Impaired reparative processes in particular related to hyaluronan in various cutaneous disorders
- A structural analysis

Ulf Bertheim
Cover illustration

Colour light micrograph of histological section from normal skin stained with HABP (brown), demonstrating the distribution of hyaluronan (HA). (Photo; Dr Ulf Bertheim)
The PD is intensely stained with an intense, uniform appearance of HA in the BM zone. The epidermis is moderately stained and only weak staining is seen in the RD
To my children Jane and Andre’, for your love, patience and inspiration
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## PAPERS I-IV
ABSTRACT

Impaired reparative processes in particular related to hyaluronan in various cutaneous disorders - A structural analysis

Ulf Bertheim

Cutaneous reparative processes, including wound healing, are highly developed procedures in which a chain of actions occurs to reconstitute the function of the wounded tissue. To prevent a delayed or excessive reparative process it is important to understand how this procedure develops and is maintained. One of the major extracellular matrix components of the skin is the glycosaminoglycan hyaluronan (HA). HA contributes to an extracellular environment, which is permissive for cell motility and proliferation, features that may account for HA’s unique properties observed in scarless foetal wound healing. The molecule is found at high concentration whenever proliferation, regeneration and repair of tissue occur.

The aims of the present studies were to analyse the distribution of HA and to investigate its possible role in various cutaneous conditions associated with an impaired reparative process like in scar tissue formation in healing wounds, changed skin characteristics in diabetes mellitus and proliferating activity in basal cell carcinomas.

Tissue biopsies were obtained from healthy human skin, type-I diabetic skin and various scar tissues. The samples were analysed in the light microscope with a hyaluronan-binding-probe, antibodies for collagen I, III, PCNA and Ki-67. Ultrastructural analyses were performed on the same tissue samples.

In normal skin HA was present mainly in the papillary dermis. In epidermis HA was located in between the keratinocytes in the spinous layer. In the different scar tissues the localization of HA varied, with an HA distribution in mature scar type resembling that in normal skin. In keloids the papillary dermis lacked HA, but the thickened epidermis contained more HA than the other scar types. Ultrastructural studies of keloids revealed an altered collagen structure in the dermal layers, with an abundance of thin collagen fibers in the reticular dermis and thicker collagen fibers in the papillary dermis. Furthermore, the keloids displayed epidermal changes, which involved the basement membrane (BM), exhibiting fewer hemidesmosomes, and an altered shape of desmosomes in the entire enlarged spinous layer. These alterations in epidermis are suggested to influence the hydrodynamic and cell regulatory properties of the wounded skin.

In diabetic patients, a reduced HA staining in the basement membrane zone was seen. The staining intensity of HA correlated to the physical properties of the skin reflected by their grades of limited joint mobility (LJM). Furthermore, the HA staining correlated with serum concentration of the HbA1c.

In basal cell carcinomas (BCC), HA occurred predominantly in the tumour stroma. The distribution was most intense in the highly developed superficial BCC type, and resembled that of the papillary dermis of normal skin. In contrast, in the infiltrative BCC type, the tumour stroma stained weakly in the infiltrative part of the tumour. Moreover, the surrounding dermal layer was deranged and devoid of HA. The findings suggest that the tumour stroma in superficial BCC causes a slow, well-regulated cell growth in which the tumour cells do not substantially disturb the normal skin function. In the infiltrative BCC type, the tumour cells cause a disintegration of the tumour stroma as well as the normal surrounding dermis, which permits further spreading of the tumour. In fact, the behaviour of the infiltrative BCC tumour, growing beyond its boundaries, resembles that of the keloid.

The mapping of the distribution of HA could be a useful tool for prognostic information, for evaluating the degree of progress and for deciding the choice of treatment in various diseases of the skin. In skin malignancies such as BCC it can be used to determine the radicality at the surgical excision of the tumour.

Keywords: Hyaluronan, scar tissue, diabetes mellitus, basal cell carcinoma, skin, wound healing
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BCC</td>
<td>Basal cell carcinoma</td>
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<tr>
<td>BM</td>
<td>Basement membrane</td>
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<td>BMZ</td>
<td>Basement membrane zone</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>GAG</td>
<td>Glycosaminoglycan</td>
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<td>HA</td>
<td>Hyaluronan, hyaluronic acid</td>
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<td>HABP</td>
<td>Hyaluronan-binding protein</td>
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<td>HYA</td>
<td>Hyaluronan, hyaluronic acid</td>
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<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<td>LJM</td>
<td>Limited joint mobility</td>
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<td>NIDDM</td>
<td>Non insulin dependent diabetes mellitus</td>
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<tr>
<td>PD</td>
<td>Papillary dermis</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>RD</td>
<td>Reticular dermis</td>
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This thesis is based upon the following publications and manuscripts, which will be referred to in the text by their respective roman numerals:


INTRODUCTION

The basics of plastic surgery rest on our understanding of the structure and function of normal skin and the management of its various pathological reactions as in various malignancies and wound processes. In spite of recent advances in our knowledge of the fundamental processes of wound healing, awareness of the factors involved in the development of chronic wounds remains limited (Falanga V et al., 1995). Progress in the treatment of chronic wounds will require a better understanding of their pathogenesis and failure to heal. Treatment principles have to be focused on investigations of and guidance concerning the connections between stimulatory and inhibitory wound healing mechanisms (Diegelmann and Evans., 2004). It is important to detect a pathologic repair process, in order to reduce the risk of infection, as well as the pain and discomfort patients may suffer (Moseley et al., 2004). Under normal circumstances the complex wound healing mechanism acts protectively, but when it fails healing may be protracted and resulting fibrosis may produce an abnormal disabling scar (Hunt et al., 1978).

By using various reconstructive techniques, pathological scar tissue can be treated but its function can rarely be completely restored to normal. The objective for the plastic surgeon is to guide the repair process toward a scar that resembles normal skin in its structure and function. An optimal reparative process, includes restoration not only of the cellular components in the dermis and epidermis but also the epidermal-dermal junction, the basement membrane (BM), the constituents of the extracellular matrix (ECM), and all the interconnecting proteins and enzymes. Interest in skin matrix components, including the glycosaminoglycan (GAG) hyaluronan (HA) and the collagens is increasing, and their various roles in the developing and restorative processes have been postulated (Gailit and Clark., 1994; Hellström et al., 1991; Grinnel., 1984). The present studies have been focused on
investigating the role and influence of these molecules in the wound healing process and how they are modified in the skin in the presence of metabolic disorders and malignant processes. Furthermore, this thesis highlights the importance of early signs of any structural disturbance that alters the tissue architecture and thereby also the possibility of an optimal reparative process.

*The Skin*

*Fig 1. Schematic drawing of the skin; Epidermis (Överhuden), Dermis (Läderhuden).*

The skin, the largest organ of the human body, constituting the body’s outer covering, provides protection against physical, chemical or microbial insults from the environment. It acts as a defensive barrier having a reparative capacity (Wood et al., 1992). In many internal disorders, different reactions in internal organs and structures as well as various types of cutaneous manifestations can be observed (Lever and Lever., 1990). The skin consists of three compartments: epidermis, dermis and subcutis. The subcutaneous tissue is not dealt with in the present thesis. The superficial epidermal layer is multilayered, consisting mainly of keratinocytes in different stages of maturation. The epidermis also contains melanocytes,
Merkel cells, Langerhans cells and lymphocytes. It is separated from the dermis by a basement membrane (BM). The deepest epidermal layer, stratum basale, consists of a single layer of keratinocytes. As the latter develop, they move towards the surface. Above the basal layer the keratinocytes form part of the spinous layer, characterized by an abundance of interconnective adhesive proteins called desmosomes. The keratinocytes of the superficial layer, stratum granulosum, contain keratoxyalin granules. At the surface, the keratinocytes become flattened, and lose their nuclei and intracellular organelles, and so adopt the characteristics of stratum corneum. The keratinocytes play an important role in the function of an active skin barrier through their interactions with the immune and inflammatory systems (Eckert, 1989; Wood et al., 1992). Nevertheless, a normal architecture—and hence normal functioning of the epidermis—relies on an undisturbed intercellular environment that not only holds the cells together, but also permits intercellular exchange of gases and nutrients. This cohesion is effected mainly by interconnecting desmosomes, which are adhesive proteins consisting of two symmetrical halves, each located in one of two adjoining cells. On the cytoplasmonic side of the plasma membrane is the desmosomal plaque, in association with the keratin filaments (North et al., 1999). Evidently, desmosomes are not static structures, rather they are dynamic units, whose composition and structure are critical for normal epidermal function (Kitajima, 2002). Intra-epidermal and dermal-epidermal cohesion are essential for the integrity of the skin (Moll and Moll, 1998).

Basement membrane (BM) is a thin layer, intersecting the connective tissue from the basal keratinocytes of the epidermis. The BM constitutes a contiguous sheet of ECM containing various proteins, separating the epidermis from the dermis. Such proteins are laminin, collagen IV and various heparan sulphate proteoglycans. Three main zones in the BM can be recognized: lamina lucida, lamina densa and sublamina densa. The key structure of the BM is lamina densa, characterized by a network of cords and intercordal spaces (Leblond and
Inoue., 1988). The hemidesmosomes attach the basal cells of the epidermis to the BM by a complicated intercellular arrangement. Together with specialized structures, primarily anchoring filaments and fibrils, they form adhesion complexes that are extracellularly connected to the cytoskeletal network of cytokeratin filaments (Jones et al 1989). As the constitution of the BM is so complex, it provides for several functions such as attachment of cells, a support or scaffolding function, regulation of permeability through the membrane, and plays a part in regulating the growth and differentiation of the keratinocytes (Briggaman., 1982; Delvoy et al., 1988; Bosman et al., 1989).

The connective tissue of the dermis consists of collagenous and elastic fibres embedded into the ECM. All three components are synthesized by fibroblasts. Collagen is by far the most abundant constituent of the dermal connective tissue. The collagen fibres form either a neatly woven network as in the superficial papillary dermis (PD) or thick bundles buried in the deeper reticular dermis (RD). A few fibroblasts are present between the collagen bundles. Another cell type present in normal dermis is the mast cell, often located perivascularly. In PD, the ECM has a loosely structure, with sparse collagen content, but numerous GAGs such as HA, heparan sulphate, fibronectin and chondroitin sulphate. The physicochemical properties of the HA polymer and interaction of the polymer with other macromolecular components is the prerequisite for rapid adaptive changes of the skin, for instance when subjected a number of disease conditions, including trauma (Juhlin., 1997; Laurent and Fraser 1992; Ono et al., 1998). As a principal molecule within ECM, the quantity of HA increases whenever rapid tissue proliferation, regeneration and repair occur (Manuskiatti and Maibachl., 1996). In RD the ECM is rich in fibroblasts and collagen that give the skin both stability and rigidity (van Zuijlen et al., 2003).
**Hyaluronan**

![Image](image.png)

**Fig 2.** Hyaluronan (HA) is a common component of the extra cellular matrix. At higher concentrations HA exhibit interesting rheological properties due to a random coil formation of a highly entangled network of flexible polysaccharide molecules with a diameter around 200 nm. (from; Smedsröd., 1989).

Hyaluronan (HA) molecules are attached to cells exhibiting specific HA receptors on the cell surface. A relatively low cell density and an abundance of ECM material characterize loose connective tissue. The extracellular macromolecular material consists of fibrous proteins such as collagen and elastin, GAGs, e.g. HA, proteins for cell attachments, and other specific soluble and insoluble proteins (Laurent, 1987). Hyaluronan (HA), previously called hyaluronic acid (Balazs et al., 1986), is a linear polysaccharide consisting of alternating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid. The molecular weight of HA is usually in the order of $10^6$–$10^7$, and its mass has a radius of about 200 nm (Laurent, 1987). The configuration of the molecule is that of a highly expanded random coil, but only about 0.1% of the content is a polysaccharide. The major constituent is water, mechanically immobilized within the coil. At a concentration exceeding 1 mg/ml the HA molecules entangle, and at higher concentrations the chain forms a contiguous flexible network. HA is synthesized in the
cell membrane of most cells by the addition of monosaccharide units supplied by UDP-
glucuronic acid and UDP-N-acetylglucosamine to the reducing end of the chain (Laurent,
1987). During elongation, the polysaccharide chain extrudes through the cell membrane into
the extracellular space. The synthesis of HA is influenced by numerous agents such as
hormones, inflammatory mediators, and growth factors (Laurent and Fraser., 1986), most
likely via regulative phosphorylation (Mian N,. 1986a). In general, agents activating adenylate
cyclase appear to stimulate the production of this polysaccharide (Mian N,.1986b).

In some tissues an HA–mediated endocytosis and intracellular deposition occur
(Hua et al .,1993). The turnover rate of HA is surprisingly rapid; its half-life in skin and joints
is about 12 h, in the anterior chamber of the eye about 60-90 min, and in the vitreous body 70
days (Reed et al, 1990). Degradation starts in the ECM of the connective tissue and the
molecules, reduced in size are transported into the general circulation to be metabolised in the
hepatocytes of the liver (Smedsröd et al., 1984). A total amount of 10-100 mg is turned over
in the circulation of an adult human each day (Fraser and Laurent, 1989).

Many physiological and cell biological functions of HA have been associated with the
physico-chemical characteristics of its polymer network and hydrodynamic capacity (Laurent
et al., 1995). Examples are lubrication, viscoelasticity, flow resistance, osmotic pressure,
exclusion properties and filter effects. HA contributes significantly to the water balance in the
body and it affects the transport and distribution of plasma proteins in the tissues (Laurent and
Fraser, 1992). Many studies have demonstrated that HA is involved in the proliferation,
recognition, and locomotion of cells. Increased concentrations of HA and the presence of HA-
receptors, such as CD-44, ICAM-1 and RHAMM (Brown, 2004) have been found in
proliferative tissue.
The total content of HA in the human body is calculated to 16g half of which is located in the skin, contributing to its normal architecture and function (Reed et al., 1988). The distribution of HA in healthy skin involves both epidermis and dermis. In epidermis HA is present mainly in the intercellular spaces around the middle and upper parts of the spinous keratinocytes in the basal layer, whereas in the granular and cornified layers little, or no HA is present (Tammi et al., 1988; Wells et al., 1990). In dermis, the most intense HA staining is seen in PD (Wells et al., 1990; Bertheim and Hellström, 1994). Nevertheless, HA is also present in the RD, showing weak staining around the coarser fibres and stronger staining in the outer lining of blood vessels, as around the adnexal structures (Wells et al., 1990, Bertheim and Hellström, 1994). The importance of HA in normal skin functioning has been asserted in numerous studies (Burd et al., 1991; Juhlin, 1997). Due to the many postulated functions of HA (Laurent et al 1995), its localization directly below the basement membrane zone (BMZ) is of the greatest interest, since changes here might affect not only the basement membrane but also the entire epidermis.

Another important role of HA is its influence on cell locomotion, which characterizes tumour invasion (Brecht et al., 1986). Tumours often have an enriched HA content and in some, increased concentrations are correlated with invasiveness (Liotta et al., 1977; Barsky et al., 1983; Zhang et al., 1995). In various tumour types, the stroma too has been shown to contain increased concentrations of HA, which would seem to correlate with the grade of malignancy and metastatic capacity of the tumour (Toole, 1981; Turley and Tretiak, 1985; Tammi et al., 1991; Rooney P et al., 1995).

In wound healing HA has been reported to effect scarless healing in fetal wounds by virtue of its increased concentration and prolonged presence in that type of tissue, possibly because it lacks degrading capacity due to absence of the degrading enzyme hyaluronidase (Longaker et
al., 1991). In normal wound healing, HA content is increased during the early wound phase, when the proliferative activity peaks (Weigel et al., 1986, Gailit and clarc., 1994). In this period it is assumed that HA together with fibrin forms the early granulation tissue (Bartolami and Donoff., 1978), which attracts inflammatory cells into the wound. CD-44 is suggested to function as an HA receptor in granulation tissue cells, such as macrophages and fibroblast (Oksala et al., 1995). This matrix modifies by the cells entering the wound as they secrete hyaluronidase and plasminogen activator into the ECM to degrade HA and fibrin (Weigel et al., 1986).

The clinical use of HA is increasing rapidly, mainly because of it has no inflammatory properties, and has rheological capacity. Today, it is used in joint diseases to promote cartilage-healing, functions as a lubricant and is now an important agent in ophthalmic surgery (Laurent et al., 1995). In the skin, it has been used as a dermal expander agent to eliminate wrinkles or fill out various skin defects. In experimental animal models it has been shown to increase the healing capacity of chronic wounds in the tympanic membrane (Hellström and Laurent, 1987; Laurent et al., 1988). Furthermore, it has been used to heal chronic diabetic ulcers (Abatangelo et al., 1983).

**Wound healing process**

Wound repair is a complex process, where a sequential occurrence with diverse cells and substances must perform their special task in coordination with each other. Several biological cell phenomena are involved in this reparative process, such as cell migration, proliferation, BM regeneration, and the formation of granulation tissue. Thus, wound repair requires continual interactions among cells, cytokines and matrix. The skin’s response to injury can be divided into four phases that begins with an orderly process of haemostasis and fibrin
deposition, which leads to an inflammatory cell cascade, characterized by neutrophils, macrophages and lymphocytes within the tissue (Diegelmann et al., 2004). Attraction and proliferation of fibroblasts and collagen deposition is forming the granulation tissue and finally deposition of matrix and remodelling of the scar tissue occur (Kirsner and Eaglstein., 1993). An injury results in extravasations of blood constituents, including mediator release, which initiates an inflammatory reaction. Activated platelets affect haemostasis by adhering to de-endothelialized blood vessel walls, by clumping and by opening coagulation pathways. Platelets also release a cascade of biologically active substances, including ECM molecules such as hyaluronan, fibronectin, fibrin/fibrinogen, and various cytokines such as growth factors, platelet-derived growth factor (PDGF), integrins, interleukin-1, epidermal growth factor, transforming growth factors (TGF-α and TGF-β), connective tissue-activating peptide-III, and keratinocyte growth factor (KGF) (Berman and Duncan., 1989; Gailit and Clark., 1994; Diegelmann et al., 2004). These molecules activate the synthesis and growth of fibroblasts, and promote cell migration into the wounded site.

Within the first few hours after an injury, abundant neutrophils infiltrate the wound site. Substantial numbers of monocytes accumulate from 24 up to 48 hours later. Both cell types are attracted to the site of tissue injury by a variety of chemotactic factors. The major function of the neutrophils is to prevent bacterial invasion. Monocytes, in their activated state as macrophages, phagocytos and eliminate pathogenic organisms, scavenge tissue debris including combusted neutrophils, and appear to be critical for the initiation of tissue repair (Hammar., 1993). Macrophages release a plethora of biologically active substances, many of which facilitate the recruitment of additional inflammatory cells or remove tissue debris and decontamination (Diegelmann et al., 2004). Macrophages also secrete growth factors, such as PDGF and TGF-β, which initiate and sustain new tissue formation. Thus the macrophages are
believed to play a pivotal role in the transition between the inflammatory phase of wound healing and the second phase and the formation of granulation tissue.

Granulation tissue consists of a dense array of macrophages, fibroblasts and neovasculature embedded in a loosely woven matrix of fibronectin, collagen types I and III and HA. As fibroblasts migrate to the wounded site, they display several distinct phenotypes, first a migratory phenotype and ultimately a contractile phenotype. In the contractile state, the so-called myofibroblasts align themselves along newly deposited ECM in the radial axes of the wound, form cell-cell and cell-matrix links, and generate tension across the wound to cause wound contraction (Clark, 1988).

Blood vessel growth, angiogenesis, into the wounded site occurs simultaneously with the ingrowth of fibroblasts and ECM deposition. Endothelial cells, lining the microvasculature adjacent to a wound, dissolve the supreme BM, emigrate through the disrupted barrier, and migrate as a cord of cells on a provisional matrix into the wounded area. As endothelial cell cords link up to form new arcades of capillaries, lumina appear in the centre of the cords and blood flow normally begins around the third day, after the trauma. BM rapidly forms between the endothelium of the capillaries and the neomatrix. When the epithelium is disrupted at the time of injury, re-epithelialization quickly ensues to re-establish tissue integrity. Within a day or two, the epithelial cells remaining at the wound margin begin to proliferate. The additional population of cells then migrate across the wound bed. If the BM has been destroyed, epidermal cells migrate over a provisional matrix of fibronectin and fibrin. A new BM grows inward from the wound margin. If the BM has not been destroyed, hemidesmosomes at the wound margin undergo dissolution, thus facilitating reepithelialization (Clark, 1988).
In the absence of firm binding at the epithelial-stromal interface is a prerequisite for epithelial mobility. Once reepithelialization is complete, the cells revert to their normal phenotype and reattach firmly to the BM by means of hemidesmosomes.

The final phase of wound repair is matrix remodelling. As with all phases of wound repair, the third phase overlaps its predecessor. In fact, matrix production and remodelling begin simultaneously with granulation tissue formation. Nevertheless, in the month that follows granulation tissue dissolution, the matrix alters continuously. Most fibronectin is rapidly eliminated from the matrix and type I collagen fibres slowly grow into large bundles that provide the residual scar with increasing tensile strength (Clark, 1988). Decorin, a small dermatan sulfate proteoglycan has demonstrated a high affinity for type I collagen and is assumed to regulate connective tissue regeneration via collagen fibril formation (Oksala et al 1995).

HA is a major component of early granulation tissue (Clark, 2001), increasing in content until 3 days after injury, before declining. As mentioned earlier, HA content continues to be increased in the fetal wound, leading to scarless healing in which no inflammatory reaction is evident (Longaker et al., 1989).

**Pathologic scar tissue formation**

When wound healing involves the BM, the healing will result in a scar formation. After tissue injury, resident fibroblasts undergo metabolic activation and exhibit increased growth and synthesis to repair the injured tissue. Under basal conditions, this activity of resident fibroblasts is limited and probably controlled by interaction with native connective tissue matrix components (Diegelmann et al 2004). When something fails in the remodelling phase,
this scar tissue formation develops into a hypertrophic scar, or a keloid, instead of a mature scar type (Ketchum 1974; Rockwell et al., 1989; Phan et al., 2002). Hypertrophic scar is difficult to distinguish from keloid, as they have similar clinical features and resemble each other histopathologically (Murray et al., 1981; Muir, 1990; Linares et al., 1972). Keloids are characterized by an abnormal rate of collagen production, and increased levels of certain ECM components such as fibronectins and proteoglycans (Babu et al., 1989; Bailey, 1975; Kischer et al., 1983), but a decrease in others, such as HA (Meyer et al., 2000). The keloidal growth pattern is characterized by an accelerated fibroblast proliferation with an abnormal, abundant deposition of collagen and ECM components, growing beyond its boundaries and invading the neighbouring uninjured tissue (Blackburn and Cosman, 1966). The exact background to this uncontrolled growth behaviour is still unknown. It has been suggested that hypoxia as a result of microvessel occlusion might be an initiating factor in the pathogenesis of excessive collagen synthesis (Hunt et al., 1978, Le et al., 2004). Several investigations have disclosed some important facts, but many mechanisms remain to be discovered. Recent studies have suggested the existence of some kind of keratinocyte-fibroblast interaction. The proliferative rate of keloid fibroblasts seems to be stimulated by certain factors secreted by keloid derived keratinocytes (Phan et al., 2002).

**Cutaneous changes in diabetes mellitus**

Cutaneous manifestations and impaired wound healing, most often in conjunction with various types of disturbance affecting the internal organs, are related to diabetes mellitus (DM) (Huntley, 1989; Lundbæk., 1957). Furthermore, changes are seen in various tissues due to their hyperglycaemic state and hormonal aberrations underlying the altered metabolic state (Lieberman et al., 1980). DM is divided into two groups depending on insulin dependency or not. In type 1, (IDDM), there is insufficient insulin production due to reduced functioning in
Langerhan island cells in pancreas. In type 2, (NIDDM) the glycaemic state is high, because of a reduced sensibility for insulin. The cutaneous disturbances, seen in DM, are correlated to the severity of the disease. The contribution to these changes of the skin concerns both the BM, which becomes more porous due to increased degradation or inadequate synthesis of heparan sulphate proteoglycan (Rohrbach et al., 1982), and the dermal connective tissue, demonstrating an increase in size and disorganization of the collagen bundles (Lyons and Kennedy, 1985). Another well-known feature in diabetes mellitus is stiffening of the periarticular connective tissue of the hands, cheiroartropathy, also named limited joint mobility (LJM). This condition is believed to identify a population exceptionally at risk of early development of microvascular complications. The LJM test can be used to evaluate the severity of the disease (Rosenbloom et al., 1981).

**Basal cell carcinomas**

Basal cell carcinomas (BCC) are usually regarded as harmless. They display a slow pattern of growth and rarely metastasize to other organs (Conley et al., 1985). Nevertheless, BCCs are malignant tumours and behave accordingly by invading surrounding tissues. The destructive consequences of invasion derive from the migration of malignant cells into adjacent tissues and from their subsequent growth in the new location. BCCs occur chiefly in Caucasians, especially in elderly individuals. Prolonged exposure to sunlight is one suggested cause of BCCs. The head and neck are the sites of predilection. Most BCCs respond well to local treatment (Pollack et al., 1982).

Other BCC types grow slowly but aggressively, infiltrating the deeper structures beneath the skin. Many clinicians and pathologists recognize from experience the aggressive form of BCC, which is either recurrent or deeply invasive into underlying muscle, cartilage and bone.
These tumours are difficult to delineate and tend to recur after treatment. Such tumours often occur in the nasolabial fold and lacrimal duct, growing downward and being difficult to circumscribe. They are most often referred to as infiltrating, morphemic, “sclerosing BCC” by the pathologist (Blackburn and Cosman, 1966).

The characterization of most BCC tumours is otherwise diverse, including the more common types: superficial, nodular, cystic, and ulcerated. The aggressive BCC types are more ulcerative and infiltrative in their behaviour, consist of small nests of tumour cells, often displaying an irregular, spiky appearance with infiltration of cells in cords. The usual peripheral palisade is absent, and the cells tend not to exhibit any differentiation. Hyalinisation of the stroma is more common in the aggressive BCC type.

The choice of treatment is often based on a preoperative biopsy, which will provide information on the histological behaviour and distribution of tumour cells in the tissue. It is difficult to predict aggressive behaviour in BCCs, but nevertheless important prior to deciding which treatment to adopt. Even after using modified surgical technique, with a microscope that provides for precise excision, the recurrence rate is high.
AIMS OF THE STUDIES

The aim of the present work was to investigate the pathogenesis of impaired skin reparative processes especially with regard to hyaluronan (HA). Studies were performed of various scar tissues, skin biopsies from patients with type-1 diabetes mellitus and basal cell carcinomas (BCC).

Against this background, the studies were designed to answer the following specific questions:

I  Does the distribution of HA in various forms of scar tissue contribute to their differing clinical characteristics?

II Does the distribution and content of HA in the skin of patients with diabetes mellitus explain the severity of their clinical manifestations?

III Does the distribution of HA in various BCC tumours correspond to their differing proliferative activity and capacity of the tumour to spread?

IV Does the ultrastructural architecture of abnormal scars, such as keloid and hypertrophic scar, provide us with further information as to their different clinical characteristics?
MATERIAL

Tissue samples from human skin were obtained for all studies. Each patient was informed of the study prior to surgery, and tissue samples were obtained with their consent.

Scar tissues
All patients, who suffered from an abnormal scar, visited the Department of Plastic Surgery, at which they were examined and diagnosed by an experienced plastic surgeon prior to treatment. The scar tissues were excised with narrow margins. In paper I; tissues from 39 patients; mature scar (n=6), hypertrophic scar (n=14) and keloids (n=12), were obtained. From one person two samples were collected: an earlobe keloid and a hypertrophic scar on the cheek, both scars a result from a dog bite 3 years earlier. Normal skin (n=8) was excised from various locations in healthy patients with other diagnoses in which skin biopsies were included. For the ultrastructural study in paper IV; scar tissue samples from 16 patients; mature scar (n=3), hypertrophic scar (n=3), keloids (n=5) and normal skin (n=5) were used.

Diabetes mellitus
Skin punch biopsies, from 23 patients with insulin dependent diabetes mellitus (IDDM) and varying grades of limited joint mobility (LJM) were used. The biopsies; diabetic skin with LJM grade 0 (n=10), LJM grade 1 (n=7), LJM grade 2 (n= 6), were taken from the dorsum of the hand, 4 cm distal to the ulnar head. Biopsies from the same area were also obtained from non-diabetic patients (n=6).

Basal cell carcinoma (BCC)
Patients, consulting a dermatologist at the department of Dermatology, or a plastic surgeon at the department of Plastic Surgery at the University hospital in Umeå, were diagnosed
preoperatively with some type of BCC tumour. They were treated with surgery and thus included in this study. Thirty specimens from 28 patients were used: superficial BCC (n=10), nodular BCC (n=10) and infiltrative BCC (n=10). Two patients had at the same time different types of BCC tumours, which were diagnosed and both included in the study. The basal cell carcinomas were surgically removed, prepared and examined by an experienced dermatopathologist to the pathologic anatomic diagnosis and ensure the radicality of the surgery. Pieces of the tumour tissue were then used for this study. As controls, normal skin was excised from various locations in healthy patients treated for other diagnoses in which skin excision was included.
METHOD

Paraffin embedded material

Papers I-IV: The excised tissue pieces were transferred to saline and within 30 minutes fixed in a solution containing 2% formaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS). For localization of HA, fixation was performed under microwave irradiation. The specimens were irradiated at 700 W up to 45°C and then transferred to PBS and kept at cold storage temperature until further processed and embedded. The tissue samples were dehydrated in upgraded series of ethanol to xylene and embedded in paraffin wax. Serial paraffin sections (5 μm) were cut and prepared for light microscopy.

Plastic embedded material

Paper IV: Pieces of scar tissue were fixed in 3% glutaraldehyde solution in 75 mM sodium cacodylate buffer with 4% polyvinylpyrrolidone and 2 mM CaCl₂ added. The specimens were postfixed in 1% OsO₄ overnight and dehydrated in increasing concentrations of acetone and embedded in an epoxy resin. Semithin sections, 0.5-1.0 μm were then stained with toluidine blue for examination in the light microscope, and ultrathin sections 70-85 nm were contrasted with uranyl acetate and lead citrate for electron microscopy.
**Histochemical staining for HA**

Papers I-IV: Following deparaffinization, the tissue sections were washed in PBS and then incubated with a fresh solution of 3% H$_2$O$_2$ in methanol 5 min at room temperature to destroy endogenous peroxidase activity. After 2 washes in PBS the slides were incubated with 1.0% bovine serum albumin for 30 min at room temperature to block non-specific binding sites. The slides were washed with PBS and then incubated with approximately 100 l of biotinylated hyaluronan binding protein probe (HABP) at a dilution of 1:40, overnight, at 4°C storage temperature. After the washings in PBS, the slides were incubated with the Vectastain-Elite avidin-biotin complex at a dilution of 1:200, for 40 min, at room temperature. After 3 washes in PBS for 10 min each, the sections were incubated for 5 min in 0.1% diaminobenzidine tetrahydrochloride (DAB) and 0.03% H$_2$O$_2$ in 0.05 M TRIS-HCL buffer, pH 7.6, at room temperature, which produced a water insoluble brown precipitate. Finally the slides were washed in tap water for 5 min and cover slipped.

**Immunohistochemical analysis**

Paraffin sections (4 m) were mounted, deparaffinized and washed in 3% H$_2$O$_2$ in methanol for 5 min at room temperature to destroy endogenous peroxidase activity. Antigen retrieval of the sections was performed for all antibody stainings in 10 mM citric acid (pH 6.0) during two microwave irradiation cycles of 5 min at 700W. In paper III, the proliferative activity was studied by use of a rabbit anti-Ki-67 polyclonal antibody in 0.05 M TBS (pH 7.4), and a rabbit anti-PCNA monoclonal antibody. In paper IV, the distribution of collagen I and III was studied by incubation in normal swine serum 1:20 for 30 min before incubation for 1 hour in a 1:400 solution of rabbit anti-human collagen type I/III, prior to application of a 1:300 dilution of biotinylated rabbit anti-mouse IgG. The immunoreactive sites were visualized with
diaminobenzidine, counterstained with Mayer Htx stain and cover slipped. Sections incubated without the primary antibody but otherwise treated identically served as negative controls.

**Quantitative analysis of HA in tissue samples**

In paper II, tissue samples were digested with pronase (Protease P-5005 5 units/2 ml buffer 0.05 M TRIS-HCL-0.01M CaCl₂, pH 7.2) for 18 h at 55 °C. The digests were kept in a bath of boiling water for 10 min and then stored frozen at 80 °C. Prior to analyses, 0.1 ml 0.2 M phenylmethanol-sulphonyl fluoride in 99% ethanol was added to inhibit the remaining enzyme activity. The solution was centrifugated for 10 min at approximately 2,000 rpm and 2 ml of the supernatant was then applied onto a 10 ml Sephadex G-25 column (PD-10, Pharmacia Fine Chemicals, Uppsala, Sweden) followed by PBS. The first 3 ml of the eluate was discarded and the subsequent 4 ml was saved for analysis of the HA content, according to the method described by Laurent & Tengblad (1980). The method utilizes purified HABP extracted from nasal bovine cartilage. The HABP is used as an antibody in an RIA-like type of assay.

**Statistical methods**

In paper II, an unpaired t-test was used for calculation of differences between the groups regarding age, duration of disease, HbA1c and microalbuminuria. A non-parametric test, the Mann-Whitney U-test, was used to calculate a possible correlation between epidermal thickness and the histological grading of the HABP staining. In paper IV, acquired data were tested with the non-parametric Kruskal-Wallis Test.
**Examination**

In papers I-IV, the specimens were examined with a Zeiss Axiophot light microscope. All images were photodocumented by means of a Zeiss Axiophot photomicroscope, using negative film, developed and processed to photopaper. In paper IV, electron microscopy was performed with a JEOL 1200EX, (Tokyo, Japan). Electron micrographs were made using negative film, developed and processed to photopaper.
RESULTS

*The architecture of normal skin*

Papers I-IV:

Epidermis is attached to the irregularly shaped basement membrane and the collagen-rich dermis. The epidermis consists of keratinocytes, which possess intercellular bridges, desmosomes. The desmosomes are large (0.37µm), elongated, spindle shaped, electron-dense plaques. The basement membrane forms a continuous sheet of extracellular matrix, with hemidesmosomes attaching the basal cells of epidermis to it. In epidermis the distribution of HA was moderate and HA was mainly seen in the interstices of the spinous layer. In the cornified and granular layers, no HA was observed. The basal layer displayed a weak, interstitial HA-staining.

Studying the proliferative activity in epidermis by the use of Ki-67 and PCNA, a moderate nuclear staining localized to the basal cells of epidermis was revealed.

Collagen, embedded with elastic fibers in the ground substance, represents by far the most abundant constituent of the dermal connective tissue. In PD, HA was present in a dense layer directly below the epidermis, with the most pronounced staining just beneath the basement membrane. In contrast, a weak HA-staining was seen in RD, distributed in an irregular, mesh-like pattern. In the superficial PD the collagen existed in a finely woven network consisting of thin calibre fibers. In the deeper RD, the collagen fibers, arranged in bundles were of a thick caliber. Regarding collagen types, the PD contained moderate amounts of collagen I and III.

In the RD, moderate amounts of collagen I were observed, but only a weak distribution of collagen III. A small number of fibroblasts were seen between the collagen bundles. Another cell type present in the normal dermis was the mast cell, generally occurring in small numbers around the vessels.
**Scar tissue**

**Papers I and IV:**

In mature scars the localization of HA resembled that of the normal skin, with the most intense HA staining in PD. An intense HA-staining of PD was also seen in hypertrophic scars. The positive staining occurred close to the basement membrane. The HA-staining of RD was scattered.

In keloids the PD was devoid of HA-staining. In contrast, a grossly thickened epidermis showed intense HA-staining. Also, a massive, bulging RD was strongly stained for HA. Ultrastructurally, the epidermis of all the scar tissues, except the keloid, closely resembled that of the normal skin. The keratinocytes of the keloids were swollen in both the basal layer and the spinous layer, and exhibited a large nucleus that almost filled out the cell cytoplasm. All scar tissues displayed a comparably thicker epidermis than that of the normal skin. The epidermal layer of the keloid was the thickest, approximately two times that of the normal skin, followed by the hypertrophic scar, and the mature scar. Changes were observed concerning the intercellular adhesion features; the hemidesmosomes and the desmosomes. Furthermore, the microfilaments were shorter and less well organized in the keloid but resembled that of the normal skin in the other scar tissues.

Both hypertrophic scars and keloids exhibited rectangular desmosomes of a shorter length axis than that of the normal skin. The desmosomes of mature scars also presented a rectangular form, but with a length axis that resembled normal skin. The width of the desmosomes was 0.2 µm, and did not vary between the normal skin and the various scar tissues. The density of hemidesmosomes, calculated as the number versus the length of the basement membrane, seemed to be reduced in all scar tissues compared to that of the normal skin. The keloid showed the lowest density, whereas the density of the hypertrophic and the mature scars did not differ, but were slightly reduced compared to normal skin.
In keloids, the major finding of the dermis was the accumulation of collagen fibers, which occupied the entire area below the basement membrane in the PD. This area corresponded to the area lacking HA in the HA-stained specimens. In the various scar tissues the fiber diameters displayed a less variation between the dermal layers compared to normal skin. Thus, the collagen fibers in the RD were thinner and in the PD thicker. The changed fiber diameter was most striking in the keloid, in which the collagen fiber diameter was almost identical in the entire dermal region.

Interestingly, the distribution of collagen I was similar in the PD of all scar tissues, whereas in the RD, the staining was stronger in the mature scar tissue and weaker in the keloids. The distribution of collagen III was more abundant in the PD in the hypertrophic scar tissues and weak in the keloids while in the RD, the distribution of collagen III was abundant in all scar tissues. The number of mast cells was more frequent in the mature scar, than in the normal skin. The keloid tissue exhibited the fewest number of mast cells.

**Skin in diabetic patients**

**Paper II;**

In the skin from patients with IDDM with no restriction of the mobility of the hands, LJM grade 0, the distribution of HA resembled that of the normal skin, except that the dense HA layer of PD, the basement membrane zone, was thinner. In IDDM patients with a moderately restricted mobility of the hands, LJM grade 1, the HA pattern in PD was even less dense. The HA pattern showed some variations, such as patches or the entire basement membrane zone devoid of HA. In IDDM patients with severe restriction in their joint mobility, LJM grade 2, the HA-staining of PD was weak with the basement membrane zone devoid of HA. The RD exhibited less HA in the IDDM patients with LJM grade 2.
In spite of these findings, a quantitative analysis of HA, did not reveal any significant difference in HA concentration between the groups.

In the LJM grade 2 group, an increased epidermal thickness as well as a pronounced epidermal HA staining was evident when compared to other LJM groups as well as the normal skin.

Significantly higher (p<0.05) HbA1c levels were observed in the IDDM patients with LJM grade 2 than in the other groups.

**Different types of BCC tumours**

**Paper III:**

In superficial BCC, the tumour islands showed a moderate HA staining. The proliferative activity was weak as indicated by a modest mitotic activity and a weak Ki-67 and PCNA immunoreactivity of the tumour islands. The surrounding tissue resembled normal skin, as no differentiated tumour stroma was observed. In nodular BCC the HA staining of the tumour strands was weak to moderate, and the tumour cells expressed an increased proliferative activity. The surrounding tumour stroma stained strongly for HA. Tumour islands of the infiltrative BCC stained weakly to moderate for HA and expressed an intense proliferation, which was present throughout the entire tumour. An intensely HA-stained tumour stroma ceased abruptly in the deeper, infiltrating part of the tumour where the adjacent connective tissue was almost devoid of HA. Furthermore, this neighbouring connective tissue also expressed a deranged appearance.
DISCUSSION

The ECM plays a vital role in governing the regulation of normal skin development and function – mainly by means of its specific arrangement of macromolecules, such as collagens, proteoglycans, GAGs, and glycoproteins. Cutaneous wound healing may be defined broadly as the interaction of a complex series of phenomena, resulting in resurfacing, reconstruction and proportionate restoration of the tensile strength of wounded skin. The process of wound repair is a sophisticated interplay between ever-changing cell populations, growth factors, proteases and matrix molecules. These complicated scenarios are closely regulated, changing in response to the environment. The cellular regulation - or dysfunction of this process - that results in the formation of abnormal scars is poorly understood (Diegelmann et al., 2004).

Both keloid and hypertrophic scar are characterized as abnormal scars, as they show aggravated, red and itchy scarring. In contrast to hypertrophic scarring, keloids do not regress with time, are difficult to treat and recur frequently. They require a different therapeutic approach even though their characteristics and morphology are alike, which indicates the importance of distinguishing between them. Several studies have in particular recognized GAGs - and especially HA – as being important for a normal wound healing process (Clark., 2001; Burd et al., 1991; Manuskiatti and Maibach., 1996). A breakthrough in the role of HA in wound healing research was the discovery of scarless wound healing coupled with the fetal ECM (Longaker et al., 1989; Longaker et al., 1991; Burd et al., 1990; Siebert et al., 1990), which is extremely riched in HA.

In postnatal wounds, other studies have shown HA to be an important feature in the healing process interacting mainly during the early events (Oksala et al., 1995; Weigel et al., 1986; Brown, 2004).
In papers I, III, IV, skin from different parts of the body of individuals of various ages, was investigated. In paper II, biopsy samples were collected from a standardized location on the diabetic patients, on the dorsum of the hand. In spite of the various biopsy sites and varying ages of the patients (in papers I, III, IV) no obvious differences in the distribution of HA could be observed. Furthermore, in papers IV, and I, the interval between the trauma and the surgical procedure (including the biopsy) varied with no significant differences in HA distribution within the scar groups.

**HA in wounded skin**

![Fig 3](image)

*Fig 3. Schematic description of the localization of hyaluronan (HA) in: (S) normal skin, (H) hypertrophic scar and (K) keloid. Ep= epidermis, BM= basement membrane, PD= papillary dermis. RD= reticular dermis.*

In the present thesis it was found that the localization of HA in the normal skin was located chiefly in PD, immediately beneath the BM. In contrast, the distribution of HA in RD was sparse, a finding that supports the results in other studies on postnatal skin (Tammi et al., 1988; Wells et al., 1990).

In paper I, a similar pattern of HA was seen in mature scar, whereas the HA in hypertrophic scar- and particularly in keloid - was lacking in the PD. In these abnormal scars, HA occurred
mainly in the bulging RD and the thickened epidermis. In paper IV, it was demonstrated that in keloid, the region of PD lacking HA, bordering the BM, was filled with densely packed collagen fibres, thicker than in the normal skin. The altered dimension of the collagen fibres indicates that they contain another type of collagen than the PD of normal skin. On the contrary, the RD of the keloid contained collagen fibres, which appeared finer than that in normal skin. The entire bulging dermis in the keloids was filled with collagen fibres of a diameter similar to that in PD. This finding contradicts the general opinion that keloids contain thick collagen fibres (Ehrlich et al., 1994). The immunohistochemical studies showed an increased occurrence of collagen type III and a decrease in type I in the RD, with a decrease in type III in the PD in the keloid, compared with the normal skin, which supports earlier findings (O’Sullivan et al., 1996). The increase in collagen III in RD, was not pronounced, however, and the altered collagen fibres of the keloid probably belong to another collagen type than collagens III and I. Considering the keloid as an uncontrolled growing scar tissue with invading capacity to reach beyond its margins, should be regarded as important. That the same, fine calibre collagen fibres are present in both PD and RD could be due to an uncontrolled production of fine calibre collagen. This collagen does not create the appropriate stability or rigidity in the dermis, and will thus allow the tissue to expand with no other restriction than the neighbouring normal skin areas. Various alterations in the architecture of the epidermal region were also noted. The keloid displayed a reduced number of hemidesmosomes anchoring to the BM, and furthermore, the desmosomes connecting the intercellular space of the spinous layer appeared different.
Fig 4. Schematic description of the mechanism behind dehydration following structural changes in the different scar tissues in relation to the distribution of HA staining in; (S) normal skin, (H) hypertrophic scar and (K) keloid. The different layers of the skin are noted as; Ep = epidermis, BM = basement membrane, PD = papillary dermis and RD = reticular dermis. The arrows represent the water transport in the skin.

It is tempting to suggest that the altered architecture of the dermis, with an ECM in the PD devoid of HA, must have influenced the changes observed in the epidermis. In view of the water attracting capacity of HA, the lack of HA in the PD will lead to an impaired function of the BM. This should in turn lead to a leakage of fluid through the BM. This act of dehydration provokes the keratinocytes to swell and to accelerate the synthesis of HA will increase, and the HA will fill out the distended intercellular space between the epidermal cells. Such a mechanism could lead to an impaired function of the adhesive proteins as an impaired epidermal barrier function, which is suggested, to be constituted by epidermal tight junctions and a mixture of lipids in the intercellular spaces of the stratum corneum (Kummi et al., 2001). These findings support an earlier report showing that an abnormal permeability barrier persists over hypertrophic scars and keloids long after the re-epithelialization of the wounds is completed (Suetake et al., 1996). Such speculations regarding the pathophysiology of keloids
have not been put forward earlier. However, these morphometric changes give support to and a theoretical explanation of the benefit of a treatment strategy with hydration and occlusion, which not only improves the clinical symptoms but also permits an improvement of the scars’ morphological properties (Perkins, 1982; Sawada et al., 1998). Regarding a possible dermal–epidermal relationship (Nowinski et al., 2002; Phan et al., 2002), these epidermal changes could implicate an impaired regulatory function on the collagen formation, emanating from the pathophysiological characteristic of the keloid.

Regarding the clinical characteristics of the abnormal scar with reddening and itching, the mast cells and macrophages have been suggested to be intimately involved in the formation of keloids and hypertrophic scars. An increase in the number of mast cells has been reported in these scar tissues (Kischer et al., 1989). Mast cells release a plethora of substances that intensifies the inflammatory response in the injured area, considered important in regulating the normal wound healing process (Noli and Miolo., 2001, Diegelmann et al., 2004).

In paper IV, mast cells were quantitated; few were noted in the PD and RD both in keloids and in hypertrophic scars, compared with those in mature scar and the normal skin. This was a surprising result, suggesting the possibility of other mechanisms than mast cells to cause the reddening and itching in the abnormal scar.

**HA in skin in diabetes mellitus**

Many patients with type-1 diabetes have restricted mobility affecting their palms, called LJM, combined with a thickened, waxy skin. This combination of symptoms, called cheiroarthropathy is associated with appreciably increased risk of microangiopathy (Rosenbloom et al., 1981). This diabetic microangiopathy of the skin includes morphologic
and functional changes with thickening of the capillary membranes, and enhanced collagen deposition around papillary microvessels (McMillan., 1966). Since the biochemical change in the skin in type-1 diabetes has not yet been identified, but is assumed to involve the ECM, we wanted to investigate the distribution of HA in the affected skin in an attempt to correlate any change in the severity of the disease. Our findings demonstrated a modified ECM composition, with changes in the distribution of HA in the diabetic skin suggesting that pathophysiological events are not related exclusively to the microvasculature.

We found significantly less HA localized in the PD of the patients most severely affected. Furthermore, the epidermis of the diabetic skin was thicker and stained more strongly for HA than the normal skin. This compartmentalization could be one explanation why the analysis of the total amount of HA showed no significant difference between the diabetic patients and the healthy controls. The thickening of the epidermis, with intensitied HA staining, and less HA in the PD, resembles the HA staining pattern in keloids.

HA is recognized not only as an important component of the structural organization of the skin with its hydrophilic capacity, but also as involved in cell migration, angiogenesis, immune reactions and phagocytosis. A loss of HA from the superficial dermis could have serious consequences for the skin’s integrity, as well as in connective tissues of other organs where similar events occur. This finding is most certainly the consequence of a structural change in the ECM and collagenous tissue, which should occur parallel with the development of microangiopathy. Increased glucose concentration in the media in which normal fibroblasts are grown in culture influences the amount, the type, and possibly the glycosylation of collagen secreted by them (Ville and Powers., 1977). When evaluating the general status of diabetic patients, all changes in the ECM should be regarded as important,
HA in tumour progression

HA affects the physical environment of cells and has been suggested to promote cell migration through its cell surface receptors. HA expression is often increased in malignant tumours, and is assumed to be associated with the invasive potential of various tumour types (Knudson et al., 1989; Toole, 1991). Several functions of HA in cell migration are described, including a weakening of the attachment to the adhesive substrata, a facilitation of the partial detachment, and the creation of a hydrated pathway that may facilitate the invasion of tissues by separating cellular and fibrous barriers (Toole, 1997). The presence of tumour-associated HA may therefore play an important role in facilitating tumour cell invasion of certain host connective tissues (Knudson et al., 1989).

In paper III, we have described the relationship between HA and various types of BCC tumours. It was found that the proliferative activity of BCC cells is associated with an increased expression of HA in the tumour stroma. In the superficial and nodular BCC types, often depicted as slow growing tumours, the HA pattern of the tumour stroma resembled that of PD in normal skin. Paradoxically, this stromal reaction with intense HA staining may represent a highly developed protective mechanism generated by the healthy tissue against the tumour cells. It can be debated whether enrichment with tumour associated HA could serve as a favourable or unfavourable prognostic factor (Knudson et al., 1989; Pirinen et al., 1998).

In the infiltrative BCC, the staining intensity for Ki-67 and PCNA of the tumour islands evidenced an intense proliferation, despite a weak staining of HA. The stroma surrounding the tumour cells showed intense HA staining. However, in the deeper, infiltrating parts of the tumour the presence of HA decreased and at some distance the adjacent connective tissue was almost devoid of HA. The modification of the tumour-associated connective tissue indicates a
close relationship between the tumour cells and the adjacent matrix. This alteration of the
neighbouring connective tissue could be of utmost importance in the pathophysiology of
tumour infiltration. We regard the modified stromal tissue as a prerequisite for tumour
progression, which also could cause a recurrence of the tumour when treating it surgically, as
these changes can reach much further than the clearly visible extension of the tumour cells.
One can assume that the loss of ECM components, paving way for the tumour invasion,
depends on an increased proteolytic activity (Leake et al., 2003), governed from the tumour
cells.
Clinical considerations

The pathophysiological implication of the observations in papers I-IV could provide us with increased knowledge and understanding of the biological mechanisms behind the formation of skin tumours, abnormal scars such as keloids and hypertrophic scars and the impaired function of the diabetic skin.

The distribution of HA in these different tissue samples representing various pathological conditions, all associated with an impaired wound healing, show various differences but mainly some similarities that indicate that they might share a pathophysiologic background.

The role for HA in the papillary dermis is vital for the structural organization and hydrodynamic constitution of the skin. A decrease can alter the architecture of the epidermis, provoked by a disturbed basement membrane function with increased leakage from the skin and an impaired function.

An abundance of thin calibre collagen content characterizes keloids. This could emanate from a sequential error with uncontrolled and disorganized production of immature collagen that never undergoes maturation.

Considering an interconnecting relationship with a down regulatory function of the keratinocytes on the collagen synthesis, the epidermal changes could be of importance for the pathogenesis of the keloid formation.

Stromal reaction in basal cell tumours could represent a highly developed protective mechanism generated from the healthy connective tissue against the adverse tumour cells and should be considered as a good prognostic sign. However in the infiltrating basal cell tumour this stroma becomes deranged and serves as a prerequisite for further tumour growth.
CONCLUSIONS

The differing distribution of HA in scar tissues contributes to their different clinical characteristics

This difference should be used as a diagnostic criteria to distinguish the scar types from each other

Specific morphologic observations of the scar tissues provide us with novel information of their pathophysiology and will also explain some of their different clinical characteristics

A reduction of the HA content in the papillary dermis leading to an impaired hydrodynamic capacity and a disturbed composition of the basement membrane with leakage of water from the dermis could be the mechanism behind the thickening of the skin in severely affected diabetic patients and in the abnormal scar

Parallel to the structural disorganisation observed in the connective tissue of the skin in diabetics similar changes should be expected concerning the connective tissue of other internal organs

Stromal reaction in basal cell tumours should be considered together with the characteristics of the tumour cells when evaluating the malignant behaviour and the choice of treatment of the tumour
SAMMANFATTNING PÅ SVENSKA

Hudens normala läkningsförmåga är en välreglerad men invecklad process som innefattar olika molekylära händelser vilka förväntas resultera i ett återskapande av normal funktion hos den skadade vävnaden. För att undvika en försenad eller abnormal läkningsreaktion är det av stor vikt att förstå denna process.

Hyaluronan (HA) är en vanligt förekommande glykosaminoglykan (GAG) som har stor betydelse för kroppens bindvävsstrukturer och vävnadsvätskor–det sk extracellulära matrix (ECM). HA har stor vätskebindande förmåga vilket bidrar till att vävnadsmiljön medger blå celltillväxt och transport. Sådana egenskaper har uppfattats ha en särskild betydelse vid sårläkning hos foster, som läker sina sår utan förekomst av synliga ärr. HA molekylen återfinns överallt där celldelning, vävnadstillväxt eller reparation sker.

Målsättningen med denna avhandling är att beskriva förekomsten och betydelsen av HA för normal hud, samt hur förekomsten av HA kan förändras vid olika sjukdomstillstånd med fördöjd eller rubbad sårläkning som följd.

Vi har studerat keloider och hypertrofiska ärr, basalcells cancer samt hud från patienter med insulin- beroende typ-1 diabetes mellitus. Analyser har utförts av vävnads prover av ärr och tumörvävnad från 50 patienter som opererats samt hudbiopsier från diabetespatienter och friska försökspersoner. Undersökningar av dessa olika vävnadsprover har skett med hjälp av ljusmikroskop och elektronmikroskop.

Resultaten visade att HA i den normala huden framförallt förekommer i den övre läderhuden, papillära dermis (PD). En mindre ansamling av HA finns även mellan cellerna i överhuden, epidermis. I de olika ärrvävnaderna fanns HA i olika grad; det morga äret (mature scar) uppvisade en likartad förekomst av HA som den normala huden. Mest avvikande var HA i keloiden där övre läderhuden (PD) saknade HA. I motsats innehöll den djupare läderhuden rikligt med HA. Det hypertrofiska äret intog en mellanständning avseende HA förekomst mellan keloid och mature scar. Detta fynd var betydelsefullt och undersökt vidare med elektronmikroskop där framförallt kollagenets struktur analyserades. Även vidhållningsstrukturer mellan basalmembran och basallameller liksom mellan keratinocyteret var förändrade i fra keloiden. Dessa avvikelser förmodas bidra till uppkomsten av en försämrad barriärfunktion hos basalmembranet (BM) med läckage av vävnadsvätska upp till överhuden som i sin tur leder till en störning av överhudens funktion med förtjockning.

I huden hos diabetespatienterna observerades en minskad förekomst av HA i övre läderhuden (PD), liksom ett ökat HA innehåll i en förtjockad överhud. Liksom hos de abnorma ären kan dessa fynd tyda på en nedsatt barriärfunktion med läckage av vävnadsvätska till epidermis. Denna ändrad struktur i huden hos diabetespatienter skulle delvis kunna förklara en försämrad sårläkning hos dessa patienter.

Vid basalcells cancer omges tumörcellerna sk tumörstromat av rikligt med HA. De ytligt växande typerna som karakteriseras av långsamt växtsätt uppvisade ett HA innehåll i basalcells-tumörstromat som inte skiljde sig från frisk bindväv i frisk läderhud. Den basalcells cancer som växer infiltrativt på djupet, uppvisade ett helt annat HA mönster. En
minskad mängd HA förekom i tumörstromat och ännu längre ut i bindväven förekom ingen HA alls.
Vi tolkar dessa observationer som att bindväven som omger tumörcellerna sk tumörstromat, genomgår en förändring av sin sammansättning under påverkan av tumörcellerna, vilket kan avläsas som en minskad närvaro av HA. Denna rubbning av bindväven har förmodligen styrt av tumören med hjälp av okänd mekanism. Bindväven kan här ha omreglerats, från tidig försvarsmekanism med uppgift att bekämpa de främmade tumörcellerna - till att bli en viktig tillväxtfrämjande struktur som tumörcellerna utnyttjar för vidare invasion i vävnaden. Dessa observationer är av betydelse för såväl prognostisk som terapeutisk värdering vid behandling av de olika basalkellstumörerna.

Dessa studier visar att förekomsten av hyaluronan (HA) i huden förändras vid olika sjukdomstillstånd och speglar vävnadens vitalitet. Kartläggningen av HA skulle kunna ha stor betydelse för bedömning av sårläkningsprocessen, om den går i rätt eller fel riktning – en sårläkningsmarkör. Detektionen av HA borde således inkluderas som ett komplement i granskningen av de hudbiopsier vi idag utför när man utvärderar eventuell förekomst av inflammation eller cancerceller som orsak till olika kroniska hudbesvär och sår.

Den kliniska nytta med denna metod skulle kunna vara betydelsefull för att förbättra vår diagnostik, liksom att följa olika sjukdomsförlopp som påverkar huden. Vår behandling av de abnorma, oönskade ärren; keloider och hypertrofiska ärr, skulle bli mer precis och effektiv, med en specifik metod att kunna skilja dem åt. Tillämpningen av HA markören på hudbiopsin vid basalcellscancer borde vara användbar vid planeringen av radikalitet vid kirurgin. Studien av basalkellscancer indikerar nämligen att vi bör utföra vår kirurgi av tumörerna med större marginal för att få med den tumöromvandlade bindväven som kan innehålla omogna ”osynliga” cancerceller som annars kan tillväxa.
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