Mast cells in cutaneous wound healing

Doctoral dissertation

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ABSTRACT

Mast cells are suggested to participate in wound healing, but their specific role has remained obscure. Mast cells are a rich source of inflammatory mediators, such as histamine, proteoglycans, the proteases tryptase and chymase, lipids, growth factors and cytokines. Among these mediators, there are many that have effects on cell growth, tissue turnover and repair. The aim of this study was to characterize the functions of mast cells in wound healing by studying mast cell activation in normal skin and the alterations undergone by mast cells in normally healing wounds, and investigating the effects of mast cell mediators on cultured keratinocytes and on epithelialization in vitro. The potential roles of mast cells and their mediators were explored also in chronic leg ulcers.

The microdialysis technique was used to monitor histamine release in skin following skin challenge with neuropeptides substance P (SP), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and capsaicin. SP and VIP, but not CGRP, caused mast cell degranulation and histamine release. Capsaicin, a neuropeptide releasing agent, did not cause any substantial histamine release, suggesting infrequent morphological contacts between mast cells and sensory nerves in normal human skin.

In normally healing wounds, the numbers of mast cells, especially those with chymase activity, decreased in number and could not be found in the epithelialization margin. In chronic ulcers, mast cells were numerous in the perilesional skin and often in contact with the epithelial margin, and chymase was partially inactivated, as detected enzyme- and immunohistochemically. The expression of stem cell factor (SCF, a mast cell growth factor) and Kit (its receptor on mast cells) showed significant alterations during wound healing. The numbers of dermal cells expressing SCF were markedly increased on day 1 after wounding and declined thereafter, whereas the expression of Kit increased steadily throughout wound healing. In chronic ulcers, most of the mast cells were Kit positive, while SCF positive cells were numerous in the wound bed. Thus, in chronic ulcers, there seems to be a potential for interaction between SCF and the Kit receptor, leading to mast cell proliferation, migration and degranulation. In contrast, only temporary SCF-mediated mast cell activation seems to occur during normal wound healing.

Significant levels of soluble tryptase activity and histamine, but low levels of chymase activity, were measured in samples washed from chronic leg ulcers. Parallel with this result, no tryptase-inhibiting activity, but clear chymase-inhibiting activity, was detected in the wash samples.

Histamine and heparin were inhibitory to keratinocytes in vitro. They inhibited both the 3H-thymidine incorporation into keratinocytes and the keratinocyte outgrowth from skin specimens. A lysate from HMC-1 mast cells inhibited keratinocytic and epithelial growth as well. Purified human tryptase had no significant effect on the growth and adherence of keratinocytes in various experimental settings. In contrast, chymase efficiently detached monolayer keratinocytes or caused marked destruction of the developing epithelium within 2 days. Since mast cells were accumulated at the epithelial margin of chronic leg ulcers, these findings provide evidence that mast cells may participate in the pathophysiology of impaired epithelialization in chronic wounds.

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Maria Huttunen
ABBREVIATIONS

$\alpha_1$-AC  $\alpha_1$-antichymotrypsin
$\alpha_1$-PI  $\alpha_1$-protease inhibitor
aFGF  acidic fibroblast growth factor
bFGF  basic fibroblast growth factor
BM  basement membrane
CGRP  calcitonin gene-related peptide
di-DFP  di-isopropyl fluorophosphate
ECM  extracellular matrix
EGF  epidermal growth factor
FceRI  high-affinity receptor for IgE
GM-CSF  granulocyte macrophage-colony stimulating factor
HMC-1  human mast cell line-1
IFN-γ  interferon-γ
IL  interleukin
KGF  keratinocyte growth factor
LT  leukotriene
MCc  chymase positive mast cell
MCt  tryptase-positive mast cell
MCtC  tryptase- and chymase-positive mast cell
NGF  nerve growth factor
PAR-2  proteinase-activated receptor 2
PDGF  platelet-derived growth factor
PG  prostaglandin
SBTI  soybean trypsin inhibitor
SCF  stem cell factor
SP  substance P
TGF-α  transforming growth factor-alfa
TGF-β  transforming growth factor-beta
TLCK  Na-p-tosyl-L-lysine chloromethyl ketone
TNF-α  tumor necrosis factor-alfa
VEGF  vascular endothelial growth factor
VIP  vasoactive intestinal peptide
LIST OF PUBLICATIONS

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


V M. Huttunen, I.T. Harvima: Mast cell tryptase and partially inactivated chymase are present in the wound base of chronic leg ulcers and chymase efficiently deteriorates keratinocyte adherence onto substratum in vitro. Manuscript.
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ORIGINAL PUBLICATIONS (I-V)
1 INTRODUCTION

Mast cells are a rich source of various potent mediators, in particular histamine, proteases, lipid mediators, cytokines and growth factors. Mast cells are traditionally viewed as the primary effector cells in allergic reactions. They are, however, increasingly being recognized also as regulators of tissue homeostasis, remodelling and repair. Wound healing is a well-orchestrated complex series of events that normally result in the restoration, structural integrity and functional properties of the damaged tissue. Mast cell-derived mediators can influence practically all stages of wound healing, as they have the ability to enhance local blood flow, recruit immune cells into tissues, inhibit keratinocyte growth, and stimulate endothelial cell and fibroblast growth. Furthermore, mast cells contain acid hydrolases and neutral proteases, which act on hyaluronic acid, collagen and the core peptides of proteoglycans, and could thus facilitate cleaning the wounded area of debris. On the other hand, mast cell proliferation is influenced by fibroblast and keratinocyte derived growth factors, such as stem cell factor (SCF). These findings imply that mast cells are involved in tissue repair processes in the dermis, but their association with epidermal regeneration is still obscure.

When a wound fails to heal, the result is a chronic ulcer. A characteristic feature of such a wound is the presence of chronic inflammation, including mast cell hyperplasia. The pathogenetic factors that impair healing of chronic ulcers are largely unknown and could be due to a number of intercellular and cell-matrix mechanisms. At present, however, knowledge of the role of mast cells in the failure of wound healing is scanty.

The goal of this work was to characterize the possible functions of mast cells and their mediators in normal wound healing. This was done by studying mast cell activation in normal skin and the alterations in mast cells during the process of wound healing, and investigating the effects of mast cells on the growth of cultured keratinocytes and epithelialization in vitro. The aim was also to gain new insights into the pathophysiology of chronic leg ulcers and to explore the potential role of mast cells and their mediators in the failure of wound healing. Special attention was paid to measuring histamine and mast cell protease activities in chronic leg ulcers.
2 REVIEW OF THE LITERATURE

2.1 Wound healing

Cutaneous wound healing is a complex sequence of events that includes inflammation, increased synthesis and remodelling of connective tissue, activation of keratinocytes and angiogenesis. These processes are all regulated by interactions between the cell and the extracellular matrix (ECM) and by various cytokines and growth factors. The phases in the healing cascade overlap, and the outcome is a quite acellular scar.

Re-epithelialization

In the unwounded situation, the epidermis is static and the basal keratinocytes are apposed to the basement membrane (BM), which isolates the basal cells from the dermal collagen, elastin, dermal proteoglycans, and large concentrations of fibronectin. In any but the most superficial wounds, the BM is usually violated and its permeability barrier function is lost. The epidermal cells may then be exposed to soluble growth mediators, which, in the stationary epidermis, may be present in lower concentrations or not at all.

Re-epithelialization is an early event in the healing process of wounds. Migration over the provisional matrix begins within hours after injury. In contrast to the usual vertical migration during differentiation, keratinocytes begin to move horizontally over a wound-bed matrix composed of collagen, fibronectin, serum and elastin. In cell culture, keratinocyte migration is stimulated by fibronectin, and, in contrast, inhibited by laminin, a BM component (Nickoloff et al. 1988). The cells that migrate to restore the continuity of the epidermis are recruited from the margins of the injury and adnexal structures. They are presumably activated by signals originating at the site of the injury.

Besides covering the wound, the migrating epidermal cells must also eventually reconstitute the BM. The BM comprises components derived from both the epidermis and the dermis (Brieggaman et al. 1971). Keratinocytes grown in culture are known to synthesize the BM components laminin and type IV collagen, as well as fibronectin (Alitalo et al. 1982, Clark et al. 1985). As the keratinocytes migrate, they express urokinase-type plasminogen activator (Gröndahl-Hansen et al. 1988) and collagenase-1 (Saarialho-Kere et al. 1993, Pilcher et al. 1997). It seems that a migrating keratinocyte can produce a matrix
that contains type IV collagen in order to attach to the matrix, pull itself in the direction of the matrix, and then release itself by means of collagenase (Woodley et al. 1985).

Migration does not require keratinocyte proliferation, which appears to be independent of migration. Proliferation is initially limited to a pool of cells some distance (50-100 cells) from the leading edge. At a later stage, the entire neo-epidermis is engaged in active cellular proliferation (Andriessen et al. 1995). Keratinocytes also undergo rapid changes in their pattern of keratinization during wound healing: keratin 1 is replaced by keratins 6 and 16 (Mansbridge et al. 1987). A number of growth factors promote keratinocyte proliferation and migration, including KGF, bFGF, EGF, TGF-α and IL-1.

Figure 1. Schematic representation of epithelialization. In contrast to the vertical migration during differentiation, keratinocytes (KC) begin to move horizontally over a wound bed matrix in response to violation of the basement membrane. Modified from Woodley et al. 1985.

**Dermal repair**

Healing of the dermis occurs concurrently with re-epithelialization, but is a lengthier and more complex process. It occurs in three sequential but overlapping phases.
1. Inflammatory / hemostatic phase

Following wounding, there is almost immediate release of inflammatory mediators from the damaged cells, platelets, resident tissue macrophages and mast cells. An initial fibrin-platelet clot is formed to close the wound. In addition to providing hemostasis, platelets produce and release growth factors, such as PDGF, TGF-β, and EGF-like growth factors, and contribute to the subsequent migration and proliferation of epidermal and dermal cells. Platelets also release proteases, which activate complement to generate the anaphylatoxins C3a and C5a. These factors increase local blood vessel permeability and attract neutrophils and monocytes.

Neutrophils appear within hours, phagocytose contaminating bacteria, and are usually gone within 3 days. Monocytes migrate into the wound and differentiate into macrophages. Their influx is critical to the wound healing process. Macrophages are found at the margins of both healing and non-healing human wounds in association with a lymphocyte population (Loets et al. 1998, Boyce et al. 2000). Macrophages secrete bFGF, TNF-α and IL-1 and are also responsible for phagocytosis of microorganisms, as well as debridement of tissue debris. Lymphocyte subpopulations show alterations during wound healing; a high T-lymphocyte CD4/CD8 ratio characterizes the initial stage and, as healing progresses, the ratio declines (Boyce et al. 2000). This implies, that CD8+ suppressor lymphocytes play a downregulatory part in the healing process, and their numerical increase during healing may indicate the requirement of an anti-inflammatory cytokine environment as the inflammatory response resolves.

Inflammation subsides as granulation tissue forms and ECM formation proceeds. Inflammation is thought to be over in about five days and has no marked role thereafter (Hung et al. 1989, Moore 1999). Both an attenuated and an excessive inflammatory reaction are detrimental to wound repair (Eaglstein et al. 1978, Hung et al. 1989).

2. Proliferative phase

Macrophages are largely responsible for initiating and maintaining the proliferative phase of dermal wound healing. Like platelets, macrophages synthesize and release PDGF and TGF-β, which are chemotactic and mitogenic for mesenchymal cells. In addition, macrophages produce IL-1 and bFGF, which are mitogenic for fibroblasts. Moreover,
bFGF is mitogenic for endothelial cells and smooth muscle cells. Granulation tissue, consisting of new capillaries and fibroblasts, is observed in the wound on day 4 post-injury. The rate-limiting step of granulation tissue induction seems to be mesenchymal cell activation (McClain et al. 1996). Fibroblasts are initially active in synthesizing fibronectin, followed by collagen types I and III. TGF-β stimulates fibroblast synthesis of collagen and causes formation of granulation tissue (Roberts et al. 1986). In contrast, TNF-α and IL-1 reduce collagen production (Solis-Herruzo et al. 1988, Rapala et al. 1997). Collagen synthesis begins during the first few days of wound healing (Petri et al. 1997). After major surgery, marked time-related increases in the aminoterminal propeptide of type III procollagen (PIIINP) and carboxyterminal propeptides of type I procollagen (PICP) have been measured in the wound fluid (Haukipuro et al. 1987, 1991). Granulation tissue fibroblasts transform into myofibroblasts, which contain actin filaments and mediate wound contraction.

Angiogenesis induces better nutritional conditions for fibroblasts and epidermal cells to eventually close the wound. Growth factors involved in the stimulation of angiogenesis include bFGF, TGF-β and VEGF. Heparin potentiates many angiogenic growth factors (Folkman et al. 1987). The low pO₂ in the wound area is also an important stimulus of angiogenesis.

In addition to growth factors, there are extensive cell-to-cell and cell-to-matrix interactions that control cellular behaviour and coordination during the proliferative phase. This phase ends when immune cells, fibroblasts and endothelial cells undergo apoptosis, while the remaining fibroblasts lay down the extracellular matrix.

3. Matrix formation and remodelling

The final phase of dermal wound repair is matrix formation and remodelling. This process starts early and lasts for months, during which there is a transformation of granulation tissue into mature scar tissue. Early in the proliferative phase, fibroblasts produce fibronectin and thus form a scaffold along which more fibroblasts and other mesenchymal cells enter the wound. During the remodelling phase, the provisional fibronectin matrix is gradually replaced by collagen fibers.

The remodelling phase is characterized by collagen breakdown and synthesis. Type III
collagen, abundant in granulation tissue, is gradually replaced by type I collagen, the predominant collagen of the mature scar as well as of the normal dermis. Type I collagen is continuously degraded, replaced by new collagen fibers, and rearranged. In wound healing, the spatial and temporal control of proteolysis is of critical importance. Although proteolysis mostly occurs within the first few days of wound healing (Ågren et al. 1992), scars continue to remodel for a long time after wounding, and cannot be considered to be in a steady-state condition until at least 2 years after wounding.

2.2 Chronic wounds

In normal skin, wound healing and re-epithelialization proceed in a highly coordinated fashion. When this coordination is lost, failure of wound healing results in a chronic ulcer. The chronic cutaneous wounds most commonly encountered are leg ulcers. In a Finnish study, the prevalence of leg ulcers was 0.6% (Launikka 1997). The prevalence increases with age and it is estimated that there are 10 000 patients currently with a leg ulcer in Finland (Jansén and Nissinen-Paatsamä 1995). Leg ulcers can have a wide variety of etiologies. Venous ulcers are thought to account for approximately 60 to 80% of cases, and most of the others are due to arterial disease, neuropathy, or a combination of these diseases.

The cause of venous leg ulcers is the underlying vascular pathology. Long-standing venous hypertension results from one or several abnormalities: faulty superficial and perforating vein valves, abnormal deep vein function, deep vein occlusion, and calf-muscle pump dysfunction (Falanga 1993). However, the pathogenic steps leading from venous and capillary hypertension to ulceration and failure to heal are quite speculative.

Histologically, a venous ulcer consists of an ulcer base covered by a fibrinous exudate, which contains polymorphonuclear leukocytes. The underlying dermis contains numerous blood vessels surrounded by pericapillary fibrin cuffs, and the remainder of the dermis consists of fibrous tissue with a variable number of inflammatory cells (Herrick et al. 1992). Extravasated red blood cells and extracellular deposits of hemosiderin result in purpura and hyperpigmentation.
One hallmark of chronic wounds is the presence of chronic inflammation. It seems that there is an apparent conversion from a resolving to a chronic non-resolving inflammatory response (Moore 1999). Macrophages are the dominant inflammatory cells in the venous ulcer bed and the surrounding tissue (Loots et al. 1998). As compared with acute wounds, reduced numbers of CD4+ T lymphocytes (T-helper cells) and large numbers of B lymphocytes and plasma cells are reported (Loots et al. 1998). The prolonged presence of ECM molecules such as fibronectin, tenascin and chondroitin sulfate is detected in chronic ulcers (Loots et al. 1998). Degradation products of fibronectin have also been found in wound fluid from venous ulcers (Grinnell et al. 1992, Palolahti et al. 1993). It seems that in chronic nonhealing ulcers, both excess depositions of ECM molecules and increased proteolytic activity against ECM molecules are present.

Impaired re-epithelialization characterizes venous ulcers: resurfacing of the wound may fail to occur for months, while the wound bed seems clean and granulating well. The prominent acanthosis regularly present at the edges of venous ulcers suggests that epidermal migration rather than proliferation has failed (Adair 1977). Indeed, the proliferation of keratinocytes at the margins of venous ulcers is not impaired (Andriessen et al. 1995), suggesting that epidermal proliferation is not a limiting factor in the healing process. Failure of keratinocytes to migrate across the wound bed points to lacking or improper signals in chronic ulcers. It remains unclear whether the defect in keratinocyte migration resides in the keratinocyte phenotype, the lack of locomotion signals, or the chronic wound bed and the structure of its matrix (Falanga et al. 1994). Another reason why chronic ulcers fail to re-epithelialize could be hypoxia, which affects the proliferation and collagen synthesis of fibroblasts (Falanga 1993). Furthermore, fibrin cuffs surrounding the blood vessels may bind growth factors and proteins, and impede oxygenation and growth factor transportation to the wound area. However, the extent to which pericapillary fibrin cuffs affect the pathogenesis of a venous ulcer is still unclear (Falanga et al. 1987).

Wound fluid reflects the microenvironment of the wounds from which it is collected and thus may offer clues to the events occurring during wound repair or its failure. Human wound fluid from acute wounds has been shown to stimulate fibroblast and endothelial cell growth (Katz et al. 1991), whereas wound fluid from venous ulcers contains degraded fibronectin and vitronectin and inhibits cell adhesion (Grinnell et al. 1992) and reduces the
growth of dermal fibroblasts (Mendez et al. 1999). Metalloproteinases are overexpressed in chronic leg ulcer exudate (Weckroth et al. 1996), and there is an imbalance between proteolytic enzymes and their endogenous inhibitors (Bullen et al. 1995, Vaalamo et al. 1999). Elevation of proteases in wound fluid would interfere with normal healing by degrading adhesion proteins and other factors necessary for repair. Taken together, these studies have led to the hypothesis that chronic wound fluid is opposed to wound repair.

2.3 Mast cells

Mast cells are effector cells in IgE-dependent immediate hypersensitivity; they also participate in many biological responses in which IgE has no role. They are reservoirs of preformed inflammatory mediators and, on activation, rapidly synthesize others. The mast cell represents the first line of defence of tissues against injury. Mast cells occur wherever the body is in contact with the environment. Numerous mast cells are localized in the skin, in the alveolar and bronchial subepithelial respiratory tissues, in the mucosa and submucosa of the small intestine, in the conjunctiva, and in the nasal mucosa. Although the number of tissue mast cells may increase (or decrease) in association with certain biological responses, normal vascularized tissues have a resident population of mature mast cells.

Mast cells are a physiological component at all levels of the dermal skin, with a density of about 7000 cells/mm², although they are most frequent in the papillary and subpapillary dermis and tend to aggregate around blood vessels, nerves and appendages (Eady et al. 1979). In normal skin, mast cells cannot be found in the epidermis. However, epidermal mast cells are found in conditions characterized by chronic skin inflammation and epidermal cell proliferation (Green et al. 1977, Bolton and Montagna 1993).

Dermal mast cells are ovoid or spindle-shaped, mononuclear cells, with a diameter of 10 to 15 μm. They are larger than eosinophils and basophils. Their major distinguishing feature is the presence of numerous, round cytoplasmic granules, which stain orthochromatically with alcin blue and safranin or metachromatically with toluidine blue or Giemsa. The cytoplasmic granules are about 0.6 μm in diameter and surrounded by a perigranular membrane.
Although mast cells are found exclusively within tissues, they are part of the haematopoietic system and originate from pluripotent bone-marrow cells. The mast cell progenitor circulating in the blood is CD34+, and expresses the receptor for stem cell factor (SCF), but lacks expression of the high-affinity IgE receptor, FceRI (Rottem et al. 1994). From the blood, mast cells progenitors invade the connective or mucosal tissue, where they differentiate into mature mast cells (Kitamura and Fujita 1989). An essential growth factor for human mast cells is the stem cell factor (SCF), which stimulates the differentiation of mast cells from human bone marrow, fetal liver, cord blood and peripheral blood (see Nilsson et al. 1996 and references therein).

Mast cells show much heterogeneity. Human mast cells can be classified by their proteinase composition into two major subpopulations: MC TC and MC T cells. MC TC cells contain tryptase, chymase, a cathepsin G-like proteinase and carboxypeptidase in their secretory granules, whereas MC T cells contain tryptase only (Table 1). MC T cells occur in the mucosal surfaces and the lungs, whereas MC TC cells are the dominant mast cell type in the serosal surfaces, the lamina propria and the skin where they constitute up to 90% of mast cells in the normal human dermis (Craig et al. 1988, Irani et al. 1989). MC TC cells do not require T cell activation and have a life-span of 6 months to a year, in contrast to MC T cells, which are T cell dependent for maturation, survive for about a month, and contain less histamine. Human mast cells are also heterogeneous with respect to their cytokine content. Although both phenotypes of mast cells contain IL-4, it is expressed by 85% of MC TC but only by 15% of MC T cells (Bradding et al. 1995). In contrast, IL 5 and IL 6 are expressed almost exclusively by the MC T subpopulation (Bradding et al. 1995). This implies that the biologic functions of MC TC and MC T cells differ from each other. In some reports, MC C cells (containing chymase and carboxypeptidase, but not tryptase) have also been described (KleinJan et al. 1996, Li et al. 1996). MC C cells can be found on rare occasions in human skin, where 0.5% of the mast cells belong to these subpopulations (Weidner and Austen 1993). In skin organ cultures also, a chymase-positive but tryptase-negative mast cell has been described. However, this cell represents a dead mast cell appearing during culture due to solubilization of tryptase (Craig et al. 1988).

Mast cells participate in acute inflammation, particularly in conditions associated with increased vascular permeability, urticaria and angio-oedema. Increased mast cell numbers
are also associated with several disorders showing features of persistent chronic inflammation and hyperproliferation. Lesions of atopic dermatitis (Järvikallio et al. 1997) and hypertrophic scars (Kischer et al. 1978) contain numerous mast cells. In psoriatic lesions, MC_T cells are increased in number in the upper dermis, and are frequently in apparent contact with the basal keratinocyte layer (Harvima IT et al. 1990, 1993). Mast cells also play a role in many regenerative processes, including angiogenesis (Azizkhan et al. 1980, Marks et al. 1986, Grutzkau et al. 1998) and wound healing (Wichmann 1955, Persinger et al. 1983, Reich et al. 1991).

Table 1. The two major proteases of human mast cells (Schwartz 1987, Caughey et al. 1988, Tam and Caughey 1990).

<table>
<thead>
<tr>
<th>Substrate specificity</th>
<th>Tryptase (basic amino acids)</th>
<th>Chymotryptic (aromatic amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH optimum</td>
<td>neutral</td>
<td>neutral</td>
</tr>
<tr>
<td>Size</td>
<td>130 -140 kDa (tetramer)</td>
<td>30 kDa (monomer)</td>
</tr>
<tr>
<td>Amount/cell</td>
<td>10 pg/T and 35 pg/TC mast cell</td>
<td>4.5 pg/TC mast cell</td>
</tr>
<tr>
<td>Regulation</td>
<td>stabilized by heparin, not inhibited by protease inhibitors in plasma</td>
<td>heparin-bound, inhibited by plasma protease inhibitors</td>
</tr>
<tr>
<td>Neuropeptides as substrates</td>
<td>CGRP, VIP</td>
<td>substance P, VIP</td>
</tr>
</tbody>
</table>

**Mast cell activation**

A typical feature of mast cells is the expression of the FceRI receptor. This receptor consists of four subunits and binds monomeric IgE with high specificity. Activation of skin mast cells can occur via FceRI or via IgE receptor-independent mechanisms (Amon et al. 1994). The specific antigen combines divalenty with IgE, leading to cross-linking of FceRI and subsequent release of mediators. In addition to the IgE receptor, mast cells have receptors for molecules such as SCF, nerve growth factor (NGF), IL-8, urokinase-type plasminogen activator (uPA), opiates, neurotransmitters and the anaphylatoxins C3a and
C5a (reviewed by Artuc et al. 1999). Other FceRI-independent mast cell secretagogues include neuropeptides, drugs (i.e. opiates) and venoms.

The mast cell is a repository of a wide range of pharmacological substances (Table 2). The mast cell secretes its pharmacological contents in response to an appropriate pathophysiological stimulus by a process resembling exocytosis (Uvnäs 1978). This brings the granule and its contents into contact with the extracellular milieu through a system of pores and channels. Stimulus-secretion coupling following immunological or non-immunological stimuli involves protein-kinase activation, and protein phosphorylation. Preformed products (within mast cell granules) and newly synthesized substances mediate the immune and nonimmune functions of mast cells. In general, mast cell derived molecules induce immediate vasodilation, smooth muscle contraction, upregulation of adhesion molecules, influx of inflammatory cells and growth promotion of resident tissue cells.

The IgE-mediated allergic reaction and mast cell activation begin within minutes. The subsequent release of histamine is a rapid process that is complete in less than 20 min and resolves in hours (Horsmanheimo et al. 1996, Petersen et al. 1996). Neuropeptide substance P causes a selective and rapid release of histamine from skin mast cells, but not from mast cells in other tissues (Church et al. 1991).

Table 2. Summary of the most important mediators constitutively expressed and stored*, or newly synthesized on stimulation of mast cells (modified from Artuc et al. 1999).

<table>
<thead>
<tr>
<th>Mediator class</th>
<th>Mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amines</td>
<td>Histamine*</td>
</tr>
<tr>
<td>Glycosaminoglycans</td>
<td>Heparin, Chondroitin sulfate E (not in skin mast cells)</td>
</tr>
<tr>
<td>Proteases</td>
<td>Tryptase, Chymase, Cathepsin G-like carboxypeptidase</td>
</tr>
<tr>
<td>Lipids</td>
<td>PGD₂, LTB₄, LTC₄, PAF</td>
</tr>
<tr>
<td>Cytokines</td>
<td>TNF-α*, IL-1β*, IL-3*, IL-4*, IL-5, IL-6, IL-10*, IL-13, IL-16</td>
</tr>
<tr>
<td>Chemokines</td>
<td>IL-8*, MCP-1, MIP-1</td>
</tr>
<tr>
<td>Growth factors</td>
<td>TGF-β, SCF*, NGF, GM-CSF, M-CSF*, PDGF, bFGF, VEGF*</td>
</tr>
</tbody>
</table>
Regulation of the growth and functions of mast cells

An essential growth factor for human mast cells is the stem cell factor (SCF), also referred to as the mast cell growth factor or the Kit-ligand (Irani et al. 1992, Valent et al. 1992, Mitsui et al. 1993). The SCF mediates a number of biological functions of mast cells. It increases mast cell proliferation, differentiation, survival, degranulation and chemotaxis (Columbo et al. 1992, Nilsson et al. 1994b, Grabbe et al. 1994), as well as adherence of mast cells to fibronectin and other ECM proteins (Dastych et al. 1994, Lorentz et al. 2002). Expression of the SCF may prevent apoptosis of mast cells (Iemura et al. 1994). Because of these properties, the SCF provides one way of regulating local mast cell numbers.

Several cell types in the skin produce the SCF. In the epidermis, it is produced by keratinocytes, where it is normally present in the cytoplasm (Longley et al. 1993). In the dermis, the SCF is produced by endothelial cells (Weiss et al. 1995), fibroblasts (Longley et al. 1993) and mast cells (Zhang et al. 1998, Welker et al. 1999). Langerhans cells are also known as a source of the SCF, based on immunohistochemical studies (Hamann et al. 1995). The SCF molecule comprises an N- and an O-glycosylated protein with a total length of 248 amino acids and is synthesized as a transmembrane protein, but is also found in soluble form. The extracellular portion of the transmembrane SCF molecule can be released from the membrane by proteolytic cleavage (Zsebo et al. 1990). Mast cell chymase cleaves the SCF to yield a soluble, bioactive form of the molecule (Longley et al. 1997). The SCF exerts its effects via Kit, a transmembrane tyrosine kinase receptor, which is encoded by the proto-oncogene c-kit and expressed in mast cell progenitors, mature tissue mast cells (Hamann et al. 1994) and melanocytes (Dipple et al. 1995). Both the soluble (SCF-1) and the membrane-bound (SCF-2) forms stimulate the Kit receptor. The expression of Kit is inversely correlated with the binding of the SCF to the Kit receptor. Kit appears to be internalized and degraded after activation by the SCF in vitro; new expression of the receptor appears to require new protein synthesis (Baghestanian et al. 1996, Shimizu et al. 1996). Also cytokines such as IL-4 and GM-CSF have been demonstrated to downregulate Kit in mast cells (Nilsson et al. 1994c, Welker et al. 2001).

Although the SCF is the best studied growth factor of mast cells, there are also other factors modulating mast cell functions. The nerve growth factor (NGF) has in vitro effects
on human mast cell differentiation (Welker et al. 2000) and its receptor is found on human mast cells (Artue et al. 1999). TGF-β induces a more powerful chemotactic response in mast cells than the SCF (Gruber et al. 1994). The granulocyte macrophage-colony stimulating factor (GM-CSF), a multipotent hematopoietic growth factor, induces proliferation of early mast cell progenitors, but interferes with mast cell maturation (Welker et al. 1997) and Kit expression (Welker et al. 2001), and thus may downregulate mast cell characteristics in more differentiated cells. All these four factors can be produced by human mast cells themselves, which provides a way of regulating mast cell influx and differentiation in an autocrine fashion.

2.4 Mediators derived from mast cells

**Histamine**

Histamine is the best known mast cell product and the only major biogenic amine present in human mast cells. Histamine is also produced by basophils, T cells, macrophages, and some other cells. It has a small molecular size of 111 Da. Histamine is formed in mast cells after decarboxylation of histidine and is stored in cytoplasmic granules. Human cutaneous mast cells have a histamine content of approximately 5 pg/cell (Benyon et al. 1987). In the secretory granules of mast cells, where the pH is acidic, histamine is probably bound to negatively charged carboxyl groups in the proteoglycan-protein matrix (Uvnäs 1978), whereas, after release into the neutral pH environment, histamine becomes less positively charged, dissociates from the complex, and is freely soluble. Both tissue mast cells and circulating basophils account for the normally low concentrations of plasma histamine (0.05 - 0.2 mg/ml). Once released, histamine is degraded either through deamination by diamine oxidase or through methylation by histamine-N-methyltransferase. The biologic half-life of histamine is short (minutes). This rapid metabolism suggests that its function is to act locally near the site of its release.

Histamine mediates its actions by means of H₁, H₂ and H₃ receptors. Stimulation of the H₁ receptor leads to venous permeability, smooth muscle contraction, neutrophil and eosinophil chemotaxis, and increased prostaglandin synthesis. H₂ receptor stimulation results in gastric acid secretion, venous permeability, decreased lymphotoxicity, increased
T cell suppressor function, inhibition of IgE-mediated basophil histamine release, and decreased neutrophil and eosinophil chemotaxis. H₃ receptors present within the central nervous system and lung appear to control histamine formation and release via negative feedback.

The intradermal injection of histamine classically produces a triple response that initially consists of localized erythema followed by an expanding area of redness (flare) and a central wheal formation (Lewis 1927). Histamine-induced whealing results from contraction of the post-capillary venules of endothelial cells, whereas the flare reaction is mediated through the stimulation of unmyelinated sensory nerves, leading to the release of neuropeptides and axon reflex vasodilation.

Histamine release from mast cells can be evoked by immunologic or by non-immunologic factors. Neuropeptides, such as SP, VIP and somatostatin are able to release histamine from skin mast cells. IgE-dependent activation of skin mast cells in vitro is relatively slow, reaching completion within 6 min after the challenge, whereas SP-induced histamine release is rapid, being complete in 20 sec (Benyon et al. 1987). SP has been stated to be one of the most potent histamine-releasing agents in the human skin (Hägermark et al. 1978). Only skin mast cells are able to mount a secretory response to non-immunologic stimulation with neuropeptides (Church et al. 1991). TNF-α can also stimulate the release of histamine and tryptase from human mast cells (van-Overveld et al. 1991).

The radioenzyme assay (REA) is a very sensitive method for assaying histamine (Harvima RJ et al. 1988). This method is based on transmethylation with purified histamine-N-methyltransferase, utilizing [³H-methyl]-S-adenosylmethionine as the methyl donor, and is optimized for low histamine concentrations. Histamine could also be assayed by HPLC (high performance liquid chromatography) using post-column derivatization, and RIA (radio immuno assay).

**Proteoglycans**

The staining of mast cells with basic dyes and the expression of metachromasia are due to the presence of highly sulfated proteoglycans that reside in the secretory granules. Two classes of proteoglycan, heparin and chondroitin sulfate E, are localized to mast cells. Heparin is associated with mast cells in the skin and in the lungs, whereas chondroitin
sulfate E is associated with intestinal mast cells. Heparin is a complex linear polysaccharide of 60 to 100 kDa, covalently attached to a core protein that is found in the secretory granules of the mast cells. The acidic pH within the secretory granule favours binding of biogenic amines and enzymes to proteoglycans. Heparin and chondroitin sulfate E stabilize mast cell proteases and alter the biological activity of many enzymes. After release from the mast cells, these proteoglycans may continue to modulate the activity of mediators that remain associated. The stabilizing effect of heparin on human tryptase activity (Schwartz et al. 1986) may be crucial for the full expression of mast-cell-mediated events.

When released from mast cells, heparin is rapidly ingested and destroyed by macrophages. Thus, heparin cannot be detected in plasma under normal circumstances. The heparin concentration within mast cell secretory granules can be estimated to be 10 mg/ml (based on Metcalfe et al. 1980).

Tryptase

Tryptase is the principal enzyme accounting for the trypsin-like activity detected in human mast cells (Table 1) (see Schwartz et al. 1981). It is the most abundant secretory granule protein in all subsets of mast cells (Schwartz et al. 1987). Tryptase is an allosteric enzyme and a tetrameric serine proteinase of 130 - 140 kDa with subunits of about 35 kDa. This enzyme is relatively specific to mast cells, since other cell types have been found to contain little or no amounts of tryptase. Tryptase is not inhibited by any of the protease inhibitors found in plasma. Instead, the enzyme is uniquely stabilized in its active tetrameric form by association with heparin, the most potent biologic stabilizer of tryptase. Tryptase is also stabilized in high-salt environments (Harvima IT et al. 1988). When free in solution, tryptase tetramers rapidly and irreversibly dissociate into inactive monomers (Schwartz et al. 1986).

Mast cell tryptase is a heterogeneous glycoprotein (Harvima RJ et al. 1999). Five different, but highly homologous tryptase cDNAs have been cloned from human skin and lung mast cell populations (Miller et al. 1989, 1990, Vanderslice et al. 1990). These tryptases can be divided into two groups, α- and β-tryptases. Tryptase is stored in cytoplasmic secretory granules mostly in the fully active β-tryptase form, whereas α-tryptase is constitutively secreted as α-protryptase. The α- and β-forms of tryptase differ in
their number of putative carbohydrate binding sites: α-tryptase has two but β-tryptase only one binding site (Miller et al. 1990). Human leukemic HMC-1 mast cell line expresses only β-tryptase (Nilsson et al. 1994a).

The immediate consequence of mast cell stimulation and degranulation is the release of active β-tryptase, along with other mediators, which then initiates the proteolytic cleavage of specific peptide and protein substrates. These substrates can be classified into three general types: neuropeptides, proteases and cell surface receptors. Tryptase and other trypsin-like enzymes are able to cleave substrates with the basic amino acid residues, lysine and arginine. Tryptase effectively cleaves 72 kDa gelatinase, fibronectin and intact type IV collagen microfibrils (Itoh et al. 1992, Kielty et al. 1993). This suggests that tryptase may function in the normal regulation of extracellular matrix turnover through a direct proteolytic mechanism. Another pathway by which tryptase may indirectly initiate extracellular matrix degradation is through the activation of matrix metalloproteinases. Tryptase can activate prostromelysin to stromelysin, which, in turn, activates latent collagenase (Gruber et al. 1989). However, tryptase does not increase matrix metalloproteinase expression in normal dermal fibroblasts (Zhang et al. 1999). Tryptase, either alone or in combination with metalloproteinases, can participate in the destruction of the BM (Kaminska et al. 1999a). The single-chain urinary-type plasminogen activator (uPA) is activated by tryptase (Stack and Johnson 1994), and can, in turn, activate plasminogen to plasmin. Tryptase cleaves fibrinogen rapidly, leading to the start of thrombin-induced blood clotting (Schwartz et al. 1985). The biological effects of tryptase also include generation of C3a from C3 (Schwartz et al. 1983). It is possible that C3a, locally generated in tissues, could promote smooth muscle contraction and increased vasopermeability.

Of the neuropeptides, tryptase cleaves vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and the peptide histidine-methionine, but not substance P (SP) (Caughey et al. 1988, Tam et al. 1990, Walls et al. 1992). Thus tryptase may regulate neurogenic inflammation in the skin (Naukkarinen et al. 1994). An additional substrate target for tryptase is the protease-activated receptor 2 (PAR-2), a G-protein-coupled receptor in keratinocytes and vascular endothelial cells (Molino et al. 1997). Tryptase and trypsin are the only two proteases currently known to activate PAR-2.
Chymase

Chymase is the principal enzyme accounting for the chymotrypsin-like activity present in human cutaneous mast cells. It is a serine proteinase and monomer of 30 kDa. Chymase is not present in all mast cells, but is selectively localized to a subset of mast cells in the skin and small bowel submucosa (Irani et al. 1986). Like tryptase, chymase is stored in mast cell granules in active form and is presumably bound to the negatively charged proteoglycan. However, chymase-heparin proteoglycan complexes are larger than tryptase-heparin proteoglycan complexes (Goldstein et al. 1992). This indicates a slower diffusion for chymase than for tryptase from the site of discharge from mast cells in the extracellular matrix. During incubation of skin specimens in vitro, chymase was found to remain at the site of release, whereas tryptase was solubilized (Kivinen et al. 2001, 2003).

Unlike tryptase, chymase is inhibited by serum protease inhibitors, such as α1-proteinase inhibitor (α1-PI), α1-antichymotrypsin (α1-AC) and α2-macroglobulin (reviewed in Harvima R 1999). Chymase is presumably inhibited by these inhibitors after its release from mast cell granules. However, it can also degrade these inhibitors efficiently (Schechter et al. 1989). Chymase is also effectively inhibited by serine proteinase inhibitors such as diisopropyl fluorophosphate (DFP), and phenylmethylsulfonyl fluoride (PMSF). When released from mast cell granules, chymase is bound to heparin, although, unlike tryptase, the stability of chymase is not substantially affected by heparin (Sayama et al. 1987).

Chymase hydrolyzes the esters of tyrosine, acetyltirosine, and acetylphenylalanine. It is a highly efficient converter of angiotensin I to angiotensin II (Wintroub et al. 1984). Chymase degrades basement membrane components at the dermal-epidermal junction, facilitating the separation of these regions (Briggaman et al. 1984). Fibronectin, in both soluble and matrix forms, is highly susceptible to degradation by rat chymase (Vartio et al. 1981). Chymase affects collagen synthesis. It cleaves type I procollagen to a fibril-forming collagen molecule (Kofford et al. 1997), and activates procollagenase (matrix metalloproteinase-1) (Saarinen et al. 1994). These abilities of chymase suggest that it plays an active role in matrix modeling and degradation. Chymase is capable of degrading SP and VIP (Caughey et al. 1988), and releasing latent TGF-β1 from the extracellular matrix (Taipale et al. 1995). Chymase also cleaves stem cell factor (SCF), an essential growth
factor for human mast cells, to yield a soluble, bioactive form of the molecule (Longley et al. 1997).

2.5 Mast cells in wound healing

Mast cells are activated in inflammatory reactions, but in addition they seem to have roles in tissue regeneration and wound healing. Studies on animal wounds show alteration in mast cell numbers during wound healing. In rat skin, early disappearance of mast cells is followed by mast cell recovery, mast cell hyperplasia, and finally, normalization of mast cell counts around incision wounds (Wichmann 1955, Persinger et al. 1983); although the early disappearance of mast cells may be due to extensive mast cell degranulation. On the other hand, an increase in mast cell number at an early stage of wound healing has been found in pigs (Reich et al. 1991). Degranulation of mast cells is also suggested to take place in wound healing in human skin (Dvorak and Kissell 1991). Numbers of MC\textsubscript{TC} cells are reported to be decreased in scars, whereas Kit-positive cells, i.e. the total number of mast cells, were increased, pointing to the existence of degranulated, so-called phantom mast cells (Hennes et al. 2000). Numerous mast cells are found in impaired wound healing. In chronic venous ulcers, mast cell hyperplasia is found primarily in the papillary dermis, but also in the reticular dermis, in the granulation tissue, and inside the epidermis. Furthermore, mast cell granules are found inside basal and suprabasal keratinocytes (Bolton and Montagna 1993) suggesting a close interaction between keratinocytes and mast cells.

Degranulated mast cells release their granules into the tissue, where they remain as intact particles and are phagocytosed by other cells (Subba Rao et al. 1983). Direct transgranulation of mast cell granules can also occur via cell-to-cell contact between mast cells and adjacent cells; these mast cell interactions occur specifically with fibroblasts and endothelial cells (Greenberg et al. 1983). Certain granule components, such as histamine, can freely dissociate from the granules under physiological conditions (Uvnäs 1978), while others remain in the granule. With these mechanisms, mast cell mediators are available in the tissue and can contribute to wound healing. In animals, mast cell degranulation elicited by compound 48/80 initiates cell proliferation (Norrby 1983) and enhances wound healing (Fitzpatrick et al. 1982).
Among mast cell mediators, there are many that have effects on cell growth, tissue turnover and repair (Table 3). Several *in vitro* studies have indicated that mast cell mediators have an effect on epidermal cell proliferation. Under cell culture conditions, histamine inhibits mitosis in pig epidermal cells (Aoyagi et al. 1981) and in human keratinocytes (Harper and Flaxman 1975). Heparin also inhibits the proliferation of keratinocytes (Cook et al. 1991), without inducing cell differentiation (Pillai et al. 1994). Heparin-induced inhibition can be reversed by exogenous growth factors such as EGF (Cook et al. 1991). In contrast, leukotrienes B₄, C₄ and D₄ stimulate human keratinocyte DNA synthesis in cell culture (Kragballe et al. 1985). Of the mast cell cytokines tested, IL-6 (Grossman et al. 1989) and IL-8 (Tuschil et al. 1992) stimulate, but TNF α, interferon γ (IFN-γ) (Pillai et al. 1989, Symington 1989) and TGF-β (Shipley et al. 1986) inhibit the proliferation of human keratinocytes in culture.

A possible additional mast cell-derived mediator that might affect cell growth is tryptase, although it is not a common mitogen. However, tryptase has been shown to be a potent angiogenic factor and a mitogen for endothelial cells (Blair et al. 1997). Tryptase is a fibrogenic factor stimulating fibroblast chemotaxis and collagen synthesis (Cairns et al. 1997, Gruber et al. 1997). Furthermore, there are data suggesting that tryptase is a mitogen for cultured fibroblasts (Ruoss et al. 1991, Hartmann et al. 1992), although, there have also been conflicting results (Forsberg-Nilsson et al. 1996). Both tryptase and chymase are inhibitory to keratinocyte growth (Algernissen et al. 1999). Nonetheless, tryptase and chymase play important roles in wound healing, in degrading extracellular matrix proteins, cleaning debris from the wounded area and releasing matrix-associated growth factors (Taipale et al. 1995).

Mast cell hyperplasia is found in several fibrotic conditions (review in Gruber 1995). Studies of cocultured mast cells and fibroblasts have shown that activation of mast cells (Dayton et al. 1989), and even as the mere presence of mast cells (Levi-Schaffer and Kupietzky 1990), stimulates fibroblasts to migration, proliferation and protein synthesis. In addition, several mast cell mediators are able to stimulate fibroblast proliferation, chemotaxis and synthesis of matrix molecules (Table 3). Histamine induces proliferation of fibroblasts (Russel et al. 1977, Jordana et al. 1988) and collagen synthesis (Hatanochii et al. 1991). Histamine has also been shown to stimulate FGF-7 secretion by human fibroblasts
via the H₁ receptor, suggesting a novel pathway for the histamine-mediated proliferative effect on fibroblasts and keratinocytes (Artuc et al. 2002). Basic FGF synthesized in mast cells is a potent mitogen for fibroblasts. Of other mast cell cytokines, IL-4 increases the production of collagen (Gillery et al. 1992) and fibronectin (Postlethwaite et al. 1992). TNF-α stimulates proliferation (Sugarman et al. 1985) and invasive migration of fibroblasts (Schirren et al. 1990), but inhibits collagen gene expression in cultured fibroblasts (Solis-Herruzo et al. 1988).

Angiogenesis is critical for normal growth and repair processes. Mast cells that are located around capillary vessels in the dermis could play a role in the regulation of angiogenesis through release of angiogenic factors. The most important mast cell-derived cytokines and growth factors in this process include bFGF, TNF-α, VEGF and TGF-β (Artuc et al. 1999). Heparin stimulates endothelial cell migration (Azizkhan et al. 1980) and aFGF-stimulated growth of endothelial cells (Thornton et al. 1983). In addition, heparin promotes the binding of angiogenic growth factors such as bFGF and VEGF to their receptors (Yayou et al. 1991, Gitay-Goren et al. 1992). Histamine (Marks et al. 1986), TNF-α (Leibovich et al. 1987) and tryptase (Blair et al. 1997) may also stimulate angiogenesis, but inhibition of in vitro angiogenesis has been reported with TNF and IFN-γ (Sato et al. 1990). On the other hand, endothelial cells in capillaries might also affect mast cell growth, maintenance and chemotaxis, since they express the mast cell growth factor SCF (Weiss et al. 1995).
Table 3. Mast cell derived mediators having potential significance in wound healing (for references, see the text).

<table>
<thead>
<tr>
<th>Angiogenesis</th>
<th>Fibrosis</th>
<th>Keratinocyte growth</th>
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<tr>
<td>VEGF</td>
<td>PDGF</td>
<td>bFGF</td>
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<tr>
<td>bFGF</td>
<td>TGF-β</td>
<td>II -6, II -8</td>
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<tr>
<td>TGF-β</td>
<td>bFGF</td>
<td>Histamine</td>
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<tr>
<td>PDGF</td>
<td>Tryptase</td>
<td>LTB₄, LTC₄</td>
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<tr>
<td>G-CSF</td>
<td>Chymase</td>
<td>TGF-β</td>
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<td>TNF-α</td>
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<td>Histamine</td>
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2.6 Interaction between nerves and mast cells

The skin is the most extensive sense organ and is therefore abundantly supplied with sensory nerves, which end in fine arborizations of free nerve endings. These sensory nerve endings are rich in neuropeptides and are in close proximity to the dermal mast cells (Wiesner-Menzel et al. 1981, Naukkarinen et al. 1993). The cutaneous neuropeptides found in human consist of more than 20 kinds of peptides, including substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), and peptide histidine-methionine (PHM) (Wallengren et al. 1987a, Weihe and Hartschuh 1988). The neuropeptides are released from nerve endings by axonal reflex and act as neurotransmitters/neuromodulators via specific receptors. They are considered to be important mediators of local inflammation, and their effects include smooth muscle contraction and relaxation, functions of secretory cells, vasodilation and increased vascular permeability. The hyperemia and edema of acute inflammation can be elicited by neuropeptides, especially SP and CGRP, which are released concomitantly from the same
nerve fibers in the superficial dermis in response to injury (Wallengren et al. 1987a). This phenomenon is called neurogenic inflammation. The term suggests that the peripheral nervous system takes part in the generation of inflammatory responses. Furthermore, cutaneous neuropeptides are thought to be involved in the pathogenesis of certain inflammatory skin diseases; e.g. in a mature psoriatic plaque, SP- and VIP-positive nerves are substantially increased (Naukkarinen et al. 1993).

Intradermal injection of picomolar quantities of SP into human skin produces flare, a wheal and itching (Hägermark et al. 1978, Wallengren and Håkanson 1987b) similar to those elicited by histamine, whereas CGRP is a potent vasodilator and produces an indurated erythema (Wallengren and Håkanson 1987b). Local pretreatment of the skin with an H1 histamine receptor antagonist or with compound 48/80, which depletes histamine from mast cells, inhibits the flare and itch evoked by the subsequent injection of SP (Hägermark et al. 1978), but the wheal response is only partly inhibited (Wallengren and Håkanson 1987b). Accordingly, recent data have demonstrated that SP is also capable of inducing histamine-independent vasodilatation and protein extravasation (Weidner et al. 2000). Nonetheless, it appears that the dermal responses of SP are at least partly mediated by histamine. The CGRP-evoked erythema does not seem to be mediated by histamine, for pretreatment with H1 histamine antagonist or compound 48/80 does not suppress it (Wallengren and Håkanson 1987b). Capsaicin, the pungent agent of capsicum peppers, is known to release and deplete neuropeptides, such as SP, VIP, CGRP and neurokinin A, from sensory nerves (C-fibers) (Holzer 1988). When injected intradermally, capsaicin evokes flare and pain, but no significant release of histamine (Petersen et al. 1997). The flare is reduced by a local anaesthetic but not by compound 48/80 or an antihistamine (Wallengren and Håkanson 1992), suggesting that histamine is not the final mediator of the vasodilatation in capsaicin-induced flare.

The prominent effects of neuropeptides on mast cells appear to be the basis for the contributions of neuropeptides to immediate hypersensitivity. SP has been demonstrated to release histamine from mast cells of human skin in vitro (Benyon et al. 1987, Ebertz et al. 1987). In fact, SP has been stated to be one of the most potent histamine-releasing agents in human skin (Hägermark et al. 1978). Like SP, VIP has also been shown to release histamine from human skin mast cells and, as in the case of SP, the histamine release is
rapid, reaching completion in 10 - 20 s. Compared to SP and VIP, the ability of CGRP to release histamine from human skin mast cells in vitro is weak (Lowman et al. 1988a). Compared to non-immunological stimulation with neuropeptides, IgE-dependent activation of skin mast cells is relatively slow, reaching completion within 6 min after challenge (Benyon et al. 1987). Sensory neuropeptides exhibit cellular selectivity, since only skin mast cells are able to mount a secretory response to neuropeptides (Lowman et al. 1988b).

The direct proximity of cutaneous sensory nerves and mast cells raises the possibility that neurogenic triggering of mast cells may be important physiologically and in certain pathologic conditions. SP released from sensory nerve fibers mobilizes histamine from adjacent mast cells, and histamine, in turn, stimulates nerve fibers to release SP (Burnstock 1977, Hägermark et al. 1978). The functional integration between C-fibers and mast cells in the skin is so intimate that the dermal response to injury can be equally blocked either by a local anaesthetic or by antihistamines (Foreman and Jordan 1983, Holzer 1988).

In addition to their role as chemical mediators of inflammatory reactions, neuropeptides are also believed to take part in the regulation of growth and repair processes of tissues. SP stimulates the in vitro proliferation of T lymphocytes (Payan et al. 1983) and fibroblasts (Nilsson et al. 1985). VIP has been shown to stimulate proliferation of cultured human keratinocytes (Hägerstrand et al. 1989), and CGRP has been demonstrated to increase the proliferation of endothelial cells (Hägerstrand et al. 1990).

After release from peripheral sensory nerves, neuropeptides are subject to continuous and rapid modification (Kikuchi et al. 1988, Katayama et al. 1991). Neuropeptides can also regulate their own metabolic rate (Brain et al. 1988). Both tryptase and chymase of mast cells are capable of degrading neuropeptides: tryptase cleaves the vasoactive intestinal peptide (VIP), the calcitonin gene-related peptide (CGRP) and the peptide histidinemethionine, but not substance P (SP) (Caughey et al. 1988, Tam and Caughey 1990, Walls et al. 1992), whereas chymase cleaves both SP and VIP (Caughey et al. 1988). Thus, tryptase and chymase are able to alter the local neuropeptide content in the skin and may regulate neurogenic inflammation. In addition, tryptase cleaves and activates the PAR-2 receptor in spinal afferent neurons, leading to the release of CGRP and SP, and thereby has the potential to induce neurogenic inflammation (Steinhoff et al. 2000).
3 AIMS OF THE STUDY

1. To study mast cell activation and histamine release by neuromediators and cutaneous nerves, using different neuropeptides and capsaicin.
2. To describe the changes in tryptase- and chymase-positive mast cells in the wound edges at different stages of normal wound healing and in chronic venous leg ulcers.
3. To describe the changes in SCF and its receptor Kit during the early events of cutaneous wound healing and to compare them with the changes in chronic ulcers and in psoriasis.
4. To measure the levels of histamine and mast cell protease activities in the wound fluid and wound bed of chronic ulcers.
5. To investigate the effect of the most important mast cell mediators on keratinocyte growth, migration and adherence in cell culture conditions.
4 MATERIALS AND METHODS

4.1 Chemicals

Partially purified preparation of trypase and chymase was obtained by using low- and high-salt extraction, octyl-Sepharose CL-4B hydrophobic affinity chromatography and heparin-Sepharose chromatography (V). The ratio of trypase activity to chymase activity in the partially purified enzyme preparation was about 13:1 (19,700 U/l trypase and 1,540 U/l chymase) in the first preparation and 11:1 (15,120 U/l trypase and 1,336 U/l chymase) in the second preparation.

In addition to the partially purified preparation, trypase was also purified to homogeneity from human lung and skin specimens, as described previously (Harvima IT et al. 1988).

Detailed information about other chemicals and supplies is presented in the original publications.

4.2 Microdialysis

Microdialysis is a technique for monitoring the chemistry of the extracellular space in living tissue (Fig. 2). The probe (0.5 mm in diameter, cutoff 20 kDa; CMA Microdialysis, Stockholm, Sweden) was inserted into the dermal skin of the dorsal aspect of the forearm as superficially as possible. No local anesthesia was used. The perfusate was conducted through the system by a microinjection pump (CMA, Sweden) at a perfusion rate of 3.0 μl/min. The baseline sample was collected 1 h after the probe insertion, and thereafter skin challenging was begun. The patients and volunteers who took part in this study are presented in Table 4.

SP, CGRP or VIP was injected intradermally about 5 mm distant from the microdialysis probe. A single SP injection was given to 5 patients. Three other patients received a second SP injection 1 h after the first one; one subject participated in this experiment twice. VIP was injected as two consecutive injections into 4 patients, who received either 100 pmol or 250 pmol VIP. CGRP was injected into 2 patients, who had two microdialysis probes simultaneously, and SP was injected as a control adjacent to the second probe. SP and
CGRP were injected as a dose of 250 pmol, and VIP was injected as a dose of 100 or 250 pmol. In cases where a second peptide injection was given 1 h after the first one, it was given at exactly the same site. Samples were collected at 15-min intervals for up to 2 h.

Capsaicin (Sigma, St. Louis, MO, U.S.A.) was injected intradermally at a concentration of 30 µM and a volume of 25 µl in close proximity to the probe. Samples were collected at 15-min intervals for up to 75 min. Capsaicin cream (0.1%) was administered topically on the probe and was covered with a compress moistened with isotonic saline solution. The compress was removed after 1 h. Samples were collected for up to 2 h.

Figure 2. Microdialysis. The "dialysis" means diffusion of molecules between the extracellular fluid and the perfusion fluid. Physiological salt solution is slowly pumped through the microdialysis probe. The solution equilibrates with the surrounding extracellular tissue solution through the dialysis membrane. As a result, it then contains a representative proportion of the tissue fluid's molecules, e.g. histamine liberated from mast cells.
4.3 Patients and samples from skin and leg ulcers

*Patients and skin biopsies for histochemistry (II, III, V)*

Table 4 shows the number of subjects and the samples that were taken in publications I-III and V. Patients were recruited from the out-patient and in-patient departments of the Department of Dermatology, Kuopio University Hospital, Kuopio, Finland. The volunteers were healthy subjects and members of the research group or staff of the Department of Dermatology. All subjects volunteered to participate in these studies and gave their consent after receiving information about the study protocol. The experimental protocols were approved by the Ethics Committee of Kuopio University Hospital.

To study normal wound healing, biopsies were taken from patients undergoing skin pinch grafting of leg ulcers. Pinch grafts were taken from the skin of the anterior thigh by lifting the skin with a needle and cutting out the partial-thickness skin graft with a scalpel. Three similar donor sites were chosen for subsequent biopsing of wound edges on day 0, and then either on days 1 and 7, or days 3 and 14. The biopsies were approximately 10 mm long and 3 mm wide, and included part of the previously made wound and some surrounding intact skin.

To study impaired wound healing, biopsies were taken from the margins of chronic venous ulcers. A wedge of tissue, approximately 10 mm long and 4 mm wide, was excised with a scalpel to include the surrounding intact skin, the ulcer edge and the ulcer base.

All biopsies were taken under local anesthesia (1% lidocaine with adrenalin). After removal, the skin biopsies were immediately embedded in OCT compound (Miles Scientific, Naperville, IL) and frozen in isopentane cooled with a mixture of absolute ethanol and dry ice. Care was taken to mount the skin biopsy in a proper orientation in OCT.

*Collection of wash samples and shave biopsies from chronic wounds (V)*

Wound fluid was collected as wash samples from 16 patients. Five patients had a venous stasis ulcer, three had venous stasis with concomitant arteriosclerosis, two had arteriosclerosis, four had diabetes and neuropathy, and two patients had rheumatoid arthritis as their ulcer etiology. The bases of the ulcers were repeatedly washed with sterile 140 mM
NaCl and 10 mM sodium phosphate buffer, pH 6.5, in small aliquots using a micropipette, and the total volume of the wash solution was 1 ml. The samples were clarified by centrifugation and stored in a deep freezer until analyzed for enzyme activity, histamine and protein. In addition, a superficial shave biopsy was taken from the base of the ulcer with a knife from 8 of these patients. After biopsing, the specimens were rinsed with saline, snap frozen in liquid nitrogen, and stored in a deep freezer until further processing. For analysis, the biopsies were weighed and 20 μl of 1.5 M NaCl and 10 mM sodium phosphate buffer, pH 6.5, per 1 mg wet weight of tissue was added. The cells in the tissue specimens were broken by freezing and thawing five times. Each specimen was centrifuged, and the supernatant was collected and analyzed.

Table 4. The number of subjects, age range and the samples taken in each publication.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Number of subjects</th>
<th>Age range (mean age)</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10 volunteers</td>
<td>21-67 years (38)</td>
<td>Microdialysis</td>
</tr>
<tr>
<td></td>
<td>10 patients</td>
<td>40-70 years (57)</td>
<td>Microdialysis</td>
</tr>
<tr>
<td>II, III, V</td>
<td>12 patients</td>
<td>29-83 years (67)</td>
<td>Biopsies from healing wounds either on days 0,1,7 (n=6) or days 0, 3, 14 (n=6)</td>
</tr>
<tr>
<td></td>
<td>6 patients</td>
<td>40-82 years (66)</td>
<td>Biopsies from chronic wounds</td>
</tr>
<tr>
<td>III</td>
<td>3 volunteers</td>
<td>35-41 years (38)</td>
<td>Biopsies from healthy skin</td>
</tr>
<tr>
<td></td>
<td>10 patients</td>
<td>22-60 years (39)</td>
<td>Biopsies from psoriatic lesions and uninvolved skin</td>
</tr>
<tr>
<td>V</td>
<td>16 patients</td>
<td>36-93 years (71)</td>
<td>Wash samples from chronic wounds and additional shave biopsies from 8 of these patients</td>
</tr>
</tbody>
</table>
4.4 Histochemical staining methods and counting cell numbers

**Histochemical staining methods for tryptase and chymase (II, III, V)**

Cryosections of 5 μm thickness were cut on poly-L-lysine coated slides which were stored at −20°C. Prior to staining, the slides were immersed in a fixation mixture of 0.6% formaldehyde and 0.5% acetic acid, pH 7.2, for 10 min. Enzyme activity of mast cell tryptase was demonstrated with 1 mM Z-Gly-Pro-Arg-MNA as the substrate, and 0.5 mg/ml Fast black K salt as the chromagen. The pH of the staining solution was adjusted to 7.5 with 100 mM Tris-HCl buffer. α1-Proteinase inhibitor (0.2 mg/ml) was included in this substrate solution. This enzyme-histochemical method has been found to stain only mast cells selectively and sensitively (Harvima IT et al. 1993). Tryptase was also stained immunohistochemically (V), using rabbit polyclonal anti-tryptase antibody (0.2 μg/ml) (Harvima IT et al. 1989b). Vectastain Elite ABC kit was used for visualizing the bound antibody.

Mast cell chymase was stained enzyme-histochemically, using 1 mM Suc-Val-Pro-Phe-MNA as the substrate and 0.5 mg/ml Fast black K salt as the chromagen at pH 7.5. Aprotinin (0.5 mg/ml) was included in the substrate solution to confirm the staining specificity, since the enzyme activity of cathepsin G, but not that of chymase, can be inhibited by aprotinin (Harvima IT et al. 1993). In addition, chymase was stained immunohistochemically (V) on acetone-fixed cryosections using mouse monoclonal anti-chymase antibody (0.1 μg/ml).

**Immunohistochemical and sequential double-staining methods (II, III)**

For immunohistochemical staining, the skin sections were fixed in cold acetone for 10 min. IL-4 (II) was stained with polyclonal antihuman-IL-4 IgG antibody (10 μg/ml) dissolved in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The bound polyclonal antibody on the skin sections was visualized using the Vectastain Elite ABC kit together with 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.04% nickel chloride, and 0.03% hydrogen peroxide. TNF-α (II) was stained similarly, using polyclonal antihuman-TNF-α IgG antibody (50 μg/ml for biopsies of acute wounds, and 15 μg/ml for
biopsies of chronic wounds). As negative controls, the primary antibody was replaced either with PRS or with nonimmune IgG from the same animal as the primary antibodies. The sensitivity of the antibodies was confirmed in control positive stainings, e.g. the anti-IL-4 antibody stained mast cells in atopic dermatitis lesions (Horsmanheimo et al. 1994).

A sequential double-staining method was used to confirm that IL-4 and TNF-α were located in the mast cells. First, the skin sections were stained using the enzyme-histochemical staining method for identifying mast cell tryptase. After that, the skin sections were photographed at ×66 magnification. In epithelializing wounds, two photographs, one on top of the other, lining the ulcer margin and extending about 0.6 mm deep into the dermis, were taken from each skin section. In chronic wounds, two photographs were taken of the ulcer base and two of the perilesional skin, from the sites where the mast cells were counted. The azo dye was then removed by overnight treatment with 15% Tween 20, and the same sections were stained immunohistochemically for IL-4 or TNF-α, and photographed at exactly the same sites as the previous pictures. The Kit receptor (III) was stained similarly, using monoclonal anti-human-Kit IgG antibody (3 μg/ml and 20 μg/ml for antibodies of Southern Biotechnology Associates and Pharmingen, respectively) and sequential double-staining. In psoriasis (III), the sections were photographed by taking three adjacent photographs along the epidermis, and extending approximately 0.4 mm into the dermis.

SCF (III) was stained with monoclonal anti-human-SCF IgG antibody (75 μg/ml) dissolved in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. The bound monoclonal antibody on skin sections was visualized using the Vectastain Elite ABC kit as described above. After immunostaining for SCF, sections were counterstained with Mayer’s haematoxylin.

*Counting the numbers of cells (II, III, V)*

Mast cells stained for tryptase and chymase were counted separately in adjacent skin sections under high magnification (×400) with the Olympus BH-2 microscope equipped with a 0.2×0.2 mm ocular grid (Elta, Graticules Ltd., Tonbridge, Kent, U.K.). The cells were counted in an area of 1.0 mm width and 0.4 mm depth immediately beneath the epidermis in the wound edge and partly in the ulcer base. In healing wounds, in the biopsies
of 3, 7 and 14-day-old wounds, i.e., when re-epithelialization and matrix formation had started, the mast cells were counted from the site of the previous wound margin, which could easily be recognized. In chronic ulcers, the mast cells were counted in an area of 1.0 mm width and 0.4 mm depth in the ulcer base (down from the surface) just beside the ulcer margin, and also in the perilesional skin 1 mm from the ulcer margin.

Dermal cells showing positive immunoreactivity for SCF were counted similarly under high magnification (×400) with the same Olympus BH-2 microscope. In wound biopsies from healing wounds, cells were counted in a similar area beneath the epidermis, as described above. In both psoriatic lesions and clinically uninvolved psoriatic skin, the cells were counted in an area of 1.0 mm width and 0.4 mm depth immediately beneath the papillary dermis.

4.5 Cell cultures and skin culture

Keratinocyte cultures (IV, V)

Human keratinocytes were isolated from healthy foreskins of infant donors undergoing circumcision. Keratinocytes were cultured with a standard method, using complete keratinocyte medium (containing epidermal growth factor (EGF) (5 ng/ml), bovine pituitary extract (BPE) (50 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) added to the basal medium). Briefly, the epidermal layer was separated from the dermis by incubation in 2.4 U/ml dispase overnight at +4°C. The epidermal layer was lifted from the dermis and incubated with 0.05% trypsin-0.02% EDTA for 20 min at room temperature to dissociate the basal cells. The action of trypsin was stopped by adding 10% fetal calf serum. The cells were spun at 1200 rpm for 10 min, and the pellet was resuspended in complete keratinocyte medium. The cells were seeded into flasks at a density of about 10,000-20,000 cells/cm². The flasks were incubated at +37°C in 5% CO₂. The medium was changed every 2-3 days. After the primary culture, the cells were passaged every 3-6 days and used between passages 3 and 8. The viability of the cultured keratinocytes was always over 90%.
HMC-1 cell culture and neuroblastoma cell culture (IV)

The human mast cell line HMC-1 (Butterfield et al. 1988) was cultured in IMDM (Iscove's MDM) supplemented with 10% FCS (fetal calf serum), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1.2 mM alpha-thioglycerol. The cells were passaged every 3-4 days.

To prepare the cell lysate, the cultures were centrifuged at +4°C and washed twice with ice-cold PBS (phosphate buffered saline). The cell pellets were stored at -70°C until lysing of the cells with a sonicator. For sonication, 3×10⁶ HMC-1 cells were suspended in 1.2 ml PBS, and were given 2×10 pulses (0.5 sec/pulse) on an ice bath. The histamine content, analyzed with the radio enzyme assay, was 2.4 nmol/10⁶ HMC-1 cells.

The human neuroblastoma cell line (SH-SY5Y) (Biedler et al. 1973) was maintained in RPMI 1640 medium containing 10% FCS and antibiotics.

¹H-Thymidine incorporation assay (IV)

Keratinocytes were plated in 24-well plates at 20,000 - 28,000 cells/well (10,000 - 14,000 cells/cm²) in 1 ml of complete keratinocyte medium. On the following day, the medium was changed to basal or complete medium. On the third or fourth day after plating, the medium was changed, and the cultures were treated with 10-20 μl of mast cell mediators (histamine, heparin, tryptase) or HMC-1 extract per well at a final concentration described in the figure legends (IV, Figs 1, 2, 3). Ten μl of 0.1 mCi/ml ¹H-thymidine (15.5 Ci/mmol) was added to each well and the cultures were incubated for about 20 hours. The wells were then washed three times with cold PBS and once with cold 6% trichloroacetic acid, and the cells were solubilized with 0.1 M NaOH containing 1% sodium dodecyl sulfate. The solubilized radioactivity was counted in a LKB 1215 Rackbeta liquid scintillation counter.

At the end of the incubation period, the cell cultures had not achieved confluence. All experiments were performed at least in quadruplicate and counts of each well were averaged. Each experiment was repeated at least twice. Thymidine incorporation has previously been found to be a valid indicator of keratinocyte proliferative growth when keratinocytes are grown as a monolayer (O'Keefe et al. 1988)
Adherence of cultured keratinocytes to cell culture plastic (V)

The cell culture wells were coated with fibronectin, and the trypsin-chymase preparation
together with 100 μg/ml heparin, inhibitor (1 mM DFP or 1 mM TLCK) and 100 mM
Tris-HCl buffer, pH 7.6, were added in varying combinations overnight. DFP (di-isopropyl
fluorophosphates) is an inhibitor of serine proteinases, and TLCK (Nα-p-tosyl-L-lysine
chloromethyl ketone) is an inhibitor of tryptic serine proteinases. In other experiments, the
enzyme preparation was added without heparin but with 0.1 mg/ml SBTI or aprotinin. On
the following day, the solution was washed away with D-PBS (Dulbecco’s phosphate
buffered saline) and non-specific binding sites were blocked with 1% heat-inactivated (30
min at +56°C) bovine serum albumin for 2 h. The wells were washed again with D PBS
and 1 ml of incomplete Keratinocyte-SFM™ (serum-free medium) was added to the wells.
After equilibration in a cell culture chamber for 30 min, about 150,000 keratinocytes in
incomplete Keratinocyte-SFM™ were added and the cells were allowed to adhere to the
plastic surface for 60 min. Thereafter, nonadherent cells were washed away with D-PBS
twice. The keratinocyte adherence was quantified by measuring the amount of solubilized
DNA on adherent cells with 0.5 μg/ml Hoechst 33258, using a fluorometer, as described
(Otto 1994). Control wells were treated similarly but without cells. The cultures and
analyses were performed at least twice using triplicate or quadruplicate wells.

Effect of trypsin and chymase on keratinocytes in high- and low-calcium cultures (V)

Four stainless steel cylinders with thick walls (inner diameter 6 mm) were placed
symmetrically and close to each other on the bottom of each well of an uncoated Falcon
6-well plate. Due to the weight of the metal cylinders, the added keratinocytes in the
suspension could not escape to any marked extent from the inside of the cylinders beneath
its thick walls. The wells were equilibrated in 5 ml of complete Keratinocyte-SFM™
medium (containing EGF and BPE) at +37°C and 5% CO₂. About 30,000 keratinocytes
were then added carefully to each cylinder, and the cells were allowed to settle down and
adhere to the plastic surface overnight. On the following day, the plastic surface was almost
completely covered with keratinocytes.

To create high-calcium conditions, the metal cylinders were removed and the complete
Keratinocyte-SFM™ medium was replaced with incomplete DMEM (without fetal calf
serum or antibiotics). After 1 h, a partially purified tryptase-chymase preparation or purified lung tryptase preparation with or without 10 μg/ml heparin and 0.1 mg/ml SRTI was added to the wells as described in Results (V). The cultures were stopped before the borders of developing epithelium reached the wall of the well. The medium was removed and 4% formaldehyde was added to the wells overnight. Thereafter, the epithelium was stained with Mayer's hematoxylin. The area of growth of the keratinocytes was measured as described (in the next chapter). The initial area of seeded cells was subtracted from the area of growth before calculating the outcome of growth. The final results are given as percentages of the growth in an unstimulated control well.

In low-calcium cultures, complete Keratinocyte-SFM™ medium was replaced with incomplete Keratinocyte-SFM™ medium (without EGF, BPE and antibiotics) after the adherence of the cells. Then, the cylinders were removed and the enzymes were added. The cultivation proceeded as described above.

Cultivation of the post-mortem skin and determining the area of epithelial outgrowth (IV)

Post-mortem skin was collected for the skin bank in Kuopio University Hospital. The skin samples used in this study were those that were left over after the allograft edges were trimmed. Skin was collected with a dermatome adjusted to cut 0.2 mm thick skin consisting of the epidermis and the upper dermis. Skin samples were obtained from healthy adults (donation criterion 14-75 years) within one day post-mortem. Previously, it has been shown that skin collected post-mortem is an acceptable source of viable epidermal cells (Hirel et al. 1996).

To test the effect of different mast cell mediators and mast cell extracts on epidermal outgrowth, the skin specimens were grown in fibronectin-coated Falcon 6-well tissue culture plates. Pieces of skin were cut with a 6 mm punch, and 4 pieces were placed dermal side down on each well. A fine wire mesh was placed on the pieces of skin to prevent them from moving. The skin cultures were maintained in DMEM, 10% FCS, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO₂. The volume in each well was 3 ml for HMC-1 lysate and 5 ml in other experiments. The culture medium was changed every second or third day and mast cell mediators (histamine or heparin) or HMC-1 mast cell extract was added to the culture medium.
After 14-21 days, the cultures were rinsed with PBS, fixed with 4% formaldehyde overnight, and stained with hematoxylin. Then, to measure the spread of the epithelialization area with digital image analysis, the skin culture specimens were immersed in liquid paraffin, put on a transilluminator and digitized with a Peltier-cooled Photometrics CH-250 camera (Photometrics Inc, Tucson, AZ, USA), Kodak KAF-1400 CCD detector (Kodak Inc, Rochester, USA), and Nikkor 50 mm/f 1.8 objective (Nikon Ltd, Tokyo, Japan). Macintosh PowerPC 7100/80 (Apple Inc, Cupertino, CA, USA) and IP-Lab software (Signal Analytics, Vienna, VA, USA) were used for camera control and image analysis. Exposure time was kept constant during the experiment.

Prior measuring the area of epithelial outgrowth, the mean area of 12 biopsy punches was measured. Each image of interest was subtracted from the blank background image of the illuminator field. After blurring the image with a 3×3 linear filter, the absolute area of the keratinocyte epithelial growth and the biopsy punch was measured. A transparent, real-time coloured overlay was projected on the segmented image and the grey values, including both the epithelial growth and punch areas, were binarized. The plug area was subtracted from the total area. Finally, pixel-based area parameters were converted into square millimeters (1 pixel = 0.0122 mm²). Each experiment was repeated at least 4 times.

4.6 Detection of enzyme activities and histamine

Detection of tryptic, chymotryptic, anti-tryptic and anti-chymotryptic activity, and total protein in the wound fluid and in the wound bed of chronic ulcers (V)

The enzyme activity of tryptase was analyzed, using 0.2 mM Z-Gly-Pro-Arg-p-nitroanilide (pNA) as the substrate, 1 mg/ml α1-proteinase inhibitor as the inhibitor to reduce possible background activity, 50 μg/ml heparin to stabilize tryptase, and 100 mM Tris-HCl buffer, pH 7.6 (Kivinen et al. 2001). The enzyme activity of chymase was analyzed using 0.2 mM Suc-Ala-Ala-Pro-Phe-pNA as the substrate in the presence of 1 mg/ml aprotinin, 2 M NaCl and 100 mM Tris-HCl buffer, pH 7.6 (Kivinen et al. 2001). The results were calculated first as U per liter of sample solution (U=μmol/min) and finally as U per mg of total protein. The protein concentration was analyzed by using
the commercially available BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) with albumin as the standard.

In comparison to trypsin and chymase activity, elastase-like activity was measured with 0.2 mM MeOSuc-Ala-Ala-Pro-Val-pNA (Grinnell et al. 1996) and plasmin-like activity with 0.2 mM D-Val-Leu-Lys-pNA (Harvima IT et al. 1989a) as substrates, each dissolved in 0.1 M NaCl and 100 mM Tris-HCl buffer, pH 7.6.

In order to measure antitryptase and antichymase activity, high-salt extract of human skin (after the preceding low-salt extraction) was used as the source for trypsin and chymase activity. The activity of trypsin in this extract was 242 U/l and 230 U/l in the absence and presence of 1 mg/ml α₁-proteinase inhibitor, respectively. The activity of chymase in the high-salt extract was 41.4 U/l and 39.8 U/l in the absence and presence of 1 mg/ml aprotinin, respectively (Kivinen et al. 2001). Hence, the ratio of trypsin activity to chymase activity was about 6:1. The activity of chymase was almost totally inhibited by 10 μg/ml of α₁-antichymotrypsin (Kivinen et al. 2001).

In the antitryptase assay, 10 μl of appropriately diluted high-salt extract was mixed with 10 μl of 1 mg/ml of heparin, 10 μl of 20 mg/ml of bovine serum albumin, 20 μl of 100 mM Tris-HCl buffer, pH 7.6, and 50 μl of wash sample from a chronic ulcer. All solutions were added to the wells of a 96-well plate. After incubation for 30 min, 100 μl of 0.4 mM Z-Gly-Pro-Arg-pNA containing 2 mg/ml α₁-proteinase inhibitor and 100 mM Tris-HCl buffer, pH 7.6, was added to the well and the remaining activity of trypsin was measured, using a micro-ELISA reader at 405 nm. The assay was controlled by omitting the high-salt extract, the wash sample, or both, from the incubation mixture. Similarly, in the antichymase assay, 10 μl of appropriately diluted high-salt extract was mixed with 10 μl of 20 mg/ml bovine serum albumin, 30 μl of 100 mM Tris-HCl buffer, pH 7.6, and 50 μl of wash sample. After 30-min incubation, 100 μl of 0.4 mM Suc-Ala-Ala-Pro-Phe-pNA containing 2 mg/ml aprotinin, 2M NaCl and 100 mM Tris-HCl buffer, pH 7.6, was added and the remaining activity of chymase was measured. The results are expressed as the percentages of the activity compared to the control.

Histamine analysis (I, IV, V)

Histamine was analysed in duplicate by using the sensitive radio enzyme assay (Harvima
RJ et al. 1988), with a detection limit of 0.5-1.0 nM.

4.7 Statistics

Statistical comparisons between paired groups in the data concerning chronic wounds and psoriasis were made, using the Wilcoxon-signed rank test. The data from healing wounds were tested for significance, using the Friedman two-way analysis of variance (ANOVA). The data from keratinocyte cultures and skin cultures were analyzed, using the Kruskal-Wallis one-way analysis of variance (ANOVA) and the data from ulcer wash samples were tested using Students paired t-test. A $P$-value $\leq 0.05$ was considered to be statistically significant. Statistical comparisons were made using SPSS v. 8.0 software (SPSS Inc. Chicago, IL, U.S.A.).
5 RESULTS

5.1 Neuropeptide- and capsaicin-induced histamine release in skin

*Neuropeptide-induced histamine release monitored with the microdialysis technique (I)*

SP and VIP injections produced wheal and flare reactions, and CGRP produced a prominent long-lasting, indurated erythema with pseudopodia. The baseline histamine level in the dialysis fractions was 4.5±4.5 nM one hour after insertion of the probe (mean ±SD, n=20). SP (250 pmol) caused rapid histamine release, which reached its maximum either in the first 0-15 min fraction (60.0±55.6 nM) or in the subsequent 15-30 min fraction (43.7±26.7 nM) after skin challenge. Thereafter, the histamine levels declined steadily and reached the baseline 60-75 min after the single SP injection, and no second rise was observed (I, Fig. 2a) within the time course of the experiment. The histamine concentration varied greatly from 7.0 to 183 nM in the 0-15 min peak fractions (I, Fig. 2a, b). In the 15-30 min fractions, the histamine concentration varied between 2.0 and 96.6 nM. In patients who received two consecutive SP injections, the histamine release following the second injection was weaker than that after the first injection in all but one subject (I, Fig. 2b). Thus, the first SP injection was sufficient to liberate most of the releasable histamine at the injection site.

The histamine release following VIP (100 or 250 pmol) injections was essentially similar (I, Fig. 3). As observed in the case of SP, the first injection released most of the histamine reservoir, and the second histamine rise was clearly lower. The amount and rapidity of the histamine release induced by 250 pmol VIP (I, Fig. 3) was comparable to that induced by 250 pmol SP (I, Fig. 2a, b). In all but one subject, the maximum histamine concentration after the first VIP injection was reached in the first 0-15 min fraction following skin provocation. The wheal and flare reactions induced by SP and VIP were seen briefly within 5-10 min after the injection, which is in accord with the observed rapid release of histamine.

CGRP (250 pmol) did not release detectable amounts of histamine up to 105 min following skin provocation in 2 subjects tested. By this time the erythema induced by CGRP was clearly developed. In contrast, the control injections of SP showed high histamine release (maximum concentrations 183 and 71.8 nM). No clear wheal or flare
reactions were observed at the injection site of CGRP.

_Capsaicin-induced histamine release monitored with the microdialysis technique (I)_

Capsaicin injection (25 \( \mu \)l of 30 \( \mu \)M) induced a very weak and almost non-significant histamine release in most subjects (I, Fig. 4a). The baseline histamine concentration was 3.4±3.1 nM (\( n=5 \)). The curve for histamine release was broad and of relatively long duration. The maximum histamine concentration (5.7±3.8 nM) was reached within 0-15 min after the injection. In every case, capsaicin evoked flare and intense pain but not a wheal. The pain subsided quickly after the injection. Application of 0.1% capsaicin cream resulted in release of histamine in 2 out of 5 patients (I, Fig. 4b) and caused a burning or itching sensation in 30 min and erythema in 60-90 min.

5.2 Alterations of mast cells and SCF in epithelializing and chronic wounds

_Tryptase, chymase and Kit positive mast cells in normal wound healing (II, III)_

Mast cells occurred in all levels of the dermis and subcutaneous tissue in the donor sites for skin grafts, but they were rarely present near the epithelialization margin. The numbers of mast cells in the wound edges of healing wounds are presented in Table 5. The total number of mast cells (tryptase-positive cells) decreased until day 3 and increased slightly thereafter, but did not reach the number of mast cells in day-0 biopsies (II, Fig.1a). Similarly, the number of chymase-positive mast cells also decreased during wound healing until day 7 and were increased somewhat in the day 14-biopsies (II, Fig. 1a). The change in the number of tryptase-positive mast cells during wound healing was non-significant, but the change in chymase-positive mast cells was significant (\( P=0.03 \) in patients who gave day-0, -1 and -7 biopsies and \( P=0.005 \) in day-0, -3 and -14 biopsies). The ratio of chymase-positive cells to tryptase-positive cells gives the percentage of chymase-positive mast cells. On day 0, 54% of the mast cells were positive for chymase activity, and the percentage decreased to 32% during wound healing, but this decrease was statistically non-significant (II, Fig.1b). In addition, the percentage of mast cells showing IL-4 and TNF-\( \alpha \) immunoreactivity was studied, but the expression of these cytokines was scanty at all
stages of wound healing, and did not show any apparent change in number during wound healing (II, Table 1).

The reactivity of mast cells with anti-Kit antibody is summarized in Table 5. In day-0 wound biopsies, which represent normal skin, only 16% of the tryptase-positive mast cells showed immunoreactivity for Kit (III, Fig 1a, b). In contrast, a clear change was observed in the expression of Kit during normal wound healing: the expression gradually increased until day 14, when 73% of the mast cells were positive for Kit (III, Fig. 1c, d). The change in Kit expression did not reach statistical significance in patients who gave biopsies on days 0, 1 and 7 (P=0.09), but the change in the biopsy series was significant in those who gave biopsies on days 0, 3 and 14 (P=0.07).

Owing to the low (16%) Kit expression in the normal skin of subjects undergoing skin transplantation, the immunoreactivity of Kit in mast cells was also studied using biopsies from three healthy subjects. Unexpectedly, Kit expression was seen in 97% (±1) of the tryptase-positive mast cells in these three subjects.

Tryptase-, chymase- and Kit-positive mast cells in chronic ulcers (II, III, V)

The number of mast cells (i.e. cells displaying enzymatically active tryptase) was higher in the perilesional skin than in the wound bed of chronic venous ulcers (141±62 and 31±20/mm², n=6, respectively)(II), as presented in Table 6. This difference in mast cell location was significant (P=0.03). Numerous mast cells were present in close vicinity to the epithelialization margin in the perilesional skin in 5 out of 6 ulcer specimens, and tryptase-positive mast cells were also seen in apparent contact with the epidermis and occasionally in the epidermis (II, Fig. 2).

In the perilesional skin, the number of mast cells with tryptase immunoreactivity was 92±38 cells/mm² (V). The numbers of cells with chymase immunoreactivity and with enzyme activity was 75±28 and 46±18 cells/mm², respectively; thus, most of the mast cells (82%) were of the MC TC type, containing both immunoreactive tryptase and chymase, and about 61% of the chymase-immunoreactive cells displayed enzymatically active chymase. In the ulcer bed, the number of tryptase-immunoreactive cells was 39±27 cells/mm², and the number of cells with chymase immunoreactivity and enzyme activity 31±23 and 17±17 cells/mm², respectively. Consequently, the percentage of MC TC cells was 79%, and the
percentage of chymase-immunoreactive cells showing active chymase was about 55%.

No immunoreactivity for IL-4 in mast cells could be detected in either the wound bed or the perilesional skin in any of the biopsies. Cells other than mast cells immunoreactive for IL-4 were seen in the perilesional skin in some biopsies. Immunoreactivity for TNF-α could be seen in a few mast cells in the perilesional skin, but not in the wound bed. Instead, numerous TNF-α immunoreactive cells other than mast cells were present, especially in the perilesional skin.

In chronic venous ulcers, mast cells were numerous in the wound edges, where 86% of mast cells were positive for Kit, as presented in Table 6. In addition, the expression of Kit was essentially similar in the wound bed, where 87% of the mast cells stained positively for Kit.

Table 5. The total number of mast cells (tryptase-positive cells) in the wound edge of acute wounds, the percentage of mast cells expressing Kit and the number of SCF-positive cells in the dermis. The values are mean±SD.

<table>
<thead>
<tr>
<th></th>
<th>Mast cells/mm²</th>
<th>Kit-positive mast cells (%)</th>
<th>Number of SCF-positive cells in the dermis (/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=12)</td>
<td>93±55</td>
<td>16±10</td>
<td>9±10</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>71±15</td>
<td>39±14</td>
<td>223±212</td>
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<tr>
<td>Day 3</td>
<td></td>
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<tr>
<td>(n=6)</td>
<td>64±34</td>
<td>35±20</td>
<td>54±29</td>
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<tr>
<td>Day 7</td>
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<td></td>
</tr>
<tr>
<td>(n=5)</td>
<td>66±18</td>
<td>61±33</td>
<td>53±61</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(n=6)</td>
<td>80±35</td>
<td>73±22</td>
<td>7±7</td>
</tr>
</tbody>
</table>
Table 6. The total number of mast cells (mast cells displaying enzymatically active tryptase) in the perilesional skin and wound bed of chronic venous ulcers, the percentage of mast cells expressing Kit, and the number of SCF-positive cells in the dermis (n=6).

<table>
<thead>
<tr>
<th></th>
<th>Mast cells/mm²</th>
<th>Kit-positive mast cells (%)</th>
<th>Number of SCF-positive cells in dermis (/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perilesional skin</td>
<td>141±62</td>
<td>86±13</td>
<td>71±33</td>
</tr>
<tr>
<td>Wound bed</td>
<td>31±20</td>
<td>87±9</td>
<td>295±152</td>
</tr>
</tbody>
</table>

**SCF immunoreactivity in epithelializing and chronic wounds (III)**

Immunoreactivity for SCF was observed in both the epidermis and the dermis. Within the epidermis, SCF-positive cells were mainly observed in the basal and spinous layers. Some of the cells expressing SCF were dendritic, and were thus probably Langerhans' cells. In normally healing acute wounds, positive epidermal cells were sparse both in the epithelialization margin and in the newly formed epidermis, and showed no clear changes during wound healing. In some of the wound biopsies, staining was either absent or very sparse. This lack of positive staining was seen occasionally in different stages of wound healing. When the epidermis was acanthotic, i.e. in the perilesional skin of chronic wounds, positive epidermal cells were more numerous (III, Fig. 2). Overall, positive epidermal cells were more often seen in chronic than in acute wounds.

The numbers of dermal cells showing SCF immunoreactivity in normally healing acute wounds and chronic wounds are presented in Tables 5 and 6, respectively. SCF immunoreactivity appeared in all the dermal layers but especially in endothelial cells and cells lining hair bulbs. On day 0 in acute wounds, the mean number of cells showing SCF-immunoreactivity was 9 cells/mm², although the variation between individuals was high (0-31 cells/mm²)(III, Table 1). An increase in the number of SCF-positive cells was seen during normal wound healing on day 1, and a decrease thereafter down to 7 cells/mm² on day 14. The change in the number of cells showing SCF-immunoreactivity during wound healing was significant on days 0, 1 and 7 (P=0.009), but non-significant on days 0, 3 and
14 (P=0.2).

In chronic wounds, the number of SCF-positive cells in the wound bed was higher than that in the perilesional skin in all biopsies (P=0.03), as presented in Table 6. No correlation between the number of SCF-positive cells and the percentage of mast cells showing positive Kit staining was observed.

*Expression of Kit receptor and SCF in psoriasis (III)*

The expressions of Kit and SCF in psoriasis were studied in comparison with chronic wounds, and a significant difference was seen between the lesional and clinically uninvolved skin. In the psoriatic lesion, 88% of the mast cells were Kit-positive, but only 46% in the uninvolved skin (P=0.004). At least some epidermal cells staining positively for SCF were seen in all the lesional biopsies, but rarely in clinically normal psoriatic skin. In the dermis, the number of SCF-positive cells was also higher in the lesional skin than in the healthy-looking skin in every patient (P=0.002). In the uninvolved psoriatic skin, the mean number of positively stained cells was low (2 cells/mm²), whereas, in psoriatic lesions, numerous SCF-positive cells (65 cells/mm²) were seen in the dermis, although the variation between individuals was great (10-208 cells/mm²)(III, Table 1).

5.3 Wound fluid and wound bed biopsies from chronic ulcers

*The levels of histamine, trypase activity and antitryptase activity (V)*

The levels of histamine, trypase activity and antitryptase activity in all 16 wash samples were 46.62 pmol/mg, 0.62 ±0.79 mU/mg and 93.416.5%, respectively. When studying those 8 patients who gave both wash samples and wound bed biopsies (V, Table 1), the amount of histamine was 297 pmol/mg in the biopsies and 97 pmol/mg in the wash samples, the level of trypase activity in the corresponding samples was 1.72 and 0.30 mU/mg, respectively, and the level of antitryptase activity in the wash samples was 95.3%. The ratio of histamine to trypase activity was higher in the wash samples (483) than in the biopsies (172), although the two groups are not directly comparable and the difference between the groups is not statistically significant (P=0.18). Thus, the wash samples
possessed tryptase activity but no detectable antitryptase activity, and histamine was presumably more efficiently solubilized in the wound fluid than tryptase activity.

The levels of chymase activity and antichymase activity (V)

The levels of chymase activity and antichymase activity were 0.017±0.024 mU/mg and 34.1±24.3 % in all the wash samples (n=16), respectively. However, the level of chymase activity in those 8 patients who gave both wash samples and wound bed biopsies, was almost undetectable (0.0014 mU/mg) in the wash samples but relatively high (0.16 mU/mg) in the biopsies (V, Table 1). The level of antichymase activity in these 8 wash samples was 32.4%, i.e., the wound fluid contained clear chymase-inhibiting activity. Furthermore, the ratio of chymase activity to tryptase activity was significantly (P=0.046) lower in the wash samples (0.016) than in the biopsies (0.12).

In order to compare and evaluate the significance of the levels of chymase and tryptase activities, other synthetic substrates of proteases were also used. The D-Val-Leu-Lys-pNA- and MeOSuc-Ala-Ala-Pro-Val-pNA-hydrolyzing activities were 0.035±0.034 mU/mg and 0.28±0.89 mU/mg, respectively, in the wash samples of all 16 patients. However, the elastase-like activity in the 8 patients especially was markedly decreased from 8.69 mU/mg in biopsies to 0.46 mU/mg in wash samples as compared to the decrease in tryptase activity in the corresponding samples (V, Table 1). The level of D-Val-Leu-Lys-pNA-hydrolyzing plasmin-like activity was low as compared to the levels of tryptase and elastase-like activity but similar to that of chymase activity, especially in the biopsies (V, Table 1).

5.4 Effects of mast cells and mast cell mediators on keratinocytes in cell culture and whole skin culture

The effect of histamine on \(^3\)H-thymidine incorporation into keratinocytes (IV)

Histamine inhibited thymidine incorporation into keratinocytes grown both in the basal (P=0.002, n=8) and in the complete medium (P=0.002, n=4) (IV, Fig. 1). In the basal medium, the inhibition of thymidine incorporation was 29% at a concentration of 1 mM histamine, and a dramatic increase in inhibition was observed at 5 mM (about 89% inhibition). In complete medium, the inhibition was comparable, being 33% and 78%,
respectively. Thus, significant inhibition was not observed until relatively high histamine concentrations. Therefore, the viability of keratinocytes was determined by trypan blue dye exclusion. The concentration of 5 mM histamine was found to be non-cytotoxic, as the decrease in viability was about 10% compared to the control.

The effect of heparin and tryptase on $^3$H-thymidine incorporation into keratinocytes (IV)

When keratinocytes were grown in basal medium, heparin inhibited thymidine incorporation into keratinocytes significantly (IV, Fig. 2) ($P=0.004, n=4$). A slight decrease in thymidine incorporation was observed already at 0.02 μg/ml. Maximal inhibition (33%) was achieved at 2 μg/ml, and higher concentrations of heparin (20 and 200 μg/ml) did not increase the inhibition level. In contrast, in complete medium, heparin showed only a minimal and non-significant inhibitory effect on thymidine incorporation (IV, Fig. 2) ($P=0.08, n=4$), and maximal inhibition (12%) was achieved at 2 μg/ml. A combination of 1 mM histamine with 1 μg/ml heparin did not increase the inhibition level from that obtained with these substances alone.

Keratinocytes grown in both basal and complete medium were also treated with skin tryptase (0.0285 to 2.85 μg/ml) alone or together with heparin (0.5 to 20 μg/ml). No significant effect on thymidine incorporation in keratinocytes could be observed even when combining 2.85 μg/ml tryptase with 0.5 μg/ml heparin, i.e. a 5-fold excess of tryptase over heparin to saturate the protein-binding capacity of heparin.

The effect of HMC-1 cell lysate on $^3$H-thymidine incorporation into keratinocytes (IV)

To further analyze the effect of mast cell mediators on the proliferation of keratinocytes, a lysate from HMC-1 cells was added at different dilutions to keratinocyte cultures grown in basal medium. HMC-1 lysate inhibited $^3$H-thymidine incorporation in a dose-dependent fashion ($P=0.003, n=4$), and maximal inhibition (47%) was reached when an extract corresponding to 16,700 lysed HMC-1 cells per well was added (IV, Fig.3). When the lysate from 50,000 HMC-1 cells was added to the wells, the histamine concentration in the well was 0.12 μM. The heparin content in HMC-1 cells was not analyzed, but an estimate of the maximum concentration of 0.06 to 0.2 μg/ml present in the culture conditions (50,000 HMC-1 cells per well) can be calculated on the basis of the study of Metcalfe et al.
(Metcalf et al. 1980). Thus, neither the histamine derived from HMC-1 cells, nor the heparin, can fully explain the inhibitory effect of the HMC-1 extract on \(^3\)H-thymidine incorporation in keratinocyte culture. HMC-1 cells did not have an effect on keratinocytes grown in complete medium.

To study whether TNF-\(\alpha\), IFN-\(\gamma\) or TGF-\(\beta\) could account for the inhibitory effect on thymidine incorporation, HMC-1 cell extract was incubated with anti-IFN-\(\gamma\) mAb (5 and 50 \(\mu\)g/ml), anti-TNF-\(\alpha\) pAb (5, 50 and 100 \(\mu\)g/ml), and anti-TGF-\(\beta\) mAb (10 and 100 \(\mu\)g/ml) for one to two hours at room temperature, and the cell extract from 50,000 and 62,500 HMC-1 cells was added to each well. These antibodies together with HMC-1 extract did not reverse the inhibitory effect of the HMC-1 cell extract on thymidine incorporation.

Extract from cultured neuroblastoma SH-SY5Y cells was used as a control for the HMC-1 cell extract to study the possibility of a non-specific inhibitory effect of a cell lysate. The cell lysate from cultured neuroblastoma cells was prepared in the same way as described for HMC-1 cells. No significant effect on \(^3\)H-thymidine incorporation could be detected when the lysate from up to 250,000 neuroblastoma cells per well was added to the keratinocyte cultures grown in basal medium.

\textit{Keratinocyte outgrowth from whole skin specimens (IV)}

Skin biopsies were grown in serum-containing medium and the mean epithelial outgrowth from the skin biopsies in the control wells was 303 mm\(^2\), consisting mainly of keratinocytes. In cases of fibroblast outgrowth, this area was subtracted from the keratinocyte growth on account of the different morphology and different staining pattern of the two cell types with hematoxylin.

Histamine inhibited the epithelial outgrowth dose-dependently at concentrations of 0.1 \(\mu\)M - 1 mM (\(P<0.0001\)) (IV, Fig. 4a), and maximal inhibition, 91\%, was achieved with 1 mM histamine. The inhibition was significant at the concentrations of 0.1 mM and 1 mM (\(P<0.01\)).

Heparin affected epithelial outgrowth in a dual way (\(P=0.01\), as shown in Figure 4b (IV). Skin cultures were grown at heparin concentrations from 0.02 to 200 \(\mu\)g/ml. At low concentrations, heparin inhibited keratinocyte outgrowth by up to 27% at the concentration of 2 \(\mu\)g/ml heparin, which parallels the results in keratinocyte culture (IV, Fig. 2). At high
concentrations (200 μg/mL), the inhibitory action was reversed, and heparin seemed to stimulate keratinocyte outgrowth (30% stimulation compared with the control).

When lysate from HMC-1 cells was added to the skin culture, no significant effect on epithelial outgrowth could be seen at low concentrations (IV, Fig. 4c). At high concentrations, the effect on epithelial spread was inhibitory ($P=0.04$), and maximal inhibition (36%) was achieved with the lysate from 200,000 HMC-1 cells (67,000 cells/mL).

The effect of tryptase and chymase on keratinocyte adherence to a plastic surface (V)

The functional significance of tryptase and chymase was evaluated using the keratinocyte adherence assay on fibronectin coated wells, since tryptase and chymase can cleave fibronectin. To study the dose-response effect, the tryptase-chymase preparation was added to the fibronectin-coated wells at 1:1 (1970 U/l tryptase and 154 U/l chymase), 1:10 or 1:100 dilution in the presence of 100 μg/ml heparin, 150 mM NaCl and 80 mM Tris-HCl buffer, pH 7.6, overnight. Consequently, the keratinocyte adherence was 122% (1:100 dilution), 56% (1:10 dilution) or 20% (1:1 dilution) as compared with the control values. Keratinocyte adherence was weak if the well was not coated with fibronectin, as demonstrated in Table 2 (V).

In further experiments, the tryptase-chymase preparation (1:1 dilution) was added to the wells together with 100 μg/ml heparin and 1 mM DFP or 1 mM TLCK. After overnight incubation, the tryptase-chymase preparation in the absence of inhibitors had decreased the keratinocyte adherence to 56±8% of that in the control wells. When the serine proteinase inhibitor, DFP, was combined with the enzyme preparation, the adherence was 97±25%, i.e., DFP completely prevented the effects of the serine proteinases, tryptase and chymase. However, TLCK, an inhibitor of trypsin, had no effect and the adherence of keratinocytes decreased to 53±7% with the tryptase-chymase preparation.

To determine whether chymase was responsible for the decrease in keratinocyte adherence, SBTI and aprotinin were used as inhibitors. SBTI inhibits both chymase and cathepsin G but aprotinin inhibits only cathepsin G and not chymase. Tryptase is not inhibited by either inhibitor (Schechter et al. 1983, 1990, Harvima IT et al. 1988). Heparin was omitted from the reaction mixture to allow tryptase inactivation in low-salt (150 mM NaCl) buffer (Harvima IT et al. 1988). The results have been summarized in Table 2 (V).
The tryptase-chymase preparation efficiently decreased the keratinocyte adherence and aprotinin could not markedly inhibit this change. Instead, SRTI almost completely prevented the decrease in keratinocyte adherence. Aprotinin and SBTI alone without the enzyme preparation could only slightly modulate the keratinocyte adherence.

The effect of tryptase and chymase on keratinocytes cultured in low- or high-calcium conditions (V)

Keratinocytes were cultivated in incomplete Keratinocyte-SFM™ or DMEM medium, since serum or tissue extracts may contain chymase inhibitors. Under these growth factor/serum-deficient low- or high-calcium conditions, the keratinocytes grew and migrated slowly and 10 μg/ml heparin even inhibited the growth to some extent (V, Fig. 1, well 2). However, 0.1 mg/ml SBTI per se had no effect on the growth (not shown). When the tryptase-chymase preparation (131 U/ml tryptase and 10 U/ml chymase) was added to the wells together with 10 or 1 mg/ml heparin, the slow growth of the keratinocytes in both culture models was virtually abolished (V, Fig. 1, wells 3 and 4). In the absence of heparin, i.e., in conditions favoring rapid inactivation of tryptase, the tryptase-chymase preparation caused effective detachment of monolayer keratinocytes and marked deterioration in the structure of the developing epithelium (V, Fig. 1, well 5). However, 0.1 mg/ml SBTI completely prevented these changes induced by the enzyme preparation in both culture conditions (V, Fig. 1, well 6). In addition, SBTI prevented the action of the tryptase-chymase preparation when 10 μg/ml heparin was present in both cultures. Furthermore, the growth-inhibitory or destructive effect of the tryptase-chymase preparation was dose-dependent in both incomplete Keratinocyte-SFM™ and DMEM (V, Fig. 2, wells 1-4).

To clarify whether histamine could also modify the effect of mast cell proteinases, 50 or 500 μM histamine was added alone or in combination with the tryptase-chymase preparation (26 U/ml tryptase and 2 U/ml chymase) using incomplete Keratinocyte-SFM™ or DMEM as the medium. Heparin was omitted from the cultures. In Keratinocyte-SFM™, the addition of histamine did not have any apparent effect on the growth inhibition of monolayer keratinocytes induced by the enzyme preparation. However, the growth inhibition induced by the preparation in incomplete DMEM was partially prevented when a
concentration as low as 50 μM histamine was present in the culture together with the enzymes (V, Fig. 3). No visible inhibition of keratinocyte growth by histamine was observed, which could have been due to the short-term nature of the cultures and the suboptimal growth conditions.

Since the experiments with the tryptase-chymase preparation did not point to any marked role for tryptase in keratinocyte growth, the effect of purified human lung tryptase was studied further. Lung tryptase (0.028, 0.28 or 2.8 μg/ml) was added to the culture together with 10 μg/ml heparin on two occasions at 2-4-day intervals, using incomplete DMEM or incomplete Keratinocyte-SFM™ medium. Tryptase had no effect on the growth and migration of keratinocytes — neither stimulation nor inhibition or detachment from the plastic surface.
6 DISCUSSION

6.1 Mast cell activation monitored with the microdialysis technique

Several assay methods are available for measuring histamine. The radio enzyme assay (REA) of histamine was chosen for this study to monitor mast cell activation and degranulation, since the challenging laboratory methodology was available and REA is one of the most sensitive methods for measuring histamine in biological samples (Harvima RJ et al. 1988). A very sensitive assay method was required, since some of the stimulating agents in this study were expected to induce only weak release of histamine.

At the time the work was performed, only sparse information was available on the direct in situ effects of neuropeptides in the skin other than observations of the clinical flare, wheal and itch reactions they induce, and histological data. SP and VIP are known to be rapid histamine-releasing agents from mast cells of human skin (Fhertz et al. 1987, Lowman et al. 1988a); moreover, SP has been stated to be one of the most potent histamine-releasing agents in human skin (Hågemark et al. 1978). VIP has been shown to have potency similar to SP for inducing itch and flare in human skin (Fjellner et al. 1981).

The results of the present study show that neuropeptides SP and VIP, but not CGRP, can induce activation of mast cells and a relatively rapid release of histamine in the skin, a result which is consistent with both the in vitro findings concerning their effects on isolated mast cells and their skin reactions at comparable concentrations. In previous studies, histamine release from mast cells in vitro induced by SP has been shown to take place rapidly, being complete within 20 sec. In contrast, activation of isolated cutaneous mast cells via IgE-dependent mechanisms is relatively slow, occurring within 6 min after stimulation (Benyon et al. 1987). In accordance, under the same experimental microdialysis conditions, the histamine release following multiple skin prick tests with the cow allergen around the microdialysis probe was clearly slower than the histamine release induced by SP (Horsmanheimo et al. 1996, Saarinen et al. 2000). However, it is noteworthy that this method, using a relatively long (15-min fraction) collection time, cannot monitor the kinetics of histamine release over a very short time period.

CGRP has been found to be a poor degranulating agent and releaser of histamine from mast cells. In addition, it is much less potent than SP for inducing both wheal and flare
reactions in human skin (Brain et al. 1986). The results of this study support these findings and conclusions since CGRP did not induce measurable release of histamine, even though the sensitive REA method was used, nor did it induce wheal or flare responses. CGRP induced only long-lasting erythema without a wheal, a finding which has also been documented earlier (Wallengren and Håkanson 1987b).

Neurons possessing C-fibers transmit nociceptive information to the central nervous system and participate in various reflex responses. These neurons carry VR1 (vanilloid receptor 1), which binds capsaicin and induces C-fibers to release neuropeptides (Caterina et al. 1997). The initial excitation is followed by a lasting refractory state, termed desensitisation, in which the neurons fail to respond to a variety of stimuli. Capsaicin applied topically to human skin induces erythema accompanied by sensations of itching, stinging and burning. Although capsaicin does not release histamine from mast cells in vitro, capsaicin treatment has been shown to cause histological changes in mast cells in vivo, suggesting degranulation (Bunker et al. 1991). These earlier observations show that it is possible that capsaicin liberates histamine from mast cells by first stimulating sensory nerves, followed by the release of neuropeptides, such as SP and VIP. Owing to its desensitisation effect on neurons, capsaicin is used in the treatment of various cutaneous disorders that involve pain, pruritus and inflammation, for example postherpetic neuralgia, psoriasis and neuralgia paresthetica (Lotti et al. 1995). In previous clinical studies, the concentration of capsaicin in topical preparations, such as creams, has been between 0.01% and 0.075%. In the present study, capsaicin was administered to the skin as a 0.1% cream under a wet occlusion to enhance penetration, or was injected into the skin as a 30-μM solution. However, despite clinical responses to capsaicin and the use of the sensitive REA method, the two stimulation methods induced little, if any, release of histamine. This suggests low levels of neuropeptides or infrequent morphological contacts between mast cells and sensory nerves in normal human skin.

The present results provide direct evidence that mast cells in the skin are readily activated when challenged with neuropeptides SP and VIP, which supports earlier in vitro observations and clinical experiments. The results concerning SP and CGRP have also been confirmed by Weidner et al. (Weidner et al. 2000). However, the inefficiency with which capsaicin induces histamine release suggests that the sensory nerves are not able to induce
substantial mast cell activation among the early events of normal wound healing. The situation is different in chronic inflammation, e.g. in psoriasis, where increased mast cell-nerve contacts have been counted (Naukkarinen et al. 1993, 1994).

6.2 Alterations of mast cells in chronic wounds and during the wound healing process

Since tryptase is the major protein stored in a fully active form in mast cell granules, histochemical staining of tryptase can easily be performed by using either enzymehistochemical or immunohistochemical techniques. Both methods are suitable and produce comparable results (Harvima et al. 1989b, Harvima et al. 1993). However, frozen biopsies and cryosections are required for enzyme histochemistry, since the enzymatic activity of tryptase does not stand the processing conditions of formalin fixation, heating and paraffin. Chymase can also be stained enzymehistochemically and immunohistochemically, since mast cells contain large amounts of this protein, though less than tryptase protein. Chymase is stored in a mostly, but not fully, active form in mast cell granules. Thus, a proper protocol for the staining of chymase includes both enzyme- and immunohistochemistry (Harvima et al. 1993, Kivinen et al. 2003).

Mast cell mediators affect the growth of several cell types in the skin, for example keratinocytes, fibroblasts and endothelial cells, which points to their significance in regenerative processes. Also, participation of mast cells in wound healing processes has been verified by findings showing alterations in mast cell counts during wound healing - although mainly in animal studies. In mouse skin, mast cells and chymase may promote the matrix remodelling phase in wound healing (Nishikori et al. 1998). Previous data concerning mast cells in human ulcers are sparse, but the results obtained so far suggest a potential role for mast cell proteases in wound healing. Decreased numbers of mast cells with chymase activity have been reported in 5-369 days old scars, whereas the total number of mast cells, i.e. tryptase-positive mast cells, was comparable to that of normal skin (Algernissen et al. 1999). The present results concerning normally healing wounds showed a significant decrease in the number of mast cells with chymase activity in the early stages of wound healing until day 7. In addition, the ratio of the number of mast cells with
chymase activity to that with tryptase activity decreased during the healing of wounds. It is likely that this decrease is due either to the extensive degranulation of mast cells and appearance of phantom cells or to the inactivation of chymase. Similarly, in the ulcer base and in the perilesional skin of chronic venous ulcers, chymase was partially inactivated in mast cells, as detected enzyme- and immunohistochemically, i.e. chymase immunoreactivity was detected but no chymase activity. Therefore, the decrease in the number of mast cells with chymase activity reflects mostly chymase inactivation. This conclusion is supported by the findings obtained in skin organ cultures ex vivo. In these experiments, it was recently shown that chymase is inactivated and only poorly diffused away from the site of mast cell death during incubation of skin specimens (Kivinen et al. 2001, 2003).

This finding of chymase inactivation is not specific for wound healing, scars or chronic ulcers, since a decrease in chymase activity has been reported in several skin diseases characterized by inflammation, such as psoriasis (Harvima IT et al. 1993), atopic dermatitis (Järvikallio et al. 1997), and cutaneous herpes zoster infection (Kaminska et al. 1996). It is likely that the observed decrease in chymase activity in this study results from the inflammatory reaction during wound healing. In addition, inflammation is a characteristic feature of chronic ulcers. A reasonable explanation is that protease inhibitors are active during these processes and inactivate chymase. Accordingly, a marked decrease in chymase activity along with increased levels of protease inhibitors, \( \alpha_1 \)-AC and \( \alpha_1 \)-PI, in the same mast cells, has been demonstrated in the psoriatic lesion and in the development of cutaneous herpes zoster infection (Kaminska et al. 1996, Harvima IT et al. 1999a). It is not known whether mast cells can synthesize these inhibitors or whether they are taken up into the cell from the circulation. Proteases in the extracellular environment are usually regulated by specific inhibitors. Mast cell tryptase seems to be an exception in this respect, since no physiological inhibitors for tryptase are known. Therefore, it appears that inactivation of tryptase is not likely to happen in wounds.

The functional characteristics of tryptase and chymase suggest that these serine proteinases are involved in degradative and regenerative processes. For instance, tryptase can release and cleave fibronectin from the pericellular matrix (Lohi et al. 1992) or from skin specimens (Kaminska et al. 1999a). Chymase can degrade basement membrane
components at the dermal-epidermal junction, resulting in separation of the epidermis from the dermis (Briggaman et al. 1984). In addition, tryptase and chymase can induce matrix degradation indirectly by first activating matrix metalloproteinases (Gruber et al. 1989, Lohi et al. 1992, Saarinen et al. 1994). Thus, these mast cell enzymes can remodel the extracellular matrix, and are able to make space for and facilitate the migration of inflammatory and malignant cells.

The present histological data suggest that mast cells are not active participants in normal epithelialization, since the tryptase-positive mast cells did not increase in number during normal wound epithelialization, nor were mast cells found in the epithelialization margin. By contrast, mast cells were numerous in the upper dermis of the perilesional skin of chronic venous ulcers and particularly in close vicinity to the epithelial margin. Moreover, occasional epidermal mast cells were encountered (II, Fig. 2), as reported earlier (Bolton and Montagna 1993). In normal skin, mast cells are dermal cells that have very few morphological contacts with keratinocytes. Nonetheless, epidermal mast cells can be encountered in conditions that are characterized by chronic skin inflammation and a hyperproliferative epidermis (Green et al. 1977), as well as in chronic ulcers, where mast cell granules have even been found inside keratinocytes (Bolton and Montagna 1993). The biological significance of these epidermal mast cells is obscure. The factor that activates and attracts mast cells towards the epidermis is not known, but one possibility could be SCF, which is produced by keratinocytes (Hamann et al. 1995).

The expression of SCF and of Kit showed significant alterations during wound healing as well as in chronic wounds and psoriasis. Epidermal immunoreactivity for SCF was sparse in normal skin, and, similarly, the expression of the Kit receptor was low - in the day-0 wound biopsies only 16% of the mast cells expressed Kit. Since previously all mast cells in normal skin have been found to be positive for Kit (Hamann et al. 1994, Ghannadan et al. 1998), three healthy subjects were chosen to be biopsied, and unexpectedly, almost all their mast cells showed positive immunostaining for Kit. This result is puzzling, but the variation in Kit expression might be explained by internalization, degradation, and/or proteolytic shedding of Kit from the cell surface (Baghestanian et al. 1996, Shimizu et al. 1996). Kit expression is also regulated by cytokines, since IL-4 and GM-CSF have been demonstrated to downregulate it (Nilsson et al. 1994c, Welker et al. 2001). Furthermore, the different
ages of the subjects, the different biopsy sites, and possible exposure to the sun could explain the variation in the percentage of Kit-positive mast cells. In a recent study, it was shown that exposure to the ultraviolet radiation has an influence on the mast cell density in the skin, and the expression of Kit correlated with the mast cell density in sun-exposed skin (Grimbaldeston et al. 2003). In this study, the wound edge biopsies were taken from the thigh skin of relatively old patients, in contrast to the non-sun exposed skin in the upper arm in much younger healthy subjects.

Previous studies concerning Kit expression in human wounds are sparse, but it has been reported that the number of Kit-positive cells is increased in scar tissue (Hermes et al. 2000). Similarly, the expression of Kit was gradually induced in mast cells during wound healing until day 14, i.e. when wounds were re-epithelialized and matrix formation was occurring. It is possible that the initial increase in the percentage of Kit-positive mast cells on days 1 and 3 is due to extensive release of tryptase and an initial decrease in the number of tryptase-positive mast cells (Hermes et al. 2000, Kivinen et al. 2001). However, since the number of tryptase-positive cells did not decline beyond 3-day samples, the stable raise in Kit expression on days 7 and 14 strongly points to Kit upregulation in mast cells.

Epidermal immunoreactivity for SCF was overall sparse and without any clear alterations during the wound healing process. In contrast, the number of dermal cells expressing SCF increased strongly at an early stage of wound healing on day 1. This increase in SCF expression suggests that its effects on mast cells can be exerted in the early phase of wound healing, regardless of the low Kit expression in mast cells at that point. As a result, mast cell activation and degranulation occur, and phantom mast cells appear. Later on, when wound healing has advanced, the expression of Kit is induced in mast cells, and conversely, the amount of SCF in the wound edges declines, and on day 14 it is at its baseline level. It is possible that the lower SCF production is nevertheless sufficient to maintain its effects together with a rise in receptor density. Alternatively, a decline in SCF expression may be needed to restrain extensive mast cell activation and to shorten the duration of inflammation, as seems to be the case in chronic wounds and in psoriasis. An opposite result, demonstrating increased expression of SCF in cutaneous scar tissue obtained from melanoma reexcisions, has been reported by Hermes et al. (2001). A possible explanation for this could be the result of the more superficial wounding (split skin grafting) and the
occlusion used in the present study.

Kit internalization and degradation seems to happen after SFC-induced activation in vitro, and new expression of the receptor seems also to require new protein synthesis (Baghestanian et al. 1996, Shimizu et al. 1996). This means that the expression of the Kit receptor is inversely correlated to the binding of SCF under the experimental conditions. During wound healing, SCF levels were already high on day 1, but the expression of the Kit receptor continued to increase thereafter. In addition, no significant correlation between the number of SCF-positive cells and the percentage of Kit-positive mast cells could be found. Hence, the SCF-induced downregulation of Kit (Baghestanian et al. 1996) may be irrelevant in in vivo situations. The different circumstances in a cell culture and a living skin tissue might offer a logical explanation for this phenomenon. A healing wound is characterized by inflammation, and several other cytokines and growth factors are rapidly expressed, together with SCF, and in vivo may play a regulatory role in Kit expression. Furthermore, chymase may modulate the SCF-Kit interaction in the skin tissue. Chymase is capable of releasing soluble SCF from the cell membrane (Longley et al. 1997). The activity of chymase in mast cells in the wound edges gradually decreased during healing. Therefore, the presence of the bioactive form of the SCF molecule can be believed to decrease throughout wound healing.

The temporary expression of SCF in normally healing wounds was contrasted with the strong SCF expression in chronic wounds and psoriatic lesions. Both these conditions are also characterized by mast cell hyperplasia and show high Kit expression. Thus, there seems to be potential for an interaction between SCF and the Kit receptor leading to mast cell proliferation, migration and degranulation. This is supported by the findings that mast cells in psoriatic lesions are in a stage of degranulation (Brody 1984) and functionally hyperreactive (Petersen et al. 1998). The strong expression of SCF may prevent apoptosis of mast cells as well (Iemura et al. 1994). The final result of these processes is the maintenance of chronic inflammation.
6.3 Effects of mast cell mediators on keratinocyte growth and epithelialization in vitro

The growth of keratinocytes in vitro has mostly been studied in low-calcium culture conditions, such as in Keratinocyte-SFM medium, using proliferating keratinocytes grown as a monolayer. The monolayer growth model with an unphysiologically low calcium concentration and exogenous growth factors, such as bovine pituitary extract, may not relate well to the growth of the epidermis in vivo, e.g., in the epithelialization of wounds and blister bases. Thus, more physiological in vitro growth models in high-calcium conditions in the presence or absence of 10% serum were also used in this study. DMEM with serum is a well characterized high-calcium medium for culturing and preparing keratinocyte epithelium - even for clinical purposes in patients with leg ulcers or burns (Harvima IT et al. 1999b, Pittelkow and Scott 1986)

Epithelialization is a process consisting of both proliferation and migration of keratinocytes. To investigate the effects of mast cells on epithelialization, keratinocyte growth and adherence to the substratum were studied in cell culture, and skin culture was used as a model for in vitro wound to study keratinocyte outgrowth from skin specimens.

Histamine dose-dependently inhibited the \(^3\)H-thymidine incorporation into keratinocytes grown both in basal and in complete medium. However, only a relatively high histamine concentration (5 mM) produced the maximal inhibitory effect. Analogous dose-dependent inhibition by relatively high histamine concentrations was shown in studies concerning the keratinocyte outgrowth from skin specimens (0.1 to 1 mM histamine). High histamine concentrations are, however, possible in vivo. Concentrations of histamine inside human mast cell secretory granules have been observed to be as high as about 100 mM (Alter et al. 1989). In that case, concentrations of 1 mM and 5 mM could be reached in the extracellular space in the vicinity of the histamine liberating-mast cells. The histamine concentrations needed to cause apparent inhibition of thymidine incorporation and epithelial outgrowth differ by about 10-fold. Since a histamine concentration of 100 \(\mu\)M had only minor effects on thymidine incorporation but notably inhibited epithelial outgrowth, the inhibitory effect may be mediated via another cell present in a whole skin specimen or histamine may markedly inhibit migration.
During the degranulation of mast cells, heparin is released into the tissue along with histamine but afterwards it is rapidly destroyed by macrophages. The theoretical content of heparin in a single mast cell is 1.2 to 4.5 pg (Metcalfe et al. 1980), and, accordingly, the heparin concentration within mast cell secretory granules approximates 10 mg/ml (10 μm cell diameter, 40% of cell volume in granules). In keratinocyte culture, heparin inhibited thymidine incorporation into keratinocytes slightly at 0.02 μg/ml heparin, and maximally at 2 μg/ml in the absence of EGF and BPE. In the presence of EGF and BPE, the inhibitory effect of heparin was minimal. A dual effect of heparin on keratinocyte outgrowth was seen in whole skin culture, i.e. inhibition at a low concentration (up to 2 μg/ml) and stimulation at a high concentration (up to 200 μg/ml), which can still occur in vivo. Therefore, mast cell-derived heparin could modulate keratinocyte growth and migration at least in conditions with a high mast cell density. The main function of heparin may lie in its ability to control the effects of other molecules; heparin stabilizes tryptase, binds chymase and modifies its catalytic and inhibitory properties (McEuen et al. 1995, Walter et al. 1996), and stabilizes and potentiates several growth factors.

HMC-1 cells are immature mast cells that lack some of the characteristics of normal dermal mast cells. Since mature mast cells are difficult to maintain in culture, HMC-1 cells are widely used as mast cell representatives. HMC-1 mast cell lysate inhibited keratinocyte growth in cell culture and epithelial outgrowth in whole skin culture. The key mediator responsible for the inhibitory effect remains unclear, but the non-specific action of cellular compounds or organelles of lysed cells is excluded, since lysed neuroblastoma cells did not show any effect on 3H-thymidine incorporation into keratinocytes. HMC-1 cells produce histamine, heparin, chondroitin sulfate, and tryptase (Nilsson et al. 1994a), and, constitutively at least, TNF-α, TGF-β, and M-CSF (Nilsson et al. 1995). The average mast cell tissue density in skin is 3,100/mm² (Craig and Schwartz 1989), i.e. 3,100,000/cm². We found that an HMC-1 extract corresponding to 16,700 cells/well, i.e. 16,700 cells/cm², clearly inhibited 3H-thymidine incorporation and 67,000 cells/cm² inhibited epithelial outgrowth. Thus, it could be hypothesized that a small portion of the mediators in cutaneous mast cells is sufficient to cause inhibition of keratinocyte growth and migration.

Tryptase is a specific mast cell marker and a unique protease, since there are no physiological inhibitors for it. Tryptase is stabilized in its active tetrameric form by
association with heparin. During wound healing, trypsin may act as a potent angiogenic factor (Blair et al. 1997) and a fibrogenic factor (Cairns et al. 1997, Gruber et al. 1997). Effects of trypsin on the extracellular matrix include activation of matrix metalloproteinases and cleavage of fibronectin and type IV collagen (Gruber et al. 1989, Lohi et al. 1992, Kiely et al. 1993). Chymase, another mast cell protease, also has many tissue modulating activities and induces degradation of the extracellular matrix and of the components of the basal membrane zone. It has been shown to degrade basement membrane components at the dermal-epidermal junction in skin specimens ex vivo (Briggaman et al. 1984). Chymase degrades both soluble and matrix forms of fibronectin (Vartio et al. 1981), cleaves type I procollagen to a fibril forming collagen molecule (Kofford et al. 1997), and activates procollagenase (matrix metalloproteinase-1) (Saarinen et al. 1994). Chymase has been reported to detach cultured keratinocytes from the culture dish and inhibit their growth (Algermissen et al. 1999). In addition, chymase has been shown to inactivate the PAR-1 receptor (thrombin receptor) on cultured dermal fibroblasts (Schechter et al. 1998), to inhibit collagen production in fibroblasts (Xu et al. 2002), to degrade the pericellular matrix of cultured airway smooth muscle cells, and to inhibit proliferation of these smooth muscle cells (Laiz et al. 2002).

Previous in vivo experiments have shown that trypsin is able to degrade fibronectin in the pericellular matrix of fibroblasts and in the basement membrane of the skin ex vivo (Lohi et al. 1992, Kaminska et al. 1999a). In this study, fibronectin was allowed to adhere to the plastic surface, followed by treatment of the adhering fibronectin with the trypsin-chymase preparation before addition of the cells. Although keratinocyte adherence decreased in a dose-dependent manner, trypsin was not likely to play a key role, since the trypsin inhibitor, TLCK, did not prevent this effect. Instead, the results suggest that chymase cleaved and released fibronectin from the plastic surface and subsequently fibronectin-promoted keratinocyte adherence was abolished. It is also possible that, once fibronectin was released from the plastic surface by chymase, trypsin also had greater potential to participate in the degradation of soluble fibronectin (Kaminska et al. 1999a). To further clarify the possible function of chymase in epithelialization, the trypsin-chymase preparation was added to low- or high-calcium culture of keratinocytes, using growth factor/serum-deficient medium. In the absence of heparin, chymase efficiently detached
monolayer keratinocytes and caused marked destruction of the developing epithelium within 2 days. One interesting finding is that heparin at 1 μg/ml or 10 μg/ml markedly inhibited these changes. Cells have numerous different proteoglycans on their cell membranes, such as heparan sulfate. These large molecules may interact negatively with heparin or cause steric hindrance to the chymase-heparin complex, preventing the enzyme from reaching the underlying pericellular components sensitive to degradation by chymase. Heparin is a sulfated glycosaminoglycan, whereas hyaluronic acid is not. This may explain the finding that heparin modified the action of the trypsinase-chymase preparation, whereas hyaluronic acid at much higher concentrations was ineffective. It is possible that this modifying effect is specific for heparin.

Purified skin trypsin had no effect on the 3H-thymidine incorporation in keratinocyte culture in the absence or presence of heparin; nor did purified human lung trypsin (stabilized with heparin) have any apparent effect on the growth or detachment of keratinocytes in culture. In other words, these findings imply that trypsin is not crucial for controlling keratinocyte proliferation and epidermal growth. However, in chronic ulcers trypsin may activate the plasminogen activator-plasminogen system (Stack et al. 1994) and matrix metalloproteinases (Gruber et al. 1989) reported to be present in wound fluids and thought to maintain chronic ulcers (Palolahti et al. 1993, Bullen et al. 1995, Ågren et al. 2000).

6.4 Tryptase and chymase activity and histamine in chronic wounds

The pathogenetic factors contributing to the non-healing state of chronic ulcers are largely unknown, but undoubtedly there are several mechanisms acting simultaneously. One of the hypotheses deals with excessive protease production in chronic wounds as well as the imbalance between proteolytic enzymes and their endogenous inhibitors. In the light of the current data, overexpression of one particular enzyme cannot be held responsible for delayed healing. The main groups of proteases in repair processes of skin wounds are the serine proteinases (e.g. neutrophil elastase, plasmin, cathepsin G) and the matrix metalloproteinases (MMPs) (see Ågren et al. 2000). Proteases not only remodel structural proteins, but also indirectly alter the migration and proliferation of cells.
Wound fluid reflects the microenvironment of the wound and thus may provide information on the events occurring during wound repair or its failure. To study the role of mast cells in chronic wounds, the levels of mast cell protease activities and histamine were measured in the wound fluid and wound bed of chronic ulcers. Histamine and trypsin activity were found to be present in both these compartments of chronic ulcers, and the magnitude of the level of trypsin activity was comparable to that of elastase-like activity. The wash samples did not contain detectable levels of trypsin-inhibiting activity, which is understandable, since no physiological inhibitors for trypsin are known. Tryptase is bound to heparin proteoglycan, apparently making the diffusion of this large molecule slower in the extracellular matrix than small molecular size histamine (Kaminska et al. 1999b, Kivinen et al. 2001). This may well explain the result that the ratio of histamine to trypsin activity was increased in the wash samples as compared to the values in the biopsies.

Clear chymase activity was detected in the superficial shave biopsies, but, in contrast to trypsin, the level of chymase activity in the wash samples was low or hardly detectable. Similarly, the ratio of chymase activity to trypsin activity was also significantly decreased. The reason for the low chymase activity in the wash samples could be inactivation of chymase by protease inhibitors (Grinnell and Zhu 1996), or alternatively, the slow diffusion rate, because chymase-heparin proteoglycan complexes are larger than trypsin-heparin proteoglycan complexes and thus diffuse slowly from the site of discharge (Kivinen et al. 2001, 2003). To elucidate this point, the level of anti-chymase activity was measured and clear chymase-inhibiting activity was detected in the wash samples. This was not surprising, since chymase can be inactivated by plasma protease inhibitors (Schechter et al. 1989), especially when the enzyme is in the soluble form (Kivinen et al. 2001).

Numerous different proteolytic enzymes are present in chronic ulcers, and therefore, elastase and plasmin activities were also measured in the wash samples and in the superficial shave biopsies from chronic ulcers to compare them with trypsin and chymase activities. Elastase is made by polymorphonuclear leukocytes. In addition to elastin, elastase cleaves a variety of matrix components such as collagens, laminin and fibronectin, while plasmin degrades laminin, fibronectin and fibrinogen. The degradation of fibronectin by chronic wound fluid can be prevented by addition of specific inhibitors of elastase in vitro (Grinnell and Zhu 1996). The detection of high elastase-like activity in the wash
samples and biopsies also supports the idea that elastase is an essential protease in the fluid of chronic ulcers. The elastase-like activity in the wash samples was markedly decreased from that in the biopsies as compared with the decrease in tryptase activity in the corresponding samples (V, Table 1). A reasonable explanation is that protease inhibitors, such as α1-PI (Grinnell and Zhu 1996), in the wound fluid have the potential to inhibit elastase-like activity, as well as chymase activity, but not tryptase activity.

Although the function of mast cell proteases and histamine in the granulation tissue of chronic ulcers remains to be shown, the presence of histamine and tryptase activity in the wash samples and wound biopsies, and of chymase in the wound biopsies, implies that these proteases and histamine may affect the ulcer margin, keratinocyte adhesion, migration and proliferation. Since mast cells accumulated close to the epidermis and epithelial margin of chronic leg ulcers and chymase caused destruction of the developing epithelium in vitro, the level of chymase activity could be sufficiently high to result in impaired epithelial adherence and growth or even in detachment of migrating keratinocytes from the ulcer base. Further investigation will be required to explore what role chymase plays in delayed wound healing. However, it is possible that the development of potent and selective chymase inhibitors will lead to useful new drugs for the treatment of chronic ulcers.
7 CONCLUSIONS AND SUMMARY

Pivotal peptides in neurogenic inflammation in the skin comprise calcitonin gene-related peptide and substance P, the latter acting at least in part via degranulation of mast cells. The results show that SP and VIP, but not CGRP, can induce activation of mast cells and release of histamine into the extracellular space. These findings are in accordance with earlier in vitro observations and clinical experiments. Capsaicin did not cause any substantial histamine release. This suggests insufficient local concentrations of neuropeptides or infrequent morphological contacts between mast cells and sensory nerves in normal human skin.

Alterations in mast cell numbers were observed during normal wound healing. The numbers of chymase-positive mast cells decreased in the early stages of wound healing, possibly because of the action of chymase inhibitors. Similarly, in the ulcer base and in the perilesional skin of venous ulcers, chymase was partially inactivated in mast cells. The tryptase-positive mast cell numbers decreased, rather than increased, during normal wound epithelialization and were absent in the epithelialization margin. This decrease may reflect extensive degranulation and the appearance of phantom mast cells in the early events of normal wound healing. In chronic venous ulcers, mast cells were numerous in the upper dermis of the perilesional skin and were often in contact with the acanthotic epidermis and the epithelial margin. These results suggest that mast cells may either stimulate the proliferation of the epidermis or attempt to take control of keratinocyte proliferation by increasing in number and migrating close to the epidermis.

The expressions of SCF and Kit showed significant alterations during wound healing. At an early stage following wounding, the dermal cells expressing SCF were markedly increased. As wound healing progressed, the expression of SCF was decreased, whereas the expression of Kit was induced in mast cells. It is possible that, in the late phases of wound healing, downregulation of SCF expression is needed to inhibit extensive mast cell activation and to shorten the duration of inflammation, as seems to be the case in chronic wounds and in psoriasis. The persistent inflammation in chronic wounds and psoriasis is demonstrated by high Kit expression in chronic ulcers and high SCF expression in the wound beds of chronic ulcers, as well as high SCF and Kit expression in the psoriasis
lesion. Thus, in chronic wounds and in psoriasis, there seems to be a potential for interaction between SCF and the Kit receptor leading to mast cell proliferation, migration and degranulation.

Marked levels of soluble tryptase activity and histamine, but low levels of chymase activity, were measured in wash samples collected from chronic leg ulcers. No tryptase-inhibiting activity, but clear chymase-inhibiting activity, was detected in the wash samples. In superficial wound bed biopsies, significant levels of tryptase and chymase activity were detected as well as histamine, although chymase was found to be partially inactivated when detected histochemically. Since mast cells had accumulated in the epithelial margins of leg ulcers, these findings provide evidence that mast cells may participate in the pathophysiology of impaired epithelialization in chronic wounds.

Histamine and heparin were inhibitory to keratinocytes \textit{in vitro}. They inhibited both the \textsuperscript{3}H-thymidine incorporation into keratinocytes and the keratinocyte outgrowth from skin specimens, heparin having a dual effect on keratinocyte outgrowth: inhibition at low concentrations (up to 2 \textmu g/ml) and stimulation at high concentrations (up to 200 \textmu g/ml). HMC-1 mast cell lysate also inhibited keratinocyte growth and epithelial growth. Purified skin tryptase had no effect on the \textsuperscript{3}H-thymidine incorporation in keratinocyte culture in the absence or presence of heparin; nor did purified human lung tryptase (stabilized with heparin) have any apparent effect on the growth or detachment of keratinocytes cultured as a monolayer or as epithelium. In contrast, chymase efficiently detached keratinocyte monolayers or caused marked destruction of the developing epithelium within 2 days.

These results show significant alterations in mast cells in human wounds, suggesting that mast cells have the potential to contribute in a major way to normal wound healing and to impairment of wound healing. It also appears that mast cells and their mediators have an inhibitory or controlling role in epidermal growth and adherence.
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