity was not significant (III, table 4). Consequently, the ratio of chymase to tryptase tended to increase, although statistically not significantly, suggesting slow spontaneous release of tryptase activity but persistence of chymase activity. In the SM culture, the number of tryptase-positive cells decreased during cultivation, especially abruptly on day 14 (III, table 4; IV, table 1). In contrast to the ALI culture, the cells with chymase activity in the SM culture tended to decrease in number and simultaneously the ratio of chymase to tryptase decreased significantly on day 7, suggesting chymase inactivation (III, table 4). The decrease in the number of cells with chymase activity was significant on day 14, when compared to cell numbers in the control biopsies, although no marked progression was noted from the cell numbers counted on day 7 suggesting stability after initial decrease. Thus, the ratio of chymase to tryptase increased again on day 14 (III, table 4). To summarize, ALI conditions appeared to maintain better the mast cells than SM conditions.

To further clarify the marked changes in tryptase- and chymase-positive mast cells during cultivation, additional SM cultures were performed and mast cells were stained immuno- and enzyme-histochemically. The number of mast cells with tryptase immunoreactivity or chymase activity decreased steadily during cultivation (IV, table 3) and the decrease in cell number was significant on day 7. Instead, the number of mast cells with chymase immunoreactivity decreased only slightly reaching significance on day 14 (IV, table 3). Furthermore, the number of cells with chymase immunoreactivity appeared to be higher than the number of cells with tryptase immunoreactivity in day-14 specimens (IV, table 3). Thus, the results suggest solubilization of tryptase from the skin tissue. Instead, chymase is partially inactivated but the chymase protein tends to persist in the tissue.

Since SCF is an essential growth and survival factor of cutaneous mast cells, exogenous SCF (20 or 200 ng/ml) was added to the SM culture. However, SCF had no influence on the number of tryptase- and chymase-positive mast cells during cultivation (IV, table 3).

Histamine and TNF-α could be released spontaneously together with tryptase during skin organ culture. Skin specimens from 3 subjects were cultured in SM conditions in the presence of histamine and/or TNF-α for 1 or 3 days (IV, table 2). After 1 day in culture, histamine had no effect, whereas TNF-α decreased maximally the number of tryptase-positive cells by 33 %. After 3 days in culture, no marked reduction in the number of tryptase-positive cells in the control culture, or further reduction in the TNF-α treated cultures, were detected. Instead, histamine alone and in combination with TNF-α significantly reduced the number of tryptase-positive cells when compared to the corresponding values in day-1 specimens (IV, table 2). Therefore, histamine and TNF-α may be mediators which impair mast cell survival during skin organ cultivation.

Since retinoic acid inhibited the development of tryptase-positive mast cells from human umbilical cord blood and reduced the number of cultured human leukemic HMC-1 mast cells (V), skin specimens from 3 subjects were cultured in SM conditions in up to 10 μM retinoic acid for up to 14 days (V, table 1). The number of tryptase- and chymase-positive cells decreased steadily during cultivation but exogenous retinoic acid had no apparent effect on these changes.

5.6.2 Alterations in the number of Kit-positive cells (IV, V)

As demonstrated in fig. 1 (IV) and fig. 6 (V), tryptase-positive mast cells are the predominant cell type staining positively for Kit in the dermis. Hence, the changes in the number of Kit-positive cells during culture reflect alterations mostly in mast cells and not in other cells. After 1-2 days, the number of Kit-positive cells was decreased in the SM culture but unchanged in the ALI culture. In the progress of the culture, the number of Kit-positive cells decreased in parallel in both ALI and SM cultures on day 7 and 14 (IV, table 1).

Similarly to tryptase-positive cells, also Kit-positive cells decreased steadily in number in the absence or in the presence of 20 ng/ml SCF during cultivation in SM conditions (IV, table 3). A significant drop in the number of Kit-positive cells was noted already on day 2 when compared to control. Addition of 200 ng/ml SCF to the culture resulted in a deep reduction in the number of Kit-positive cells on day 2, but without affecting the number of tryptase-positive cells. This reduction reached maximum on day 2 without progressing further (IV, table 3).

In the SM cultures treated with histamine and/or TNF-α, the changes in the number of Kit-positive cells paralleled those of tryptase-positive cells in day-3 specimens (IV, table 2). Histamine or TNF-α reduced the number of Kit-positive cells when compared to the control value.

In the SM cultures treated with up to 10 μM retinoic acid, the exogenous retinoic acid could not affect the changes in the number of Kit-positive cells during the entire culture period (V, table 1). This is in contrast to the results obtained in leukemic HMC-1 mast cells which showed a reduction in Kit expression by 1 μM retinoic acid (V, fig. 5), or to the results obtained with 200 ng/ml SCF (IV, table 3).

5.6.3 Alterations in cells positive for stem cell factor (IV)

In the control skin, 2 specimens out of 3 revealed only occasional weakly stained cells and endothelial-like positivity in the dermis (IV). During cultivation in ALI or SM conditions, no apparent changes in SCF immunostaining, or any staining difference between ALI and SM cultures, were seen during the course of culture. In one subject, no SCF immunostaining was detected in the dermis of all skin specimens. Despite the low SCF expression in the dermis, every subject exhibited positively stained dendritic cells in the epidermis indicating that the staining method was competent.

5.6.4 Alterations in apoptosis in chymase-positive mast cells (IV)

Since chymase persists better in the skin tissue than tryptase and Kit during skin organ culture (IV, table 3), chymase immunohistochemistry was used in the double-staining of apoptotic mast cells (IV, fig. 2). As demonstrated in table 1 (IV), the apoptosis index of chymase-positive cells increased rapidly and significantly from 6.1% to 27.2% already in day-1-2 skin specimens in the SM culture. Later on day 7, the index declined back to baseline. In contrast, the apoptotic process in mast cells was apparently delayed in the ALI culture since the apoptosis index was unchanged in day-1-2 skin specimens but was increased to 10.0% in day-7 specimens although statistical significance was not reached.

5.7 Inhibition of chymase activity by α_1-antichymotrypsin (III)

Serum contains protease inhibitors which may inactivate chymase. To study the sensitivity of chymase activity to inactivation, skin sections from 3 control skin, i.e. healthy-looking, specimens (day 0 specimens, III, Table 3) were preincubated in diluent control or 0.1, 0.3, 1.0 or 3.0 mg/ml α_1-AC at room temperature for 60 min. Thereafter, chymase activity was stained enzyme-histochemically using Suc-Val-Pro-Phe-MNA. The number of chymase-positive cells decreased dose-dependently and it was $54.0\pm28.1\%$, $51.0\pm44.9\%$, $15.3\pm22.7\%$ and $5.5\pm7.0\%$ of the cell number counted in the skin sections treated with diluent control by 0.1, 0.3, 1.0 and 3.0 mg/ml α_1-AC, respectively. There was, however, high individual variation in the sensitivity of chymase activity to α_1-AC and 0.3, 1.0 or 3.0 mg/ml α_1-AC was needed for almost complete inactivation of chymase activity on skin sections.

In contrast, preincubation of the high-salt extract with 0, 0.01, 0.03, 0.1, 0.3 or 1.0 mg/ml α_1-AC in the presence of 0.1 mg/ml heparin, 1.0 mg/ml bovine albumin, 0.2 M KCl and 0.06 M Tris-HCl buffer, pH 7.6, at room temperature for 30 min resulted in practically complete inhibition of the chymase activity toward 0.2 mM Suc-Ala-Ala-Pro-Phe-pNA by already 0.01 mg/ml α_1-AC (III). Thus, chymase activity in solution was considerably more sensitively inactivated by α_1-AC than chymase activity immobilized onto skin sections.

5.8 Alterations in mast cells after topical treatment of normal skin with retinoic acid cream (V)

The number of tryptase-positive mast cells increased in the upper dermis during the treatment of normal skin of 3 subjects with 0.1% retinoic acid cream, but the number of mast cells showing chymase immunoreactivity or chymase activity, i.e., MC_{TC} cells, remained unaffected (V, table 2). This suggests that the number of MC_T cells increased during the treatment. The percentage of TNF-α-positive mast cells showed no apparent changes (V, table 2). Furthermore, no alterations in the percentage of Kit-positive mast cells were observed during the entire treatment period (V).

6. DISCUSSION

A variety of factors can affect the growth, migration, survival and differentiation of mast cells into a specific subtype in different tissues. Although the density of mast cells is pronounced in the upper dermis beneath the epidermis, the mast cell–epidermis association is even more obvious in chronic skin inflammation, such as in psoriasis (86). The factors attracting mast cells towards the epidermis may include, e.g. SCF derived from the epidermis (229, 360), TGF-β (361, 362), dense capillary network and its endothelial cells (297), and sensory nerves (363). Nevertheless, the skin is normally exposed to air, which is essential for the maturation of the epidermis. Since there is a continuous cross-talk between the epidermis and the dermis, it could be possible that the air exposure may be important to the maturation or survival of mast cells in the dermis.

Many different mediators and cells are functioning together in pathological processes at the tissue level, and therefore it is reasonable to elucidate the co-effect of potent mediators in addition to studying the effect of a mediator alone. Histamine and TNF-α reside in the secretory granules of cutaneous mast cells, and they are biologically powerful and clinically relevant mediators in humans. When liberated simultaneously from the mast cell granules, histamine and TNF-α alone can possibly inhibit keratinocyte growth and these mediators may function synergistically. The concentration of histamine in the secretory granules of mast cells is about 100 mM (77) and in the dermal skin about 50-100 μM (124). Therefore, the concentrations of histamine used in this study are likely to occur in the microenvironment of cells in the skin tissue. The concentration of TNF-α used was 1-50 ng/ml that can be considered to be relevant when compared with TNF-α concentrations used previously (145, 219).

6.1 The skin organ culture model (I-V)

Skin organ cultures have widely been used as an experimental tool. Nowadays many modifications of the model of Trowell (283) are used for unique experimental needs. The development of synthetic skin as dressings for burn wounds and chronic skin ulcers has been the great stimulus for developing such models by different research groups. Nevertheless, all models have omitted important structures that may affect cell–cell and cell–matrix interactions. Especially mast cells have received very weak attention in this respect. Also, the purification and cultivation of human skin mast cells *in vitro* has been very challenging. The mediators of mast cells are well known but their role *ex vivo* or *in vivo* is still poorly known. The effect of air exposure to the epidermis has been studied and shown that *ex vivo* cultivation should maintain epidermal structures over 11 days (364). It has also been found that keratinocytes increase in number up to 18th day when growing at air-liquid interface (364). Our studies (I) demonstrated that the epidermis of skin specimens becomes loose after the 7th day as its consistency and gross morphology is altered. By prolonging the cultivation time to 14 days even more destructed morphology is seen and it is not beneficial to continue cultivation beyond this time point (I). There has been an evident need for a model that could make it possible to study the sequence of events of mast cell survival and death and their relation to epidermal physiology.

To detect immunohistochemical staining intensity, there has been commonly used +/- signs to show the results. To measure in an objective way the changes in the staining intensity, and to get numerical values that are reproducible and non-dependent on the investigator, a method was developed from a commercially available software together with the development of skin organ culture model. Involucrin was used as a marker and control for testing the method and also for the characterization of the skin organ culture model in this study. In normal human epidermis, involucrin is expressed in the upper stratum spinosum (365). The expression of involucrin serves as a marker of differentiation and epidermal homeostasis but not as a marker of the degree of cornification (354). Our method showed that local expression of involucrin can vary physiologically within the same section but the averaging the data yields consistent and reliable results - even with 3 measurements per section of 3 different sections for one sample case. Since the method is based on artificial division of the epidermis and staining gradient into subzones, it may be insensitive to small focal changes, so it could be reasonable to concentrate on certain cell layers — the averaging and division may introduce bias. Although the detection method was used in skin histochemistry it can be applied to analysis of any tissue. Our results show that even 3 measurements are sufficient for reliable results for histological analysis. With small sample size false positive measurements are rare, but false negative measurements may cause problems. With the grayscale-OD -calibration equation it was possible to handle 99.64 % of the gray scale values which is assumed to be sufficient (I, Figure 2). Thus, it was possible to convert light intensity into OD units. This allowed reliable order scaled comparisons. It is important to notice that by this method it is easier to compare different experiments and safe measuring limits can be better found.

6.2 The involucrin and TNF-α immunoreactivity in keratinocytes in the different culture models (I, II)

In skin organ culture model ALI seemed to resemble normal skin up to 7 day, showing in upper epidermis immunoreactivity. In SM samples involucrin immunoreactivity shifted already on day 2 towards mid or basal epidermis and showed incresed immunostaining possibly due to disturbed homeostasis and attempts to resist chemical and physical stress (I). Similar results were obtained in the second experiment, where involucrin labelling intensity increased in SM culture in day 3 (II). In the same experiment, addition of TNF-α to the culture seemed not to prevent this increase in day-3 samples (II). TNF-α had also no marked effect on the apoptotic index of cultured monolayer keratinocytes. Involucrin staining has been found to markedly increase after UVB-exposure reaching down to level of basal keratinocytes (215). This possibly indicates the formation of sunburn cells that do not express differentiation markers like involucrin (216). The epidermal expression of TNF-α in ALI and SM specimens was found to be very faint, only single occasional dendritic cells were stained in the epidermis. Already in day-1-2 specimens cultivated in ALI conditions, an increase in TNF-α immunoreactivity was observed and it increased during the cultivation. Instead, no such increase in TNF-α immunoreactivity was seen in SM samples. In one study, TNF-α could induce differentiation of keratinocytes *in vitro* as indicated by the stimulation of cornified envelope formation though it had strong antiproliferative effect

(366). In our study, TNF-α had only slight, if any, inhibitory effect on the increase in involucrin immunostaining, but 0.25-1 mM histamine in turn showed clear and significant inhibition (II). Interestingly, the combination of 1 mM histamine and 18 ng/ml TNF-α resulted in an additional increase in the inhibition of involucrin immunostaining during skin organ culture (II, fig. 7). When skin specimens from 3 subjects were cultured in SM conditions in the presence of histamine and/or TNF-α for 1 or 3 days, TNF-α inhibited the increase in involucrin immunostaining by only 0-5%, whereas histamine inhibited it by 15-17% compared to control values. By combining histamine with TNF-α, the inhibition was 25%. The increased TNF-α staining in the ALI culture together with spontaneously released histamine possibly prevented the increase in involucrin staining seen in the SM cultures. Furthermore, this may suggest that TNF-α cause cytostasis, not differentiation. Thus, histamine and TNF-α may induce more profound changes in the epidermis of whole skin specimens when used in combination than when used alone. Interestingly, the epidermal expression of involucrin in the ALI culture reflected more normal skin expression up to 7th day. Histamine prevented the increase in involucrin immunostaining during SM cultivation, a change which may reflect increased apoptosis and death of keratinocytes.

As a conclusion, the SM cultivation was associated with pronounced involucrin staining in the epidermis, but the ALI cultivation with increased TNF-α staining. In normal human skin *in vivo,* TNF-α may need other counteracting stimulus like histamine secretion for resulting its inhibitory effects (81, 216, 250), and the normal production of TNF-α from keratinocytes may be insufficient to cause any cytotoxic or apoptotic changes. TNF-α can also induce mast cell degranulation and release of histamine (219, 345) and elevated histamine levels have been found in suction blister fluids of UVB exposed skin (148, 149) showing their importance in epidermal pathology.

6.3 The effect of TNF-α and histamine on proliferating keratinocytes and developing keratinocyte epithelium (II)

The primacy of cytokines in eliciting cutaneous immune responses makes them a highly attractive target for new biological response modifiers. In inflamed skin, keratinocytes and inflammatory cells both produce large amounts of tumour necrosis factor TNF-α. TNF-α is also a key cytokine in innate immune responses and has many effects, ranging from inflammation to apoptosis. These effects are reviewed to better understand the role of TNF-α as it relates to the pathogenesis and treatment of inflammatory skin diseases like psoriasis. TNF-α increases production of pro-inflammatory molecules (e.g. IL-1, IL-6, IL-8, NF-kappa β, vasoactive intestinal peptide) and adhesion molecules (e.g. intercellular adhesion molecule-1, P-selectin, E-selectin) (367). TNF-α also promotes apoptosis through binding to the TNF-receptor 1; however, psoriatic lesions are hyperproliferative despite an increase in TNF-α (367). This paradox is partially explained as NF-kappa β activation seems to inhibit TNF-α-induced apoptosis. The importance of TNF-alpha and apoptosis in psoriasis is shown through the review of clinical trials using anti-TNF-α immunobiologics (e.g. etanercept, infliximab) and apoptosis-inducing treatments that result in clinical improvement of the

disease (368-370). Blockade of this proinflammatory cytokine by a monoclonal anti-TNF-α antibody might be effectively used in the treatment of inflammatory skin diseases especially in the management of psoriasis but this approach is also effective for a variety of other dermatological conditions including pyoderma gangrenosum and Behcet's syndrome (371). Targeting of cytokines is still in its infancy for therapy of skin disease. However, blocking tumour necrosis factor alpha by infliximab (372, 373) or etanercept (368, 370) has shown particular promise.

In a previous study (11), histamine at the concentration of 100 μM was found to inhibit significantly the [^{3}H]-thymidine-incorporation of proliferating monolayer keratinocytes in low-calcium culture and the outgrowth of keratinocyte epithelium from whole skin specimens. Similarly, 100 μM histamine was needed for significant inhibition in the [^{3}H]-thymidine-incorporation of monolayer keratinocytes in that same study (II). Furthermore, histamine inhibited the growth of keratinocyte epithelium in a dose-dependent manner paralleling the results obtained in the [^{3}H]-thymidine-incorporation assay. The mechanism for the growth-inhibitory effect of histamine was also clarified. The results of the MTT assay suggest that histamine was not cytotoxic to keratinocytes (11). The increase in the apoptotic index from 2.8% to 12.9% by 0.5 mM histamine suggests that the growth inhibition by histamine is mediated at least in part via increased apoptosis and death of keratinocytes.

An in vitro-epithelialization model was also developed to study the growth inhibitory effect of histamine and TNF-α alone and together. The growth of keratinocyte epithelium was inhibited dose-dependently up to 25% by TNF-α. In other experiments, TNF-α inhibited keratinocyte proliferation dose-dependently without noticeably affecting viability (9, 366), and the cytostasis was increased by combining TNF-α with gamma interferon (9, 217). The cytostatic effect was also reversible (217). Our results are in accordance with previous studies which claim that TNF-α alone is insufficient to induce apoptosis of keratinocytes (374) and the synergy with another mediator may be needed. However, in one case the subcutaneous injection of TNF-α DNA plasmid has been found to cause apoptosis in epidermal keratinocytes (375). In our study, in the high-calcium in vitro-epithelialization model the combination of histamine with TNF-α in turn led to potentiation in inhibition, up to 87% inhibiton in epithelium growth. MTT assay revealed that the potentiation in growth inhibition by simultaneous effect of histamine and TNF-α is due to greatly enhanced cytotoxicity. However, histamine and TNF-α alone were not cytotoxic under experimental conditions used.

Expression of ICAM-1 by keratinocytes is an important activation event in the pathogenesis of T cell-mediated immune reactions. Both histamine and TNF-α have been shown to induce ICAM-1 expression in cultured human keratinocytes. In addition, these mediators function synergistically leading to increased ICAM-1 expression (8, 245). The treatment with TNF-α markedly induces the expression of ICAM-1 on the cultured epidermal keratinocytes (217). In previous studies, histamine has been shown to inhibit the mitosis of keratinocytes by using both H1- and H2-receptors (148, 149). Histamine promotes TNF-α mediated induction of keratinocyte ICAM-1 expression probably through H2-receptors (245) but also suppresses gene expression and synthesis of TNF-α via H2 receptors (146) implying the dualistic role of histamine on the epidermis (142). However, ICAM-1 expression in keratinocytes may not only lead to T

cell activation but to increased lysis of keratinocytes by cytotoxic T cells (248). ICAM-1 has also been found in many tumor cells (376).

The simultaneous action of histamine and TNF-α on keratinocytes in this study resulted in potentiation in growth inhibition and increased cytotoxicity. Previously, treatment of cultured keratinocytes with 1 μg /ml paraphenylenediamine for 3 h has been found to induce ICAM-1 expression probably due to slight membrane damage, but already 2 μg/ml paraphenylenediamine induced cytotoxicity (377). Thus, ICAM-1 expression and cytotoxicity seem not to be completely separate events. Possibly, during the simultaneous action of histamine and TNF-α on keratinocytes, these mediators could first activate and thereby induce ICAM-1 in keratinocytes but then cause cytotoxicity (Figure 9). To clarify this, keratinocytes were first cultured with either histamine or TNF-α, i.e. in conditions shown to induce ICAM-1 (8, 245) but not cytotoxicity. Therefore, preactivation of keratinocytes by histamine or TNF-α renders the cells more susceptible to subsequent cytotoxic effects by TNF-α or histamine. Figure 10 shows a hypothetical model for possible cytotoxic keratinocyte death. ICAM-1 is only one of the multiple surface markers that are expressed during keratinocyte activation.
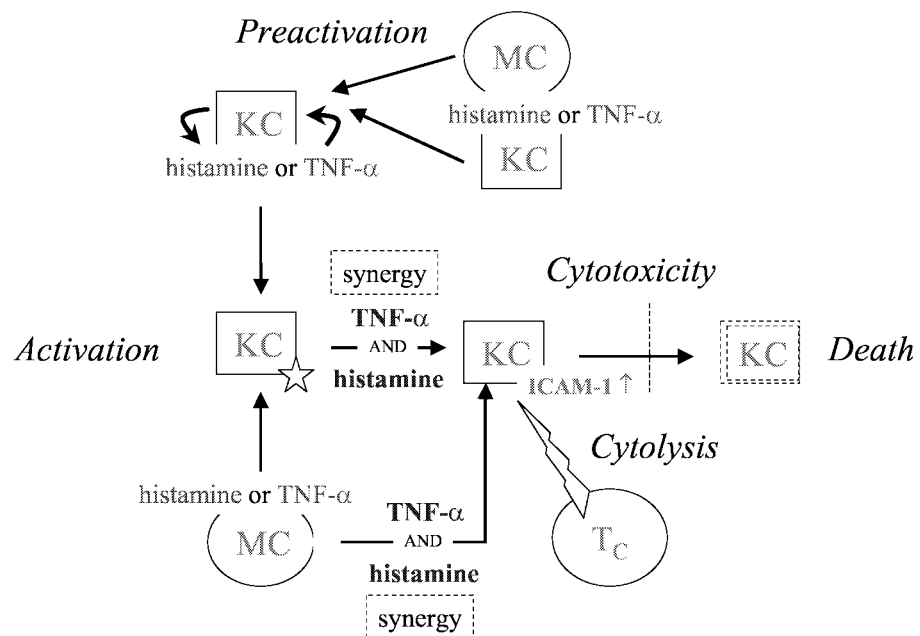
Figure 10. A hypothetical schematic picture of the sequence of events leading to cytotoxicity in keratinocytes. Either histamine or TNF-α from an adjacent keratinocyte, mast cell or autocrinically from target keratinocyte itself may preactivate keratinocyte. Upon activation, the keratinocyte expresses ICAM-1. After that, histamine and TNF-α either separately, sequentially or both in combination act synergistically leading to increased cytotoxicity. *In vivo*, also cytotoxic T-cells interact via ICAM-1 with keratinocytes to induce cytolysis of keratinocytes. MC = mast cell, KC = keratinocyte, T_c = cytotoxic T-cell.

6.4 Sequence of events leading to death or survival of mast cells (III, IV, V)

The present results indicate that after mast cell degranulation tryptase activity is released and the enzyme complexed to heparin proteoglycan can diffuse through the extracellular matrix to exert its activity near the degranulated mast cell and also at more distant sites like in the basement membrane zone (2, 171). Opposite to tryptase, chymase-heparin proteoglycan complexes do not diffuse to the same extent and chymase is partially inactivated after degranulation, especially if it is in the soluble form. Thus, chymase can mostly affect cells and different matrix structures that are located close to the mast cell. Chymase activity persisting locally might contribute to switching off the degranulation of mast cells (378) or promoting the growth and activation of mast cells by cleaving soluble bioactive stem cell factor from cell membranes (197).

In the skin, blisters can be formed in the intraepidermal or subepidermal layers. Proteolytic enzymes participate in the blister formation by degrading structural components of basement membrane and connective tissue in the dermis. In the first step of the formation of blisters, mast cells degranulate their mediators to blistering area of skin. Degranulated mast cells are a prominent feature of the skin blisters of individuals affected with e.g. bullous pemphigoid (379), and mast-cell-derived chemoattractants are present at high concentrations in blister fluids *in vivo* (147). Mast cell tryptase have also shown to participate in the destruction of basement membrane, or at least fibronectin (171).

When incubating the high-salt extract containing soluble chymase activity with varying concentrations of α_1-AC extensive inhibition of chymase activity was measured by a low 0.01 mg/ml dose of α_1-AC. Clearly higher concentrations (0.3, 1.0 and 3.0 mg/ml in the 3 specimens) of α_1-AC were needed for practically complete inactivation of chymase activity on skin sections. This suggests that when tightly packed into the mast cell granule chymase is relatively well protected from the action of α_1-AC. In contrast to chymase, similar inactivation of tryptase has not been detected (6, 86). It is of interest that in day-7 and day-14 skin specimens the number of cells with chymase immunoreactivity was higher than the number of cells with tryptase immunoreactivity, i.e., there were chymase-positive and tryptase-negative cells. Thus, these chymase-positive cells resemble the previously identified MC_C (chymase-positive, tryptase-negative) mast cell type (38, 39).

After freezing the skin, α_1-PI-resistant soluble tryptase activity was present in the blister fluids on day 1 and 2 (III, table 1). This sustained activity over 2 days agrees with the findings that there are no known physiological inhibitors for tryptase. Thus, the involvement of tryptase in the blister formation is possible. On the other hand, the results suggest that chymase is inactivated and/or chymase is not sufficiently diffused to the blister fluid. The 6-mm specimens incubated for up to 2 days in Ringersteril® alone showed no significant changes in the number of chymase-positive cells meanwhile a tendency toward decreased numbers of tryptase-positive cells and increased ratio of chymase to tryptase was noted suggesting spontaneous release of tryptase activity but not chymase activity (III, table 3).

When incubating skin specimens in compound 48/80 (III, table 3) the number of chymase-positive cells as well as the ratio of chymase to tryptase decreased significantly

on day 1. An explanation for this decrease in chymase-positive cells may be that a proportion of chymase was inactivated during mast cell activation and degranulation. After the incubation in compound 48/80 for 2 days, the number of tryptase-positive cells decreased and the ratio of chymase to tryptase increased significantly, which suggests extensive solubilization of tryptase activity (III, fig. 1). Similarly, the ALI cultivation for up to 14 days resulted in significantly decreased numbers of tryptase-positive cells but no significant decrease in mast cells with chymase activity (III, table 4), although serum and its protease inhibitors were present throughout the culture and the skin biopsies were considerably destructed in morphology.

The more physiologic ALI culture preserved mast cells in the upper dermis better than the SM culture In the SM culture (III, table 4), the changes in mast cell numbers were similar with those obtained with compound 48/80 (III, table 3) suggesting that these less physiologic culture conditions lead to mast cell degranulation and partial inactivation of chymase. However, one explanation for the surprisingly high persistence of the remaining chymase-positive cells, but not tryptase-positive cells, after 14 days in the SM culture may be that chymase was entrapped by α_2-macroglobulin preventing further inactivation by large protease inhibitors but allowing detection with the small synthetic substrate used (9). Tryptase and histamine are also spontaneously released during cultivation of skin specimens (III, fig. 1; IV, table I-III) and it has earlier been found that 0.1 mM histamine strongly inhibits the outgrowth of keratinocyte epithelium in whole skin culture using the same culture medium as in this study (11). In addition, an enhanced expression of ICAM-1 on cultured keratinocytes has been detected by simultaneous action of 0.5 mM histamine and 500 U/ml TNF-α (245). Since the concentration of histamine in mast cell granules and in the dermal skin is high, about 100 mM and 50-100 μM, respectively (8, 125, 380), relatively high histamine concentrations (0.25 mM and 1 mM) were used in this study (IV, table 2) assuming that these concentrations can exist at least temporarily in the microenvironment of cutaneous mast cells. Histamine at 0.25 mM reduced the number of tryptase- and Kit-positive cells suggesting a possible mechanism for the decay in mast cells during skin culture. Whether this is a direct or indirect effect of histamine on mast cells is unclear. Similarly to histamine, exogenous TNF-α reduced the number of tryptase- and Kit-positive cells (IV, table 2). TNF-α can induce the death of cells by different mechanisms (44, 45). Since TNF-α is preformed in mast cell granules (92, 207, 219), the result obtained provides another possible means for the decline in mast cells during culture, but the detailed mechanism remains to be clarified. The number of Kit-positive cells showed high variation in relation to the number of tryptase-positive cells in control skin specimens, i.e., the percentage of Kit-positive mast cells varied in the range of 50-100% (IV, table 1-3). One explanation may be the present finding that Kit is sensitively regulated and it disappears rapidly during skin organ culture.

The differences in the changes of the number of mast cells with tryptase or chymase immunoreactivity (IV, table 3) suggest that tryptase protein is solubilized but chymase protein remains in the tissue. In contrast, the decrease in the number of tryptase- and chymase-positive cells did not reach the same extent in the ALI culture (IV, table I). This result indicates that mast cells remain longer intact in skin specimens cultured at the ALI than in those cultured in SM conditions. Skin organ culture specimens undergo destruction during prolonged cultivation and the differences in the number of tryptase-

and chymase-positive cells between ALI and SM cultivation were not feasible until day 7 (IV, Table 1). The results on Kit-positive cells revealed differences already in day-1-2 skin specimens where the number of Kit-positive cells decreased in the SM culture but was unchanged in the ALI culture (IV, table 1). This suggests that the decay in mast cells in the SM culture started early on day 1-2 meanwhile mast cells in the ALI culture remained viable. Most importantly, the apoptosis index of chymase-positive cells increased substantially on day 1-2 in the SM culture but not in the ALI culture. It is likely that the apoptotic process was over on day 7 in the SM culture and mast cells were mostly dead since no marked apoptosis staining was detected anymore. In the ALI culture, an apparent increase in the apoptosis index was noted on day 7 being congruent with the results on tryptase- and Kit-positive cells (IV, table 1). These experiments suggest that tryptase is solubilized from the skin specimens. Instead, chymase activity is partially inactivated and the rest of the activity, as well as most of the protein, remains close to the site of mast cell degranulation. The parallel release of both histamine and soluble tryptase activity from whole skin specimens is also described. The more rapid changes in mast cells in the SM culture may be due to less physiologic conditions, faster destructive changes in the skin, and liberation of cytotoxic or apoptosis-inducing molecules from the cultured cells.

SCF has been shown to be an essential factor for inducing proliferation or preventing apoptosis in mast cells in culture (112). To further clarify the significance of SCF in preventing the decay in mast cells in the SM culture, SCF was added to the culture medium at the concentration of up to 200 ng/ml which should be sufficient to exert its activity on mast cells (381-383). The enhanced decrease in the number of Kit-positive cells by 200 ng/ml SCF already in day-2 specimens (IV, table 3) indicates that SCF had reached and affected mast cells in the skin specimens. The decrease in the number of Kit-positive cells by SCF may be due to SCF-induced down-regulation or internalization of Kit in mast cells or proteolytic shedding of Kit from the mast cell surface (384, 385). In fact, these mechanisms could explain the disappearance of Kit-positive cells during culture in general. Nevertheless, despite its evident action on mast cells during culture, SCF could not prevent or accelerate the decay in tryptase- and chymase-positive cells (IV, V). Possibly, the apoptotic process in mast cells began rapidly in SM conditions and therefore exogenous SCF had no chance to prevent it. Alternatively, other cytokines, growth factors or neuronal supply besides SCF are required (35, 363, 383, 386).

As an important modulator of hematopoietic cells, atRA had no effect on mast cell survival (V). Up to 10 μM concentration were used but the number of Kit-positive mast cells remained constant in skin organ culture. Though the cutaneous mast cells are mainly MC_{TC}-type, these results indicate that atRA does not affect on mature mast cells in tissue. This can also be seen when treated 0.1% topical atRA cream with normal human skin *in vivo*, atRA had no effect on MC_{TC}-type mast cells and on the expression of Kit and TNF-α on mast cells (V).

6.5 The significance of the present results

In human skin *in vivo,* TNF-α may need other concurrent mediators, like histamine, for sufficient inhibitory effects (81, 216, 250). The implications of the present findings for poor epithelialization or epidermal pathology, e.g., in chronic leg ulcers (7), bullous pemphigoid (147), toxic epidermal necrolysis (101), and UV-induced sunburn skin (148, 149), are of interest. Furthermore, elevated histamine levels have been measured in suction blister fluids of UVB-exposed skin (148, 149). TNF-α can also induce mast cell degranulation and release of histamine (219, 345). The present results show that histamine and TNF-α can have profound effects on keratinocytes when functioning together, either simultaneously or sequentially. This synergistic effect could be shown in different experimental conditions; in monolayer cultures of keratinocytes, in epithelium cultures of keratinocytes, and in the epidermis of cultured whole skin specimens. Therefore, mast cells can be inhibitory, and even cytotoxic, to keratinocytes in the microenvironment between mast cells and keratinocytes.

Parenterally-administered histamine dihydrochloride boluses have been used for H2-receptor-mediated tumour growth inhibition in rat Leydig cell sarcoma (387, 388) or in malignant glioma (389). Furthermore, combination of histamine with cytokine immunotherapy may result in potentiation in the antitumor effect of interleukin-2 and interferon-α (390). This study shows for the first time a great potentiation in the inhibitory action of histamine and TNF-α on epithelium growth when these mediators are combined together. Therefore, this finding may provide an experimental background for possible future studies and perhaps for therapeutic use.

Mast cells contain large amounts of tryptase protein. After released enzymatically active tryptase can relatively easily diffuse through the dermal matrix reaching local and distant skin sites, such as the epidermis. In chronic inflammatory skin diseases, such as in psoriasis, tryptase-positive mast cells are increased in number in the upper dermis. Therefore, this enzyme is an interesting target for developing enzyme inhibitors for possible therapeutic use. In contrast to tryptase, chymase is more carefully controlled and the enzyme is inactivated at least partially after extrusion of granules from mast cells. When in the soluble form, chymase is inactivated rapidly and sensitively by protease inhibitors, such as α_1-AC. In addition, chymase protein complexed to heparin proteoglycan diffuses slowly through the matrix. This strict control may be necessary in physiological situations since chymase can have powerful effects in the skin, such as dermal-epidermal separation. The present findings may explain why an urticarial wheal does not lead to blister formation every time the mast cells are activated and degranulated. In pathological conditions, e.g., in pemphigoid, the control mechanisms may fail and chymase can freely destruct or affect surrounding structures.

Previously, a MC_C type of mast cell (chymase-positive, tryptase-negative) has been described immunohistochemically. Nevertheless, the present results suggest that this cell type may represent a dead mast cell in tissues – tryptase is solubilized away but chymase immunoreactivity remains at the site of granule extrusion.

7. CONCLUSIONS

1) The epidermal expression of involucrin in the ALI culture resembled more that of normal skin for up to 7 days in culture when compared to the increased involucrin staining in the SM culture. Addition of histamine and especially the combination of histamine and TNF-α prevented the increase in involucrin immunostaining during SM cultivation. Epidermal TNF-α staining was more pronounced in the ALI culture than in the SM culture after 1-2 days in culture, a change which may explain the result of lower involucrin staining in the ALI culture.

2) The cultivation of skin specimens in SM conditions induced rapid apoptosis and subsequent decay in tryptase-, chymase- and Kit-positive mast cells whereas simply the air exposure in the ALI culture was able to delay these changes considerably. This suggests that air exposure to the epidermis is essential for the homeostasis and survival of mast cells in the dermis. The more rapid changes in mast cells in the SM culture may be due to less physiological conditions and faster destructive changes in the skin.

3) SCF alone was not sufficient to prevent the rapid decay in mast cells during SM cultivation. Possible factors promoting the decay in mast cells during skin organ culture may be directly or indirectly associated with released histamine or TNF-α.

4) Active tryptase together with histamine is released from mast cells to extracellular matrix and further out from the skin tissue. Instead, chymase is partially inactivated and the rest of the activity as well as chymase immunoreactivity tend to remain close to the site of mast cell degranulation. The decrease in chymase activity during cultivation is possibly due to protease inhibitors. The previously immunohistochemically described MC_C mast cell (tryptase-negative, chymase-positive) may represent a dead mast cell from which tryptase is depleted.

5) Histamine and TNF-α function synergistically impairing epidermal viability. The simultaneous or sequential action of histamine and TNF-α on keratinocytes resulted in potentiation in growth inhibition and increased cytotoxicity. Interestingly, histamine caused an increase in the apoptotic index *in vitro* showing a possible way for mast cells to regulate keratinocyte growth in various skin conditions.

6) Up to 10 μM atRA could not affect the survival or death or Kit expression of mast cells in skin organ culture. *In vivo* 0.1% atRA cream increased the number of MC_T-type of mast cells, but the number of MC_{TC} was unaffected as was the expression of Kit and TNF-α in cutaneous mast cells.

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LIST OF ORIGINAL PUBLICATIONS

I Kivinen PK, Hyttinen M, Harvima RJ, Naukkarinen A, Horsmanheimo M, Harvima IT. Quantitative digital image analysis applied to demonstrate the stratified distribution of involucrin in organ cultured human skin. Arch Dermatol Res 1999: 291: 217-223.

II Kivinen PK, Hyttinen M, Lappalainen K, Harvima IT. Increased growth inhibition and cytotoxicity by simultaneous or sequential action of histamine and tumor necrosis factor-α on cultured human keratinocytes. submitted

III Kivinen PK, Kaminska R, Naukkarinen A, Harvima RJ, Horsmanheimo M, Harvima IT. Release of soluble tryptase but only minor amounts of chymase activity from cutaneous mast cells. Exp Dermatol 2001: 10: 246-255.

IV Kivinen PK, Hyttinen M, Harvima R, Naukkarinen A, Horsmanheimo M, Harvima IT. Mast cell survival and apoptosis in organ cultured human skin. Exp Dermatol 2003: 12: 53–60.

V Hjertson M, Kivinen PK, Dimberg L, Nilsson K, Harvima IT, Nilsson G. Retinoic acid inhibits *in vitro* development of mast cells but has no marked effect on mature human skin tryptase- and chymase-positive mast cells. J Invest Dermatol 2003: 120: 239-245.