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Epidermal-dermal crosstalk during burn wound scar maturation

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CHAPTER 1

General aspects of wound healing and burn wound healing

1 GENERAL ASPECTS OF WOUND HEALING AND BURN WOUND HEALING

1.1 BURN INJURIES

Burn injuries are among the worst traumas which can happen to man. The larger a burn injury, the more severe the consequences and the higher the chance of an adverse outcome or even death. In The Netherlands each year 40,000 people visit a general practitioner for treatment of a burn wound and 1600 people require in-hospital care primarily for burns.
Approximately 80% of the burn accidents happen in or around the house, mainly in the kitchen. Scalds, usually due to hot water, are the most common cause of burns. Water at 60°C will create a deep dermal or full-thickness burn in three seconds, and at 70°C the same burn will occur in one second [1]. The temperature of freshly brewed coffee from a percolator is generally about 80°C, which is hot enough to cause a full-thickness burn in less than one second. Children are particularly at high risk to burns. Hot beverages, particularly coffee and tea, are the predominant cause of scald burns in children [2]. One study showed that 81% of the burn injuries in children under the age of 5 were due to scalds [3]. Cooking oil, when hot enough to use for cooking, may be in the range of 150-180°C and can consequently cause very severe burns.
Other causes of burns are fire, electricity, chemical substances and even sunshine.
In The Netherlands around 200 people die of their burn incident each year, mostly at the place of the accident. The case fatality rate of scald injury is low; instead most deaths occur in residential fires, commonly caused by careless smoking, by arson or by defective or inappropriately used heating devices [2]. Burn injury can also be self-inflicted, an attempt to commit suicide, most often caused by throwing and igniting a flammable liquid over the victim.

1.2 SKIN

The skin consists of two morphologically different layers that are derived from two different germ layers. The more superficial layer, the epidermis, is a specialized epithelial tissue derived from surface ectoderm. The deeper and thicker layer, the dermis, is composed of vascular dense connective tissue derived from mesenchyme [4].

1.2.1 Epidermis

In recent years the concept of the epidermis has gradually been changing from that of an innocent bystander, that in its stricted sense protects the body from the loss of fluids and electrolytes, and the penetration of harmful substances, into that of an active participant in several important processes. The epidermis is formed of stratified squamous epithelium. It adheres to the dermis partly by the interlocking of its downwards projections (epidermal ridges) with upward projections of the dermis (dermal papillae). Desmosomes and hemidesmosomes link the epidermal cells to each other and to the basal membrane [5]. The epidermis does not contain blood vessels. In the deepest cell layer (basal cell layer), the cells are dividing. The basal layer contains two types of proliferative keratinocytes: stem cells, which form about 10% of the immature epidermal cells [6], and have unlimited self-renewal capacity, and transit amplifying cells, cells that after a few cell cycles will finish proliferation and start terminal differentiation and move to more superficial layers [7,8]. The basal stem cells are distinguished from other basal cells by their characteristic expression of high levels of the adhesion molecule α6, a member of the integrin family, and low levels of a proliferation-associated cell surface marker recognized by the monoclonal antibody 10G7 [6]. Integrins mediate adhesion of the keratinocytes to the underlying extracellular matrix, and also regulate the initiation of terminal differentiation [9,10]. A newly formed cell moves to the surface, passing through the spinous and granular cell layers and is dead by the time it arrives in the horny cell layer. As the epidermal keratinocytes move through the epidermis after loosing their attachment to the basal lamina, they undergo a process of differentiation to produce the stratum corneum. The journey from the basal layer to the surface takes about 60 days [11].
Keratinocytes make up about 85% of cells in the epidermis, but three other cell types are also found there: melanocytes, Langerhans cells and Merkel cells [11].
Melanocytes migrate into the basal layer of the ectoderm from the neural crest. They are the only cells that can synthesize the pigment melanin. Melanin is made within melanosomes and is taken up by neighboring keratinocytes. It protects the underlying tissue against ultraviolet radiation damage by absorbing and scattering the rays and by scavenging free radicals.
The Langerhans cell belongs to the dendritic cell family. It plays a key role in many immune reactions. It takes up exogenous antigen, processes it and presents it to T-lymphocytes either in the skin or in the regional lymph nodes.
Merkel cells are located in or near to the basal layer of normal human epidermis and probably act as transducers for fine touch.

1.2.1.1 Keratins

The stratum corneum cells (corneocytes) have lost their nuclei and other recognizable organelles and comprise 65% insoluble, cysteine-rich, disulfide cross-linked proteins [12], called keratins, derived from the Greek word *keras* (horn). The series of changes

whereby keratin filaments aggregate into bundles through the action of a protein called filaggrin have thus been termed keratinization. Epidermal differentiation involves the synthesis of a highly insoluble cornified envelope [13]. Epithelial keratins are expressed in a cell-specific and tissue-specific manner [14]. They play a role in maintaining the shape, the internal organization and the movement of the cell. They fall into two groups: the basic keratins (numbered 1 to 8) and the acidic keratins (numbered 9 to 19). For filament formation one member of the basic and one of the acidic keratins must form a pair.

In the skin, the major keratins in the basal cell layer are keratins 5 and 14. In the epibasal cell, one cell away from contact with the basal lamina, the keratinocytes synthesize a new keratin pair: keratins 1 and 10, a characteristic of epidermal differentiation [13].

In hyperproliferative circumstances, like wound healing, the patterns of keratin expression change. In the suprabasal compartment, the keratin pair 1 and 10 are replaced by keratins 6 and 16 [15]. The induction of keratin 16 occurs within 6 hours after injury. One function of keratin 16 that has been suggested, could be enabling the differentiating keratinocyte to become competent for re-epithelialization [16]. This is in contrast to keratins 5 and 10, which function to impart mechanical strength and to establish the cytoarchitecture in epidermal cells [17].

Filaggrin and transglutaminase are both expressed in differentiated keratinocytes. As the keratinocyte moves upward into the stratum corneum, filaggrin is cleaved from profilaggrin by specific enzymes. It interacts with keratin filaments causing them to aggregate and form the interfilamentous matrix in the corneocyte [13]. During the process of cornification specific proteins synthesized in the granular layer assemble under the cell membrane and are cross-linked into an insoluble cornified cell envelope. The cross-linking is catalyzed by keratinocyte transglutaminase [18]. This enzyme is expressed specifically in the granular layer. Substances that promote keratinocyte differentiation induce the expression of transglutaminase, whereas inhibitors of differentiation inhibit the expression of this enzyme [5].

1.2.2 Dermis

The dermis is situated between the epidermis and the subcutaneous fat. It supports the epidermis structurally and nutritionally. Its thickness varies, being greatest in the palms and soles and the least in the eyelids. With aging the dermis becomes thinner and loses elasticity. The dermis interdigitates with the epidermis, so that the upward projections of the dermis, the dermal papillae, interlock with downward ridges of the epidermis, the rete ridges. Like all connective tissue, the dermis has three components: cells, fibers and amorphous ground substance [11].

The bulk of the dermis consists of a network of fibers, principally collagen, but also reticulin and elastin, packed in bundles. Those in the papillary dermis being finer than those in the deeper, reticular dermis. The amorphous ground substance of the

dermis consists largely of two glycosaminoglycans: hyaluronic acid and dermatan sulfate, with smaller amounts of heparan and chondroitin sulfate [11]. The function of the ground substance is that it binds water, in order to allow nutrients, hormones and waste products to pass through the dermis. It also is a lubricant between the collagen and elastic fiber network during skin movement and it provides bulk, allowing the dermis to act as a shock absorber.
The dermis also contains muscles, both smooth and striated, and vessels [11]. Blood vessels are not only necessary for feeding, but also for regulation of the body temperature. Besides that, blood vessels play a role in allowing transendothelial migration of immune cells, by expressing adhesion molecules that bind to receptor molecules on the immune cells [19]. This transmigration process allows immune cells into the tissue to do their surveillance work.
Lymphatic vessels, beginning as blind-ended capillaries in the dermal papillae, pass to either the superficial lymphatic plexus in the papillary dermis, or to the deeper horizontal plexuses. They play a role in water homeostasis of the dermal tissue and also in the recirculation of immune cells.

1.2.2.1 Cells of the dermis

The main cells of the dermis are the fibroblasts, besides small numbers of macrophages, dendritic cells, T-lymphocytes and mast cells [11].
The main role of the fibroblast is the synthesis of collagen, reticulin, elastin, fibronectin, glycosaminoglycans and collagenase. The macrophage is mainly a phagocyte and can destroy bacteria. It can also produce a variety of cytokines and growth factors. Dendritic cells are antigen presenting cells. They have a role in processing and presentation of antigens to other immune cells. T-lymphocytes are normally present in the skin. They are part of the skin immune system, helping to prevent the penetration of infectious agents and to modulate the responses to foreign antigens.
Mast cells can be stimulated by antigens, complement components, nerves or other substances to release their mediators, including histamine, prostaglandins, leukotrienes, tryptase, cytokines and chemotactic factors for T-lymphocytes, eosinophils and neutrophils. Macrophages, dendritic cells, T-lymphocytes and mast cells are, by virtue of their multiple potent biological properties, important regulatory cells in the process of wound healing [20-24].

1.2.2.2 Neuropeptides

The skin is supplied with sensory nerve fibers. Their afferent function is to signal the presence of nociceptive stimuli to the central nervous system, where they will be interpreted as pain, itch, etc. Their efferent function is to contribute to the local defense against harmful stimuli. Neuropeptides are involved in both functions [25]. They are synthesized and released predominantly by small unmyelinated afferent neurons (C-fibers) and small myelinated Aδ-neurons (Aδ mechanoheat receptors) [26]. A large proportion of the afferent nerves express peptides, including Calcitonin

gene-related-peptide (CGRP), Substance P (SP), Neurokinine A (NKA) and Vasoactive Intestinal Peptide (VIP) [27]. The skin is also supplied with parasympathetic and sympathetic nerve fibers. Blood vessels and sweat glands are regulated by sympathetic fibers. Neuropeptide Y (NPY) is expressed in a large population of the sympathetic neurons [27].
Nerve fibers are in close relationship with Langerhans cells and mast cells in the human skin [28,29]. Both cell types are involved in modulation of immune reactions, and these functions are regulated by specific neuropeptides [30-33].
Skin innervation plays an important role in mediating normal wound healing. Released neuropeptides may modulate key aspects of normal wound healing, such as cell proliferation, cytokine and growth factor production, and neovascularization [34].

1.3 WOUND HEALING

Wound healing is the consequence of a continuous sequence of signals and responses in which epithelial, vascular, hemopoietic and connective tissue cells come together outside their usual domains, interact, repair the damage and having done so turn back to their normal functions [35]. The purpose of wound healing is to restore the functions of the skin, such as protection of the body against harmful environmental entities, prevention of entry of microorganisms and loss of plasma, the regulation of body temperature, the processing and interpretation of environmental information through the neurosensory system and a social-interactive function [36].
Vertical cutaneous injuries, such as surgical incisions which have a minimal loss of tissue, will essentially heal through the formation of a blood clot, rapid epithelialization, and fibroblast proliferation. Progressive collagenization and increased strength, which reach normal levels within weeks, will complete the healing process and leave discrete scarring, in most cases. On the other hand, cutaneous wounds with a predominant horizontal loss of tissue, like burn injuries, exhibit a healing which proceeds through a series of complex, biological mechanisms according to the extent and level of the involved structures. A burn wound becomes ischaemic, hypoxic, and highly edematous. Therefore burn wound healing follows a much slower course compared with the healing of other types of wounds.
The wound healing response can be divided into three distinct, but overlapping phases: 1) hemostasis and inflammation; 2) dermal and epidermal proliferation; and 3) maturation and remodeling (Figure 1) [37], which will be discussed separately in the next paragraphs.

1.3.1 Hemostasis and inflammation

The first response after disruption of tissue integrity, is to control the damage produced to the vascular system. A hemorrhage means immediate danger to the body, which reacts with prompt vasoconstriction, platelet aggregation and activation of the

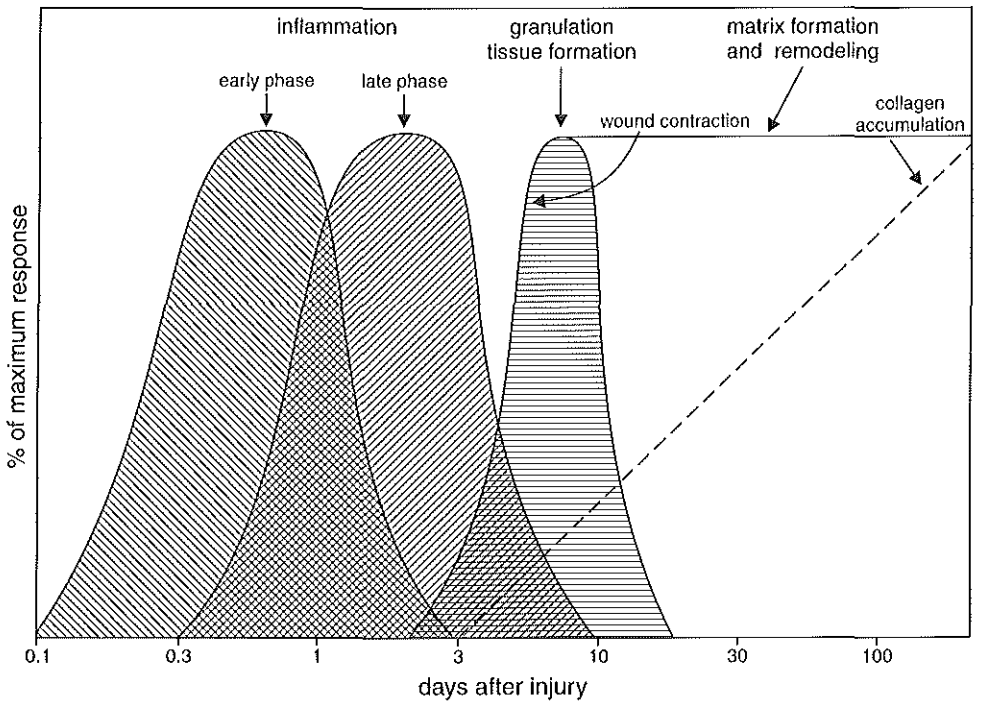

Figure 1. The phases of wound healing. The amount of granulation tissue parallels the amplitude of wound contraction. Collagen deposition starts early after first granulation tissue formation and increases gradually during matrix formation and remodeling. (*modified from:* Clark, R.A.F. Cutaneous tissue repair: Basic biologic considerations I. J Am Acad Dermatol 1985;13:701-725.)

coagulation system. The initial response to trauma involves a transient 5- to 10-minute period of intense vasoconstriction that aids in hemostasis. This is followed by active vasodilation that usually becomes most pronounced approximately 20 minutes after the injury and is accompanied by an increased capillary permeability. Histamine is believed to be a key chemical mediator responsible for the vasodilation and the changes in vascular permeability [38]. Shortly after wounding, platelet adhesion occurs at the site of the trauma. Platelets function to initiate the formation of a clot that helps to achieve hemostasis. The contact between the extracellular matrix and platelets, as well as the presence of thrombin and fibronectin, results in the release of growth factors and vasoactive substances such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF- β), fibroblast growth factor (FGF), epidermal growth factor (EGF), bradykinin, prostaglandins, prostacyclins, thromboxane, histamine and serotonin [39]. Platelet degranulation also initiates the complement cascade with the formation of C3a and C5a, which are potent anaphylatoxins promoting the release of histamine by basophils and mast cells. This highly coordinated series of events ultimately leads to uncomplicated wound healing [40,41].
Granulocytes, in a rapid response to signaling by platelets and also through factors

produced by the activation of the complement system, form the first line of defense against local bacterial contamination. In the absence of bacterial contamination, the granulocyte has been claimed to be non-essential to the wound healing process. Usually within 24-72 hours, the granulocytes are gradually replaced by monocytes that acquire the characteristics of tissue macrophages and become central coordinators of the inflammatory and repair process. Macrophages not only help to clean the wounded area of undesirable debris and bacteria, but they also promote the build up of the new connective tissue. Through growth factors and cytokines like TGF-β, PDGF and EGF, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interferon-γ (IFN-γ), through enzymes like collagenase and arginase, and through prostaglandins, they regulate the matrix synthesis by affecting either fibroblast chemotaxis or proliferation, or collagen synthesis [42]. Macrophages also play a role in mediating angiogenesis [43] and in the recruitment and activation of other immune cells [44].
It has been demonstrated that activated T lymphocytes, following the influx of granulocytes and macrophages, enter a wound area by day 4 or 5 and become important modulators of the healing process [42]. An intact T-cell immune system is essential, at least indirectly, for a normal healing outcome [23,42].
Other cells, like mast cells, and their major protease, chymase, also play a role in the wound healing process by promoting capillary outgrowth and collagen formation [24,45]. It has also been suggested that dermal dendritic cells participate in wound repair by initiating the inflammatory response and by stimulating epithelial proliferation and restoration of epithelial architecture [46].

1.3.2 Dermal and epidermal proliferation

1.3.2.1 Granulation tissue

Granulation tissue forms the framework for the repair process and supports the resurfacing epithelium. It begins to form a few days after the injury. The name *granulation tissue* is derived from the granular appearance of newly formed blood vessels in the new tissue. One of the main developing features of granulation tissue is the formation of a capillary network. This network is formed by endothelial cells that proliferate from intact venules close to the wound. The primary stimuli for neovascularization are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [47]. When VEGF is experimentally removed from wounds, the granulation tissue nearly completely disappears [38].
Besides new blood vessels, granulation tissue consists of macrophages, fibroblasts and extracellular matrix [48]. Fibroblasts first appear in significant numbers in the wound on the third day after injury and achieve peak numbers around the seventh day [49]. They migrate into the wound site from the surrounding tissue, attracted by cytokines like IL-1, TNF-α, TGF-β and PDGF, that were produced and released initially by platelets and subsequently by macrophages and lymphocytes.
Fibroblasts synthesize many of the components of the extracellular matrix. Early in

granulation tissue, fibroblasts deposit a matrix of fibronectin and hyaluronic acid which promotes cell migration and proliferation. Fibroblasts also produce glycosaminoglycans that form the ground substance of the matrix [38]. Fibronectin precedes the appearance of collagen, which is also produced by fibroblasts and which aids in increasing the tissue tensile strength and resilience [50,51]. Fibronectin gradually disappears as soon as mature collagen bundles are formed [52].
Collagen is initially synthesized as a monomer. After it has been secreted into the extracellular wound environment, it polymerizes into collagen fibers. These collagen fibers are covalently cross-linked to increase their tensile strength. The signal that stimulates production of collagen appears to be a combination of growth factors, which are secreted as the result of both hypoxia and products of anaerobic metabolism such as lactic acid [53].
The cellular and molecular structures that initially broke down and eliminated the debris of wounded tissue gradually change function and proceed to a new task, the build up of new connective tissue (cells and extracellular matrix). The reconstructed connective tissue will first serve as a provisional instrument of tissue continuity and later as a permanent fibrous bridge between the edges of the wound (scarring).

1.3.2.2 Re-epithelialization

Within hours after injury, epithelial cells from the wound margins or adnexal structures will initiate a series of specific mechanisms, directed at covering the denuded surface. Re-epithelialization and granulation tissue formation take place simultaneously.
The early phases of the re-epithelialization process are marked by the migration of epithelial stem cells from the margins of the wound and from residual hair follicles and eccrine ducts [54]. A main source of epithelial stem cells is considered to reside in the outer root sheath of the hair follicle [7], explaining the observation that when the hair follicles are destroyed as a result of the injury, re-epithelialization is greatly retarded.
The migration of epithelial stem cells is followed within 24-48 hours by the proliferation of new cells behind the migrating front to provide an additional population of cells to cover the gap [55]. The rate of epithelial resurfacing varies from 0.1 mm to 1 mm per day, highly depending on local conditions. The initial stimulus is largely unknown, but several mechanisms have been suggested. The migration may be induced by loss of attachment to neighboring damaged cells, active contact guidance, or the presence of a gradient of soluble mediators. Epithelial proliferation is assisted by growth factors [56,57]. In particular, EGF, TGF-β, PDGF, bFGF, keratinocyte growth factor (KGF) and insulin-like growth factor (IGF-1) have been shown to promote epithelialization [42,58,59].
Through phenotype modulation, the epidermal basal cells lose their desmosomes and hemidesmosomes links to the basal membrane. The keratinocyte intracellular tonofilaments are retracted, form peripheral actin filaments (pseudopodia) and ex-

press fibronectin receptors [57,60]. The cells start their movement over the provisional matrix while secreting plasminogen activators and collagenases in order to open a way through the tissue. During re-epithelialization, keratinocytes use specific cell receptors, integrins, for binding to connective tissue components, but discussion on integrins is beyond the scope of this thesis.
The mechanisms involved in the epithelial movement are diverse and controversial and include the "leapfrog model", whereby cells above and behind the leading cell stream over the latter to attach to the wound bed [61], and the model of the formation of a chain of cells which advances while individual cells maintain their original position in the chain [57], or possibly a combination of both.
The epithelial migration ceases when the advancing epithelium meets its counterpart growing from the opposite direction. The cells which were migrating in a lateral motion across the wound surface then regain the normal vertical direction of differentiation (the keratinocyte maturation process) toward the surface and give rise to cornified cells.

1.3.3 Maturation and remodeling

When the migrating epithelium has completed resurfacing the new connective tissue matrix, the formation of granulation tissue stops. The signals involved in this suppression are unknown [35,62]. The granulation tissue now becomes true scar tissue. In the maturation phase the scar flattens, softens and blanches. The main feature of the maturation phase is the deposition and remodeling of collagen in the wound. From a clinical viewpoint this is the most important phase of healing because the rate, quality and total amount of matrix deposition, i.e. collagen, determine the strength of the scar. The tensile strength, which initially is low, increases in subsequent weeks as the scar matures, although it never regains the strength of the original tissue [63]. Collagen remodeling depends on both continuous synthesis and breakdown of collagen. Collagen breakdown begins early in the wound healing process. The degradation of wound collagen is controlled by a variety of collagenases derived from granulocytes, macrophages, keratinocytes and fibroblasts [48]. These specific enzymes are able to degrade the triple helical structure of the collagen at specific sites [39]. The expression and activity of collagenases is tightly controlled by cytokines. Many cytokines, like TGF-β1, not only stimulate collagen synthesis, but also inhibit the production of matrix proteinases, like collagenase, and stimulate the production of proteinase inhibitors [64]. Also the matrix itself influences the outcome of the wound healing process, as it can regulate fibroblast parameters such as migration [39].
The remodeling of the collagen meshwork includes reorientation of the collagen fibers in response to mechanical stress. The latter is a characteristic feature of normal scarring in which collagen bundles of the dermis run in a distinct parallel orientation with respect to the surface of the skin.

The mature scar is relative acellular and avascular. The fibroblasts and endothelial cells involved in the wound healing process disappear mainly by apoptosis, i.e. programmed cell death [62]. The inflammatory cells present in the early phases of the wound healing process probably also disappear by apoptosis [65]. Apoptosis in fibroblasts and endothelial cells starts at the time of wound closure. If the wound is closed with a skin graft, apoptosis starts within 6 hours [66], suggesting that there is an interaction between epidermis and dermis in initiating apoptosis in cells of the extracellular matrix. Little is known about the mechanisms involved in the induction of apoptosis. In vitro studies suggest that the expression of the c-myc proto-oncogene and the interaction of the apoptosis signal transducer Fas with Fas ligand play a role in fibroblast apoptosis [67,68]. Apoptosis signals can also induce collagenase activity. It was found that p53, a transcription factor with growth-suppressing functions, binds to the promotor of collagen type IV collagenase, and thus increases collagenase activity [69].
Clinically, the original redness, elevation, and firm consistency of the new scar tissue gradually evolves into a mature scar, with pale, flat, soft scar tissue which is at the same level as the adjacent skin surface. This phase of maturation and tissue remodeling can take as long as 2 years.

1.4 BURN WOUND HEALING

1.4.1 Effect of heat on cells and tissue

Thermal energy is a manifestation of random molecular kinetic energy. This energy is easily transferred from high energy molecules to those with a lower energy status during contact, for example in living tissues. Both the temperature and the time period for which this temperature is sustained determine the degree of damage to a cell [1]. At temperatures between 40 and 44°C, various enzyme systems begin to malfunction, and early denaturation of protein occurs. Cellular functions become impaired, one of which is the membrane Na^+ pump. This results in a high intracellular Na^+ concentration and concomitant swelling of the cell. As the temperature increases, damage accumulation outruns the cell's inherent repair mechanisms and leads to eventual necrosis. The production of oxygen free radicals is part of this damage process. These highly reactive molecules are capable of promoting further cell membrane abnormalities, leading to cell death [70].
If the heat source is suddenly withdrawn, damage accumulation will continue until the cooling process brings cells back down to a normal temperature range. Cooling determines the difference between cell survival and cell death.
As the temperature increases, protein coagulation takes place, which causes destruction of the protein architecture. New aberrant bonds are formed, creating macromolecules not similar to the original structures. The cell necrosis is complete, usually beginning at the skin surface, where the heat energy was absorbed most directly,

extending downward. This zone is called the *zone of coagulation* [71]. The *zone of stasis* lies deeper and peripheral to the zone of coagulation. In this zone the damage is less and most cells are initially viable. However, the blood flow becomes progressively impaired and finally stops. This development of ischaemia results in necrosis of the already affected cells. Peripheral to this zone lies the *zone of hyperemia*, which is characterized by minimal cellular injury and prominent vasodilatation with increased blood flow, due to vasoactive mediators that were produced as part of the inflammatory response. Complete cellular recovery usually happens in this zone [71].

1.4.2 Burn wound depth

Burns can be divided into different categories, based on the depth level of the tissue damage [36]. First degree burn injury involves damage only to the epidermis and is rarely clinically significant other than being painful. The involved area is initially erythematous due to vasodilatation. Eventually desquamation happens, but this is followed by complete scarless healing within 7 days.
Second degree burns are partial-thickness by definition and are further categorized into superficial and deep. In superficial injuries, the epidermis is destroyed as well as varying superficial portions of the dermis. These lesions are usually painful because the nerve endings in the mid and superficial dermis survive. Blistering is often present. Healing generally occurs rapidly and completely through migration to the surface of epithelial stem cells which survive in deeper portions of the hair follicles as well as the sweat and sebaceous glands [5]. Relatively little scarring occurs in a superficial injury, due to the limited inflammatory phase, which is cut short by wound closure (re-epithelialization) occurring within 2 weeks. In deep partial-thickness wounds most of the dermis is destroyed and only in the deepest parts of the hair follicles, sweat and sebaceous glands few epithelial cells remain. As the epithelial cells have to migrate from the depth, and due to the loss of stem cells, re-epithelialization is greatly retarded in these wounds.
Heat kills the superficial nerve endings, so the wound is relatively insensitive. As the deeply situated pressure receptors may survive, pressure sensation can still be present. Blistering is usually absent due to the thicker adherent overlying eschar which prevents the lifting by the edema. Due to the long period before wound closure, the inflammatory phase is prolonged, which gives rise to extensive collagen deposition and consequently abundant scar formation.
In third degree or full-thickness burns necrosis of the entire thickness of the skin occurs. As there are no epithelial appendages left, healing can only occur by re-epithelialization from the wound edges, or, in case of small wounds, by contraction of the wound edges. So third degree wounds are routinely treated with excision and skin grafting, serving as a source of new stem cells. As no nerve endings are left, this type of wound is insensitive.

1.4.3 Long-term problems

Apart from all kind of acute problems associated with a large trauma, a burn patient can also suffer from several long-term complications, due to the decrease of the normal functions of the skin or the affected body parts.
Physical complications include problems with thermoregulation in case of excessive loss of sweat glands, neurosensory malfunction of the skin and development of cancer in the burn scars, the Marjolin's ulcer [72]. Psychological and social problems are also common after, for example, burning and scarring of the face. Burning can

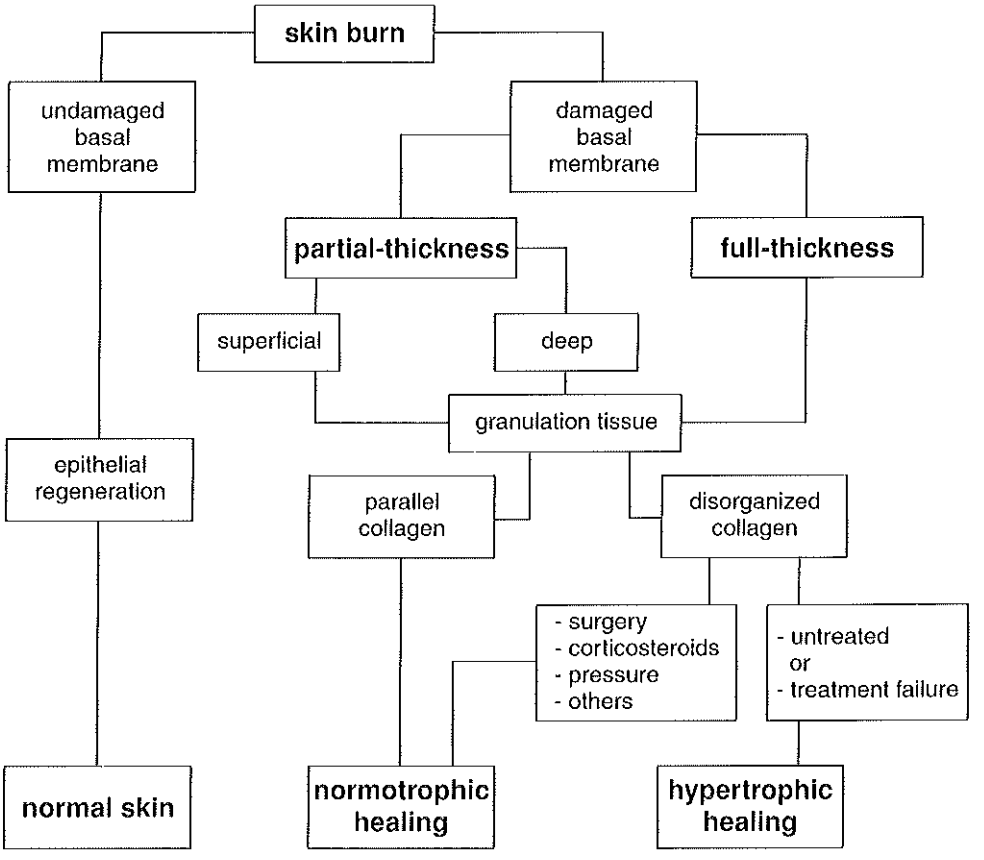

Figure 2. This figure shows the possible outcome of the wound healing process in spontaneously healed burn wounds. In case of an undamaged basal membrane the wound heals by epithelial regeneration. In untransplanted partial- and full-thickness wounds the regenerated collagen can be organized in a normal, parallel manner. This results in a normotrophic scar. Disorganized regeneration of the collagen will result in a hypertrophic scar if no treatment is applied and in case of treatment failure. (*modified from:* Linares HA. Pathophysiology of the burn scar. In: Herndon DN, ed. Total Burn Care. London: W.B. Saunders; 1996:383-397.)

cause a post-traumatic stress disorder. In The Netherlands one third of the burn patients suffers from a post-traumatic stress disorder 1 to 2 years after the accident [73].

1.4.4 Hypertrophic burn scars

A common complication of burns is the formation of a hypertrophic scar, a phenomenon unique to man (Figure 2) [74]. The lack of an experimental animal model of hypertrophic scars explains why there is almost no fundamental biological information about this distressing phenomenon, which occurs regularly in injuries of the reticular dermis, but with unpredictable and highly variable severity. The incidence of hypertrophic burn scars has been sporadically reported; the highest incidence, more than 50%, occurs in children under the age of 5 [75,76].
Hypertrophic scars are tumor-like lesions elevated above the level of the surrounding skin. The edges are usually prominent and end abruptly. They typically remain within the borders of the original wound. This is in contrast to keloids, which enlarge even beyond the margins of the original wounds [77]. Hypertrophic scars produce symptoms of burning, stinging and itching. The itching can be quite disturbing, and scratching may cause damage and bleeding of the recently healed surface. The origin of this specific form of itch is still unknown.
Histologically, the typical feature of hypertrophic healing predominantly resides in the reticular layer of the dermis. By the time a wound is completely re-epithelialized and the granulation phase is completed, the collagen fibers in both the papillary and the reticular dermis will have a predominant parallel orientation, characteristic for normotrophic scars. In hypertrophic scars, however, there is extensive collagen deposition and the collagen fibers have a tendency to run in a haphazard direction and show a whorl-like pattern [70]. Immunohistochemical examination has revealed that hypertrophic scars contain whorls of connective tissue in nodular structures containing α-smooth muscle actin-positive fibroblasts with small blood vessels and fine, randomly orientated collagen fibrils [78]. These structures never involve the subcutaneous tissue [70]. The epidermis shows the usual features of scarring; the epidermal ridges and dermal papillae are often absent or not as deep and as numerous as in normal skin [70].

1.4.5 Rating the burn scar

For the evaluation of burn scars the Vancouver Scar Scale can be used, as is shown in Table 1. This scale was developed to reflect the easily recognized characteristics of the burn scar. The components which are considered are vascularity, pliability and height. Vascularity is always altered in the early phase of wound healing, with active scars being hyperemic as a result of the increased angiogenesis and consequently blood supply. Initially, a scar is red or pink ("immature"), but after a period of time,

Table 1. The Vancouver Scar Scale for the assessment of burn scars, allowing objective comparison of the scars. *(from:* Sullivan T, et al. Rating the burn scar. J Burn Care Rehabil 1990;11:256-260.)

Grade	*Vascularity*	*Pliability*	*Height*
0	normal (color that closely resembles the color of the rest of one's body)	normal	normal (flat)
1	pink	supple (flexible with minimal resistance)	<2 mm
2	red	yielding (giving way to pressure)	<5 mm
3	purple	firm (inflexible, not easily moved, resistant to manual pressure)	> 5 mm
4	-	banding (rope-like tissue that blanches with extension of scar)	-
5	-	contracture (permanent shortening of scar producing deformity or distortion)	-

months to years, the scar matures, i.e. normalizes [70]. Vascularity is assessed by deciding on the amount of redness in the scar. Pliability relates to the functional mobility of the scar as related to contracture and the elastic texture of the scar. Height is related to the overall collagen content of the scar, as well as the relative edema of the tissue.

1.4.6 Etiological aspects of hypertrophic burn scars

The biological mechanisms responsible for the deviation of the normal healing process toward an excessive reparative response are largely unknown. Evidently, the most visible feature of a hypertrophic scar is the excess deposition of collagen, suggesting that the balance between collagen synthesis and degradation is out of control. The predominant cell present in hypertrophic scars is the fibroblast [77], the cell that is responsible for collagen synthesis. Comparison of the rate of proliferation of fibro-

blasts of keloids or hypertrophic scars and normal skin generally shows no significant difference [79,80]. However, apoptosis-inducing protein levels, such as Fas and IL-1 converting enzyme, are decreased, whereas the level of bcl-2 proto-oncogene was increased in fibroblasts of hypertrophic scars compared to those of surrounding non-injured skin, suggesting a desequilibrium in the signaling mechanism mediating programmed cell death [81]. It has also been suggested that the production of a mutant form of the p53 gene, which was found in cultured fibroblasts from skin lesions of patients with keloids, prevents induction of apoptosis in keloid fibroblasts, and thus may be a causal factor in the formation of keloids [82].

Excessive biosynthesis of extracellular matrix proteins by fibroblasts has been proposed as one of the potential contributing factors to the accumulation of excessive matrix. Indeed, comparison of hypertrophic scar fibroblasts and normal fibroblasts has demonstrated that hypertrophic scar fibroblasts showed a higher collagen type I and type III mRNA expression and produced more collagen than normal skin fibroblasts [83,84]. Fibroblast recruitment, proliferation and production of the extracellular matrix are influenced predominantly by the amount and the balance of the fibrogenic growth factors IGF, PDGF, TGF-β and bFGF [77,85]. Fibroblasts of hypertrophic scars also showed an increased synthesis of collagen in response to low doses of TGF-β1, compared with normal fibroblasts [86], possibly due to the overexpression of TGF-β receptors by the hypertrophic scar fibroblasts [87].

Excessive matrix accumulation may occur 1) in case of increased synthesis of extracellular matrix proteins or 2) in case of a reduction in matrix degradation. Reduced collagenase activity of hypertrophic scar fibroblasts may be responsible for excessive accumulation of collagen [88,89], possibly due to the activity of collagenase inhibitors such as α2-macroglobulin which was found in the extracellular matrix of the hypertrophic scars [90].

Glycosaminoglycans affect the ultimate physical characteristic of the mature collagen in the scar because of their intervention in the extracellular formation of collagen fibrils. They regulate the aggregation of collagen monomers [91]. There is evidence that the composition of glycosaminoglycans in wound healing and hypertrophic healing differs from normal skin [77,92-94]. While in normal skin the dermis shows a much greater amount of decorin than chondroitin sulfate, the opposite occurs in granulation tissue and hypertrophic scars [95], possibly because fibroblasts from post-burn hypertrophic scar tissue synthesize less decorin than normal dermal fibroblasts [96]. This has implications for the development of hypertrophic scarring, as decorin is involved in tissue reorganization and may also play a role in modulating the activity of fibrogenic cytokines [96]. Furthermore, the strong association between collagen and glycosaminoglycans in hypertrophic scars may prevent collagenase from breaking down collagen [97,98].

Although the predominant cell present in hypertrophic scars is the fibroblast, other cell types, like mast cells, may also be involved. Clinically, the release of histamine by mast cells likely contributes to the common patient complaint of itch, although the

absence of sufficient neutralization of the complaints with anti-histamines contradicts this possibility. However, mast cells are able to release many other substances, like chymase, tryptase, prostaglandins and leukotrienes, that can possibly influence the process of hypertrophic scarring, but which are not neutralized by anti-histamines.
Genetic factors have also been suggested in influencing hypertrophic scar formation. Black and Asian people are 10 times more susceptible to keloids and hypertrophic scars than whites [99]. People carrying the HLA-DR-β16 gene have a relative risk of 12 for developing hypertrophic scars after thermal injury [100].
Until recently, research on the pathogenesis of hypertrophic scarring was focussed on the dermal compartment. Research is now beginning to focus also on the epidermal part as it is known that hypertrophic scars most commonly occur after delayed re-epithelialization. Burn wounds that epithelialize in less than 2 weeks rarely develop hypertrophic scars. When a burn takes between 2 and 3 weeks to heal, however, one third of the wounds develop scar hypertrophy, and wound closure taking more than 3 weeks leads to a 78% rate of hypertrophic scarring [75]. This suggests that the epidermis or epidermal factors may play an active role in the process of hypertrophic scarring under specific conditions as occurring during delayed wound healing. This is supported by the observation that keratinocytes or factors derived from keratinocytes are able to regulate the proliferation of fibroblasts [101], fibroblast collagen synthesis [102] and collagenase activity [103].

1.4.7 Treatment of hypertrophic burn scars

Therapeutic solutions to hypertrophic scars have been met with the same degree of controversy as its etiopathology. The lack of a basic understanding of the etiology of hypertrophic healing is the reason for the lack of effective treatments. The one widely used therapy for hypertrophic burn scars is the near-continuous wearing for many months of elastic compression garments [104]. Under pressure the collagen fibers in the dermis will modify their disorganized orientation and will adopt a more parallel arrangement which is characteristic of normotrophic healing. The scar also becomes less vascular, and express less glycosaminoglycans and has less collagen deposition [70].
Surgical treatment of hypertrophic scars is associated with a high rate of recurrence, varying from 50-80% for simple excision [77], but it may be the treatment of choice in case of contractures. Other suggested treatments include silicone gel sheets [105,106], electric stimulation of the scar [107] and application of cryotherapy to the lesion and/or followed by intralesional triamcinalone injection, a corticosteroid [108] known to inhibit the transcription of matrix proteins like collagen and fibronectin, and reducing the synthesis of α2-macroglobulin, an inhibitor of collagenase activity [77]. Triamcinalone injection is an effective treatment for small scars, but for appli-

cation on large surfaces it is difficult, time-consuming and even contraindicated due to the systemic effects of the injected corticosteroids.
Fortunately all hypertrophic scars will flatten, soften and blanchen after a period of time, although this can take up to several years .

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CHAPTER 2

Aims of this thesis

2 AIMS OF THIS THESIS

2.1 QUESTIONS UNDERLYING THE EXPERIMENTAL WORK

A very disturbing outcome of the burn wound healing process is the hypertrophic scar. The aim of this study was to search for factors in the burn scar, which can be responsible for the formation of a hypertrophic scar and which may explain the clinical complaints such as pain and itch.
We asked five questions in this study:

1. Is epidermal activation and maturation altered during the development of hypertrophic scarring?
Until recently it was thought that both the cause and the consequences of hypertrophic scars were situated in the dermis. The reason for this is that microscopically the only difference between normotrophic and hypertrophic scars lies in the dermis. There is an extensive collagen deposition in the hypertrophic scar and the collagen fibers are not arranged regularly. We wanted to investigate whether the epidermis is involved in the hypertrophic process, by determination of different markers for epidermal activation and maturation in burn scars.

2. Can keratinocytes influence the process of hypertrophic scarring by producing growth factors with pronounced effects on the dermal compartment?
If keratinocytes are involved in the process of hypertrophic scarring, is this by producing and releasing growth factors, like TGF-β, bFGF or VEGF?

3. What is the contribution of immune cells in the healing process of normotrophic and hypertrophic scars, and can this be expressed in terms of number and functional state?
Some studies claimed the upregulation of the number of immune cells in hypertrophic burn scars, but little is known about the kinetics of the immune cells during the tissue remodeling phase of burn wound scars. The contribution of immune cells in the healing process is interesting as it is believed that those cells may contribute to itch, which often accompanies hypertrophic scarring.

4. Are neuropeptides involved in the process of post-burn tissue remodeling?
As post-burn hypertrophic scarring is often accompanied by symptoms such as pain and itch, we wanted to get more insight in the expression level of neuropeptides, representing the messenger molecules of the nervous system.

5. Are elastic fibers regenerated in human burn wounds, and at which pace? Does the production of collagen normalize in the course of time?
No long time follow-up data are available on fibroblasts and their products in spontaneously healed partial-thickness burn wounds. To obtain these data we monitored the regeneration of elastic fibers and the production of collagen I in both normotrophic and hypertrophic burn scars.

2.2 STUDY DESIGN

The five questions raised in chapter 2.1 form the lead of the experimental work of this thesis, which is reported in the next chapters.
In our study, patients were included which were treated at the Burn Center of the Red Cross Hospital in Beverwijk, after approval of the protocol by the Medical Ethical Committee of this hospital. The main criteria for inclusion were: 1) patients older than 18 years; and 2) patients suffering from partial-thickness burns that were conservatively treated (i.e. no grafting).
One month post-burn, i.e. when re-epithelialization was normally completed, 3 mm skin biopsies were taken of two partial-thickness burn-sites and of a control skin-site not exposed to burn. The locations of the two biopsies of the burn wounds and the patient characteristics are given in Table 1.
After 3 and 6 months, biopsies were taken of the same burn-site as the 1-month post-burn biopsies. The appearance of the scar was graded using the Vancouver Scar Scale (Table 1, chapter 1). Scars were considered hypertrophic, when the score for vascularity and height were 2 or 3 and pliability was 3, 4 or 5.
To investigate the involvement of the **epidermis** in burn wound healing and in particular in hypertrophic burn wound healing, we performed immunohistochemistry and in situ hybridization on the frozen biopsies. Immunohistochemical detection of keratins 5, 10, 16 and 17, filaggrin, transglutaminase and CD36 was used to study the maturation and activation of the keratinocytes in the burn wound scars (question 1; chapter 3.1). Chapter 3.2 describes the presence and production of the growth factors TGF-β1, -β2, -β3, bFGF and VEGF in the epidermis, which was analyzed by using both immunohistochemistry and in situ hybridization (question 2).
Alterations in the **dermis** in burn wound healing are discussed in chapter 4. The participation of immune cells in burn wound healing was investigated by using immunohistochemistry and markers to detect Langerhans cells, monocytes, macrophages, granulocytes, T helper and T cytotoxic lymphocytes, B lymphocytes, NK cells and mast cells (question 3; chapter 4.1). We studied the outgrowth of nerves in the burn wound scars by using immunohistochemistry and the general nerve marker Protein gene product 9.5 (PGP 9.5). For detection of neuropeptide containing nerves we used markers against SP, NKA, CGRP, VIP and NPY in the Zamboni fixed biopsies (question 4; chapter 4.2). Finally, in chapter 4.3 we describe the deposition of elastic

fibers and the production of collagen by fibroblasts by using both electron microscopy and immunohistochemistry (question 5).
Results of the different studies described in chapters 3 and 4 are discussed in chapter 5. We present our view on (epi)dermal factors, immune cell participation and neuropeptides in burn wound healing, in particular in relation to hypertrophic scar formation.

Table 1. Characteristics of the patients included in this study.

patient	age	sex	total burned surface area	the 2 biopsy sites
1	32	m	20	abdomen / abdomen
2	28	m	33	back / upper extremity
3	66	m	18	lower extremity / lower extremity
4	24	m	13	upper extremity / upper extremity
5	22	m	62	lower extremity / lower extremity
6	58	m	46	upper extremity / upper extremity
7	39	m	12	lower extremity / lower extremity
8	58	m	5	back / back
9	31	m	23	chest / upper extremity
10	40	m	7	lower extremity / lower extremity
11	50	v	15	abdomen / abdomen
12	22	v	11	lower extremity / lower extremity
13	52	m	5	upper extremity / upper extremity
14	62	m	5	upper extremity / upper extremity
15	74	m	10	back / back
16	49	m	17	abdomen / abdomen
17	42	m	12	back / back
18	19	m	5	chest / lower extremity
19	54	v	5	upper extremity / upper extremity
20	26	v	11	lower extremity / lower extremity
21	26	m	12	abdomen / abdomen
22	37	m	10	lower extremity / lower extremity
23	50	m	20	lower extremity / lower extremity
24	22	v	34	lower extremity / lower extremity
25	20	m	8	lower extremity / lower extremity
26	37	m	13	lower extremity / lower extremity
27	21	m	78	lower extremity / lower extremity
28	49	m	18	back / back
29	45	m	17	chest / chest

CHAPTER 3

Epidermal aspects of normotrophic and hypertrophic burn wound healing

3.1 EPIDERMAL PARTICIPATION IN POST-BURN HYPERTROPHIC SCAR DEVELOPMENT*

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ABSTRACT

The reconstruction of epidermal architecture over time in normotrophic and hypertrophic scars in untransplanted, spontaneously healed partial-thickness burns has scarcely been studied, unlike the regeneration of epidermal grafts used to cover burn wounds and the regeneration of the dermis during hypertrophic scarring. The expression of markers of epidermal proliferation, differentiation and activation in normotrophic and hypertrophic scars in spontaneously healed partial-thickness burns was assessed and compared with the expression of these markers in normal control skin of healthy persons, to determine whether hypertrophic scarring is associated with abnormalities in the phenotype of keratinocytes. Punch biopsies were taken both of partial-thickness burns after re-epithelialization and of matched unburned skin. At 4 and 7 months post-burn, biopsies were taken of normotrophic and hypertrophic scars that had developed in these wounds. The biopsies were analyzed using immunostaining for markers of keratinocyte proliferation, differentiation and activation (keratins 5, 10, 16 and 17, filaggrin, transglutaminase and CD36). We observed a higher expression of markers for proliferation, differentiation and activation in the epidermis of scars at 1 month post-burn, than in normal control skin of healthy persons. There was a striking difference between normotrophic and hypertrophic scars at 4 months post-burn. Keratinocytes in hypertrophic scars displayed a higher level of proliferation, differentiation and activation than did normotrophic scars. At 7 months post-burn all keratinocyte proliferation and differentiation markers showed normal expression, but the activation marker CD36 remained upregulated in both normotrophic and hypertrophic scars. Surprisingly, in matched unburned skin of burn patients, a state of hyperactivation was observed at 1 month post-burn. Our results suggest that keratinocytes may be involved in the pathogenesis of hypertrophic scarring.

INTRODUCTION

During wound healing, a sequence of inflammation, tissue synthesis and reorganization results in the formation of mature scar tissue. Skin repair after burning is essentially similar to healing after simple trauma, yet burn-related ischaemia, hypoxia and edema slow down wound healing [1]. The end-result of burn wound healing can be normotrophic healing or abnormal wound healing resulting, for example, in chronic ulcers or hypertrophic scars. Besides disabling contractures and disturbing cosmetic aspects, hypertrophic scars also cause a variable degree of discomfort, such as severe itching [1]. The etiology of hypertrophic scarring is still not known, and until now research has been mainly focused on the dermal aspects of those scars (review: [1]), neglecting the epidermis. As hypertrophic scars most commonly occur after re-epithelialization has been delayed [2], one could hypothesize that hypertrophic scarring results from abnormalities in the epidermal-dermal crosstalk rather than from isolated defects in the dermis. Keratinocytes can regulate the collagen synthesis of der-

mal fibroblasts [3], and conversely keratinocyte growth is supported by keratinocyte growth factor produced by fibroblasts [4]. In addition, activated keratinocytes are a source of specific pro-inflammatory cytokines and fibrogenic and growth factors, and as such have importance in inflammation [5] and wound healing [6]. Further study of epidermal maturation in hypertrophic scarring is worthwhile.

Antibodies to specific markers, such as keratins, filaggrin, transglutaminase are useful in study of the keratinocyte phenotype in tissue remodeling during burn wound healing, because they are expressed in different layers of the epidermis in a differentiation-specific manner [7-9]. Expression of CD36 in normal keratinocytes is absent, occurring only in response to specific immunological and nonimmunological stimuli [10-13]. Research has previously been focused on regeneration of the epidermis in burns treated with grafts [14-18], and so we aimed to describe keratinocyte maturation in ungrafted, spontaneously healed partial-thickness burns. We focused on keratins, filaggrin, transglutaminase and CD36 as a starting point for further studies on keratinocyte maturation in hypertrophic burn wound scars. We wished to determine whether hypertrophic scarring is associated with abnormalities in the phenotype of keratinocytes.

We monitored the expression of epidermal maturation markers immunohistochemically in both normotrophic and hypertrophic burn wound scars during a 6-month follow-up.

MATERIALS AND METHODS

Twenty-two patients aged 19-74 years (mean age: 41 years) were treated for burns at the Burn Center of the Red Cross Hospital in Beverwijk, The Netherlands. The extent of the total burn injury in individual cases varied from 5% to 62% (mean: 16%) of the total body surface area. This study was approved by the Medical Ethics Committee of the Red Cross Hospital.

After informed consent had been obtained from each patient, 3-mm punch biopsies were taken of two partial-thickness burns at three time points: 1 month, after re-epithelialization has been completed (mean: 4.8 weeks, standard error (SEM): 0.32 weeks), 4 months (mean: 17.8 weeks, SEM: 0.77 weeks) and 7 months (mean: 30.6 weeks, SEM: 0.71 weeks) post-burn. Matched unburned skin of the same patients was obtained only at 1 month post-burn. At 4 and 7 months post-burn, the scars were classified as normotrophic or hypertrophic using the Vancouver Scar Scale, a classification system based on consistency, elevation and color [19].

Serial 5-μm cryostat sections were cut from Tissue-tek (Miles, Elkhart, USA) embedded biopsies and attached to glass slides coated with poly-L-lysin and fixed in 100% acetone for 10 minutes. All incubations were performed at room temperature. An alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method was used as described by Schaumburg-Lever [20]. In brief, sections were preincubated with 10% normal rabbit serum in phosphate-buffered saline (PBS) followed by incu-

bation with antibodies against keratins (keratin 5: Euro-Diagnostica, Malmö, Sweden; keratin 10: Monosan, Uden, The Netherlands; keratin 16: Sigma, Zwijndrecht, The Netherlands; keratin 17: DAKO, Glostrup, Denmark), filaggrin (Biomedical Tech., Stoughton, Massachusetts), transglutaminase (Biomedical Tech.) and CD36 (CLB, Amsterdam, The Netherlands). The sections were rinsed, incubated for 30 minutes with rabbit anti-mouse-immunoglobulin antibodies (DAKO) and incubated for 30 minutes with APAAP (DAKO). The immunoreaction was visualized by using a solution containing new fuchsin (Chroma-Gesellschaft, Köngen, Germany), sodium-nitrite, naphtol phosphate, dimethylformamide and levamisol in TRIS-HCl buffer (pH=8.0). Slides were counterstained with Mayer's haematoxylin and mounted in glycerol-gelatin (Merck, Darmstadt, Germany). The negative controls involved concentration-matched mouse IgG (Becton Dickinson, San Jose, CA, USA) and omission of the first and second step.

Two investigators (T.E.H. and V.A.) independently assessed the staining intensity and compared it with expression in normal control skin from healthy persons undergoing plastic surgery. For each of the four epidermal layers (basal, spinous, granular and horny layers) the staining intensity of each marker was scored, using a semi-quantitative scale ranging from 0 to 3 as described previously (0 = not detectable, 1 = light staining, 2 = moderate staining and 3 = strong staining) [21]. On most markers there was consensus, but in case of any discrepancy the mean value was calculated.

For all markers, the mean summary score (referred to from this point on as the *mean score*) of the four epidermal layers was used in the statistical analysis. Differences in the score between normal control skin from healthy persons with burned and matched unburned skin, differences over time, and differences between normotrophic and hypertrophic scars were statistically analyzed using the Mann-Whitney U-test, Wilcoxon matched-pairs signed-ranks-test, Chi-square-test or ANOVA-test (SPSS version 5.0.2, SPSS, Chicago, USA, 1993). A P-value equal to or lower than 0.05 was considered statistically significant.

RESULTS

Biopsies from 22 burn patients were examined. Four patients who initially joined the study were lost to follow-up. This resulted in 38 biopsies taken at 1 month post-burn, 34 biopsies (23 normotrophic and 11 hypertrophic scars) taken at 4 months post-burn, and 36 biopsies (24 normotrophic and 12 hypertrophic scars) taken at 7 months post-burn.

In the haematoxylin-eosin-stained sections, the epidermis of burn scars showed a normal architecture except for the epidermal ridges and dermal papillae which were not as deep or as numerous as in normal control skin. The epidermis of burned skin at 1 month post-burn contained only sporadically infiltrating cells, which were disappeared at 4 and 7 months post-burn. In the epidermis of matched unburned skin and

in normal control skin inflammatory cells were absent.
The dermis of the normotrophic scars showed a normal architecture, whereas the hypertrophic scars showed the typical dermal collagen organization of whorls and nodules.
At 1 month after burning, the expression of keratins 5, 16 and 17 (figure 1), filaggrin, transglutaminase and CD36 was clearly higher than in normal control skin of healthy persons (Mann-Whitney U test: $P<0.01$). No alterations were observed in keratin 10 expression.

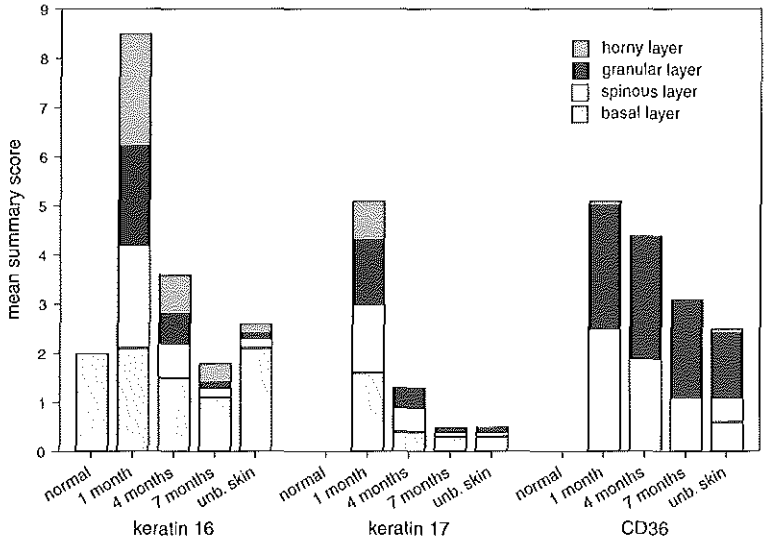

Figure 1. Keratin 16 and 17 and CD36 expression in the epidermis in both normal control skin of healthy persons, in burn scars at 1, 4 and 7 months post-burn time, and in matched unburned skin of the patients.

The matched unburned skin of every patient taken at 1 month post-burn showed a normal expression pattern for all markers except CD36 (figure 1), 19 of the 22 showing an upregulation of this marker (figure 2A). The difference between this elevated expression in matched unburned skin and the absence of CD36 expression in the normal control skin of healthy persons (figure 2B) was highly significant (Wilcoxon test: $P<0.01$). There was no relation between the distance from the biopsy site of the matched unburned skin to the burned area and the CD36 expression (Chi-square test, $P=0.28$). No relation was observed between total body surface area and CD36 expression in the matched unburned skin biopsies (ANOVA test, $P=0.27$).
At 4 and 7 months post-burn keratin 16 expression differed significantly between the epidermis of normotrophic (figure 2C) and of hypertrophic (figure 2D) scars. There was still an upregulation of keratin 16 in 9 of the 10 hypertrophic scars (*mean score* 6.4) as against 5 out of 21 normotrophic scars (*mean score* 2.6; Mann-Whitney U

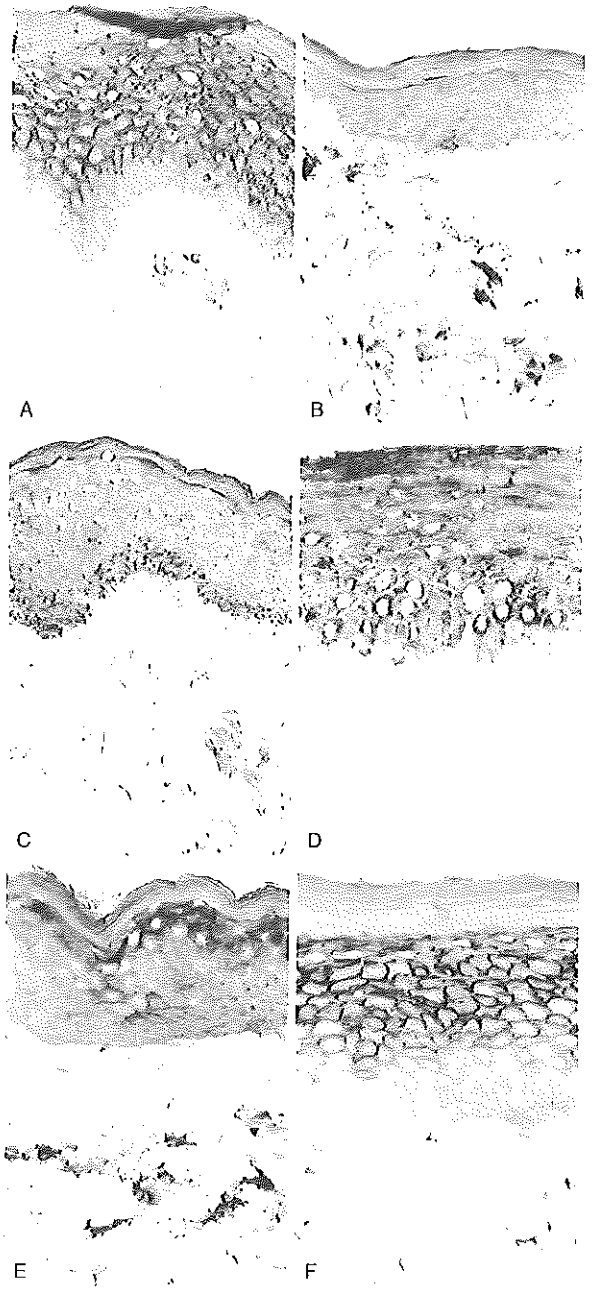

Figure 2. Immunohistochemical localization of keratin 16 and CD36 in partial-thickness burn scars at 4 months post-burn time, matched unburned skin and normal control skin of healthy persons.
CD36 in matched unburned skin of a burn patient (**A**) and in normal control skin of a healthy person (**B**). Keratin 16 in a normotrophic scar (**C**) and a hypertrophic scar from the same patient (**D**), and CD36 in a normotrophic scar (**E**) and a hypertrophic scar from the same patient (**F**). Original magnification ×400.

test: P<0.01; figure 3). At 7 months post-burn the expression of keratin 16 had reverted to normal in both normotrophic (*mean score* 1.7) and hypertrophic scars (*mean score* 2.1; Mann-Whitney U test: P=0.83).

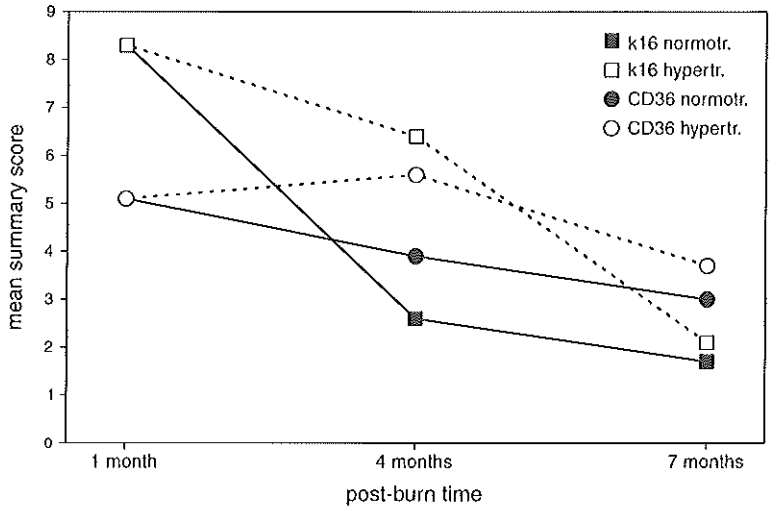

Figure 3. Keratin 16 and CD36 expression in normotrophic and hypertrophic burn scars.

Keratin 17 was still slightly upregulated in 11 of the 33 scars at 4 months, with a small, but statistically insignificant, difference between normotrophic (*mean score* 0.7) and hypertrophic scars (*mean score* 2.2; Mann-Whitney U test: P=0.24). At 7 months no scars showed keratin 17 expression, as expected.
CD36 remained expressed by keratinocytes at both 4 and 7 months post-burn. At 7 months, 34 of the 36 scars still showed CD36 expression. This upregulation was significant compared with normal control skin of healthy persons (Mann-Whitney U test: P<0.01). At 4 months post-burn the difference between normotrophic scars (*mean score* 3.9; figure 2E) and hypertrophic scars (*mean score* 5.6; figure 2F) was significant (Mann-Whitney U test: P=0.03; figure 3), but there was no longer a significant difference at 7 months post-burn (*mean scores* normotrophic scars: 2.9, hypertrophic scars: 3.7; Mann-Whitney U test: P=0.38).
The expression of keratins 5 and 10 and filaggrin and of transglutaminase in scars was not different from that in normal control skin at 4 and 7 months post-burn.

DISCUSSION

Our data show that keratinocytes in burn wounds in which re-epithelialization just completed is, have entered an alternative pathway of differentiation and are expressing an activated phenotype compared with those in normal control skin from healthy persons. This is shown by the upregulation of keratins 5, 16 and 17, filaggrin,

transglutaminase and CD36 in the 1-month-old burn scar. These results agree with those of previous studies on epidermal maturation in grafted burn wounds [16-18,22] and show that the first aim of the newly formed epidermis is a rapid migration and proliferation to cover the denuded area. This is followed by enhanced differentiation to restore the barrier function of the skin. With regard to the chronic phase of spontaneously healed burn wounds, our study clearly demonstrates a time-dependent difference in keratinocyte phenotype between normotrophic and hypertrophic scars. This indicates that the distinction between normotrophic and hypertrophic scars is located in both the dermis and epidermis.

A recent study has also revealed an upregulation of keratin 16 in the epidermis of hypertrophic scars [23]. However, the workers concerned did not observe time-dependent expression, probably owing to the unknown depth of the original wound and to dissimilar treatments, in particular grafting.

Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have been shown to upregulate suprabasal keratin 16 expression specifically in hyperproliferative epidermis [24,25], and should be regarded as important growth factors in the pathogenesis of hypertrophic scarring. Potential sources of EGF or TGF-α in hypertrophic burn scars are the immunologically active cells present in the dermis or epidermis and keratinocytes themselves. The latter play an important part in the expression of keratin 16, as it has been shown that keratinocytes can produce and be stimulated by TGF-α in an autocrine loop [26]. Alternatively, induction of keratin 16 in hypertrophic scars may be caused by the continuous upward mechanical forces to which the epidermis is exposed, because of the tumor-like growth of the reticular dermis. Indeed, keratin 16 is known to be involved in the reorganization of the keratin filaments to facilitate mobility by keratinocytes [27].

The reasons for the prolonged CD36 expression by keratinocytes, the cause of its expression and its function in burn wound scars are all unclear. It is known that CD36 is upregulated by keratinocytes in response to some immunological and non-immunological stimuli [10-13]. CD36 can be expressed by various other cell types, such as platelets, monocytes, macrophages, erythrocytes and endothelial cells [28]. It belongs to a family of integral membrane glycoproteins that recognize a wide range of ligands and can be expressed by platelets, monocytes, macrophages and endothelial cells [29-32]. It may serve as a signal transduction molecule [33] and as a general adhesion molecule [34]. The biochemical features of the CD36 molecule expressed on keratinocytes of hypertrophic scars are identical to those on other cell types [35], suggesting that the function of CD36 on keratinocytes might resemble its role in other cell types.

In macrophages, CD36 has been identified as an adhesion molecule in the clearance of apoptotic neutrophilic granulocytes [34]. CD36 may serve as an adhesion molecule in keratinocytes, whether for inflammatory cells, or for adjacent keratinocytes to form a tight connection when the epidermis is mechanically challenged in scar formation. It may be that Langerhans cells upregulated in the burn scars (personal,

unpublished, results) use the CD36 molecule as an adhesion factor to re-enter the epidermis after re-epithelialization.

In platelets CD36 is a receptor for both thrombospondin [29] and collagen [30]. In erythrocytes it is implicated in the binding of malaria-infected cells to endothelium [31], and in macrophages in the binding and internalization of oxidized LDL [32]. It is unlikely that CD36's main function in keratinocytes is to be a receptor for thrombospondin-1, as it has previously been shown that CD36-positive keratinocytes in a variety of skin diseases remained thrombospondin-1 negative [12].

In platelets and monocytes, CD36 serves as a signal transduction molecule [33], as it may be in keratinocytes. After burn wound healing and during tissue remodeling, dermal-epidermal crosstalk is necessary for the formation of a mature scar.

We do not have an explanation for the surprising observation that CD36 was expressed by the keratinocytes in the matched unburned skin. However, it suggests that this molecule is probably a marker for keratinocyte activation rather than for keratinocyte differentiation, as has previously been suggested [10,36]. IFN-γ is one of the cytokines that have been reported to induce CD36 expression by keratinocytes [11,12,37,38]. However, neither ICAM-1 nor HLA-DR, both of which are also known to be directly upregulated by IFN-γ [39], was present on the keratinocytes of the burn scars (data not shown). This suggests that IFN-γ is not involved in the upregulation of CD36 in burn scars.

The upregulation of CD36 is likely to be influenced by the presence of an hormonally active peptide, as it is also upregulated in the matched unburned skin of the patients. No long-term follow-up data are available on cytokine levels that lead to activation of the noninvolved epidermis, so that there are no data to support this suggestion. Neuropeptides regulated by the central nervous system and secreted by nerve endings are another possible source of centrally located messenger that might influence both the keratinocytes of the burn scars and the keratinocytes of the matched unburned skin. When the skin is injured, signals elicited by the sensory nerves from this tissue reach the central nervous system, and in addition to this so-called orthodromic response, the sensory nerves are capable of a second, efferent impulse to the skin, the antidromic response [40]. With this response, neuropeptides are released into the skin, which can influence keratinocyte activation. Neuropeptides can also modulate keratinocyte functions, such as cytokine production [41] and possibly CD36 expression. Nerves reach both the burned and the unburned skin and can thus influence keratinocytes of the whole skin.

We conclude that the development of hypertrophic scarring is not only an isolated dermal defect, but rather the result of a defect in the interaction between dermis and epidermis and also influenced by systemic neuro-hormonal systems.

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3.2 TGF-β1, -β2, -β3, bFGF AND VEGF EXPRESSION IN KERATINOCYTES OF BURN SCARS

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ABSTRACT

Keratinocytes are increasingly recognized as key regulators of skin inflammation and remodeling, as they are capable of producing growth factors and cytokines that are important mediators in the wound healing process. We investigated the expression and distribution of TGF-β1 mRNA by mRNA in situ hybridization and of TGF-β1, TGF-β2, TGF-β3, bFGF and VEGF protein expression using immunohistochemistry in spontaneously healed partial-thickness burns and compared this with the expression of these markers in matched unburned skin. This was done to assess their role in the remodeling phase of burn wound healing. Punch biopsies were taken from both partial-thickness burns after re-epithelialization and from matched unburned skin. At 4 and 7 months post-burn, biopsies were taken of normotrophic and hypertrophic scars that had developed in these wounds. We observed a higher expression of all mentioned growth factors in keratinocytes in scars at 1 month post-burn compared with matched unburned skin. At 4 months keratinocytes still displayed a higher expression of TGF-β3 and bFGF, but the expression of TGF-β1, TGF-β2 and VEGF was normalized. The expression of TGF-β3 in the epidermis of hypertrophic scars was slightly higher than in normotrophic scars. At 7 months post-burn all growth factors studied showed a normal expression on keratinocytes.
Our results suggest that keratinocytes are not only involved in re-epithelialization, but also in the scar maturation. The data support the idea that keratinocytes not only respond to cytokines and growth factors in an autocrine fashion, but also exert regulatory paracrine effects on contiguous cells.

INTRODUCTION

Keratinocytes are increasingly recognized as key regulators of skin inflammation and remodeling, as they are capable of producing a variety of factors and cytokines that promote chemotaxis and activation of macrophages and other inflammatory cells, angiogenesis, and the proliferation of fibroblasts [1-3]. Their role in wound healing, however, has not been studied so extensively as that of immune cells like macrophages and other leukocytes [1].
Transforming growth factor-β (TGF-β) is such a modulator of wound healing. It is constitutionally present in platelets, but it is also produced by several cell types present in wounds, including activated macrophages, granulocytes, fibroblasts and keratinocytes [1,4,5]. In mammals three TGF-β isoforms have been identified: TGF-β1, TGF-β2 and TGF-β3, which share 70-80% amino acid homology and also many biological activities. The genes for each of the isoforms are located on different chromosomes and their differential expression may occur through distinct cell-specific regulatory mechanisms [6].
The many fibroblast growth factors stimulate the proliferation of almost all major cell types involved in wound healing [7]. Basic fibroblast growth factor (bFGF) stimu-

lates angiogenesis and mitosis of fibroblasts, resulting in acceleration of collagen deposition in the dermis and, moreover, is mitogenic for keratinocytes [7-9]. In mouse skin, keratinocytes were the only cells in a full thickness wound model to express bFGF, indicating an important role for keratinocytes in the production and release of this growth factor [10].

Vascular endothelial growth factor (VEGF, also named vascular permeability factor) is a heparin-binding glycoprotein occurring in four molecular forms, consisting of 121, 165, 189 or 206 amino acids, respectively, generated by alternative RNA splicing [11]. Only the two smaller isoforms (VEGF121 and VEGF165) are secreted, the other two remain intracellular. Human keratinocytes express the 121, 165 and 189 amino-acids forms [12] and are the main source of VEGF in the skin [13]. VEGF acts specifically on vascular endothelial cells, since the receptors for VEGF are mainly expressed on these cells [14].

We reasoned that the kinetics of TGF-β1-3, bFGF and VEGF expression in human epidermis might reveal their potential role in the tissue remodeling involved in burn scar maturation. Therefore we investigated the expression and distribution of these growth factors in the epidermis of burned skin shortly after re-epithelialization and compared this with the expression in unburned control epidermis.

MATERIALS AND METHODS

Patients and biopsies

Twenty-two patients, aged 19-74 years (mean age: 41 years), were treated for burns at the Burn Center of the Red Cross Hospital in Beverwijk, The Netherlands. The extent of the total burn injury in individual cases varied from 5% to 62% (mean: 16%) of the total body surface area. This study was approved by the Medical Ethics Committee of the Red Cross Hospital in Beverwijk.

After informed consent had been obtained from each patient, 3-mm punch biopsies were taken of two spontaneously healed partial-thickness burns at three time points: 1 month post-burn, i.e. after re-epithelialization has been completed (mean: 4.8 weeks, standard error (SEM): 0.3 weeks), 4 months post-burn (mean: 17.8 weeks, SEM: 0.8 weeks) and at 7 months post-burn (mean: 30.6 weeks, SEM: 0.7 weeks). Matched unburned skin of the same patient was obtained only at 1 month post-burn. At 4 and 7 months post-burn, the scars were classified as normotrophic or hypertrophic using the Vancouver Scar Scale, a classification system on the basis of vascularity, pliability and height [15].

In situ hybridization

In situ hybridization was performed on 5 μm thick frozen sections. We used a 500 bp *Sma*I-*Bam*HI fragment of TGFβ1 cDNA cloned into pBluescript KS (Stratagene, La Jolla, CA, USA) [16]. The specific cRNA probes were labeled with digoxigenin following the manufacturer's protocol (Boehringer, Mannheim, Germany). The in situ

hybridization was performed as described previously [16,17]. Briefly, after pretreatment the sections were hybridized with 50 ng of the relevant probe per slide during 16 hours at 62°C. Subsequently, sections were washed in 2x standard saline citrate (SSC) with 50% formamide at 50°C, then in 0.1x SSC with 20 mM β-mercaptoethanol at 62°C, and finally treated with 2U/ml RNAse T1 (Boehringer) in 2x SSC plus 1 mM EDTA at 37°C. The immunodetection of digoxigenin-labeled hybrids was done using nitro blue tetrazolium (NBT) as chromogen and bicholylindolyl phosphate (BCIP) as coupling agent (Boehringer). The sense riboprobes were included as negative controls and did not show any staining.

Immunostaining

Serial 5μm cryostat sections were cut from Tissue-tek (Miles, Elkhart, USA) embedded biopsies and attached to glass slides coated with poly-L-lysin and fixed in 100% acetone for 10 minutes. All incubations were performed at room temperature. The sections were preincubated with 10% normal goat serum in phosphate-buffered saline (PBS) followed by incubation with antibodies against TGF-β1, TGF-β2 and TGF-β3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), bFGF (Serotec, Oxford, England) and VEGF (Oncogene, Cambridge, MA, USA). The antibodies against TGF-β1, TGF-β2 and TGF-β3 showed no cross-reactivity with each other, as determined by the manufacturer by Western blotting, immunoprecipitation and immunohistochemistry.

The sections were rinsed, incubated for 30 minutes with biotinylated goat anti-rabbit immunoglobulin (Biogenex, San Ramon, CA, USA) and incubated for 30 minutes with streptavidin-alkaline phosphatase (Biogenex). The immunoreaction was visualized by using a solution containing new fuchsin (Chroma-Gesellschaft, Köngen, Germany), sodium-nitrite, naphtol phosphate, dimethylformamide and levamisol in TRIS-HCl buffer (pH=8.0). Slides were counterstained with Mayer's haematoxylin and mounted in glycerol-gelatin (Merck, Darmstadt, Germany). The negative controls involved concentration-matched rabbit IgG (Becton Dickinson, San Jose, CA, USA) and omission of the first or second step.

Assessment of sections

Two investigators (T.E.H. and V.A.) independently assessed the staining intensity and compared the expression in burned skin with the expression in matched unburned skin. For each of the 4 epidermal layers (basal, spinous, granular and horny layer) the staining intensity was scored, using a semi-quantitative scale ranging from 0 to 3 as described previously (0 = not detectable; 1 = light staining; 2 = moderate staining; and 3 = strong staining) [18]. If no consensus was reached, the mean value was calculated.

For all markers, the mean summary score (further referred to as *mean score*) of the 4 epidermal layers was used in the statistical analysis as the individual layers gave only marginally significant results. Differences in the score between burned and matched

unburned skin, differences over time, as well as differences between normotrophic and hypertrophic scars were statistically analyzed using the Mann-Whitney U-test or Wilcoxon matched-pairs signed ranks test (SPSS version 5.0.2, SPSS, Chicago, USA, 1993). A P-value equal to or lower than 0.05 was considered statistically significant.

RESULTS

Histopathology

Biopsies from 22 burn patients were included. Four patients who initially joined the study, were lost to follow-up. This resulted in 38 biopsies taken at 1 month post-burn, 34 biopsies (23 normotrophic and 11 hypertrophic scars) taken at 4 months post-burn, and 36 biopsies (24 normotrophic and 12 hypertrophic scars) taken at 7 months post-burn.

In the haematoxylin-eosin stained sections, the epidermis of burn scars showed a normal architecture, except for the epidermal ridges and dermal papillae which were not as deep and as numerous as in normal control skin.

The dermis of the normotrophic scars showed a normal scar architecture, whereas the hypertrophic scars showed the typical dermal collagen organization of whorls and nodules [19].

The expression of TGF-β1-3

TGF-β1 mRNA was abundantly expressed by both basal and suprabasal keratinocytes of the burn scars at 1 month post-burn (Figure 1A+B). The expression in the epidermis decreased at 4 and 7 months, and reached levels comparable with matched unburned skin. In matched unburned skin TGF-β1 mRNA was expressed mainly by the basal and only a few suprabasal keratinocytes.

TGF-β1 mRNA was also expressed, in both the burned and the matched unburned skin, by endothelial cells, immune cells and fibroblasts in the dermis of the burn scars. However, the expression in keratinocytes was more abundant. The expression in fibroblasts and endothelial cells of the burn scars was clearly higher compared with matched unburned skin, even at 4 and 7 months post-burn.

TGF-β1 protein expression showed a comparable pattern. At 1 month post-burn, it was clearly expressed by basal and suprabasal keratinocytes. In matched unburned skin TGF-β1 was expressed in the basal layer of the epidermis (*mean score* burn scar: 4.1; unburned skin: 2.1; Wilcoxon signed ranks test: $P<0.01$; Figure 2). At 4 and 7 months the expression of TGF-β1 had returned to levels comparable with the expression in the epidermis of matched unburned skin. In the dermis of the burn scars a few cells of the infiltrate, fibroblasts and endothelial cells were positive, as was also the case in matched unburned skin.

At 1 month post-burn TGF-β2 expression was marginally increased in the basal, spinous and granular layer cell layers (*mean score*: 4.7; unburned skin: 4.2; Wilcoxon signed ranks test: $P<0.05$). In unburned epidermis TGF-β2 was expressed in the

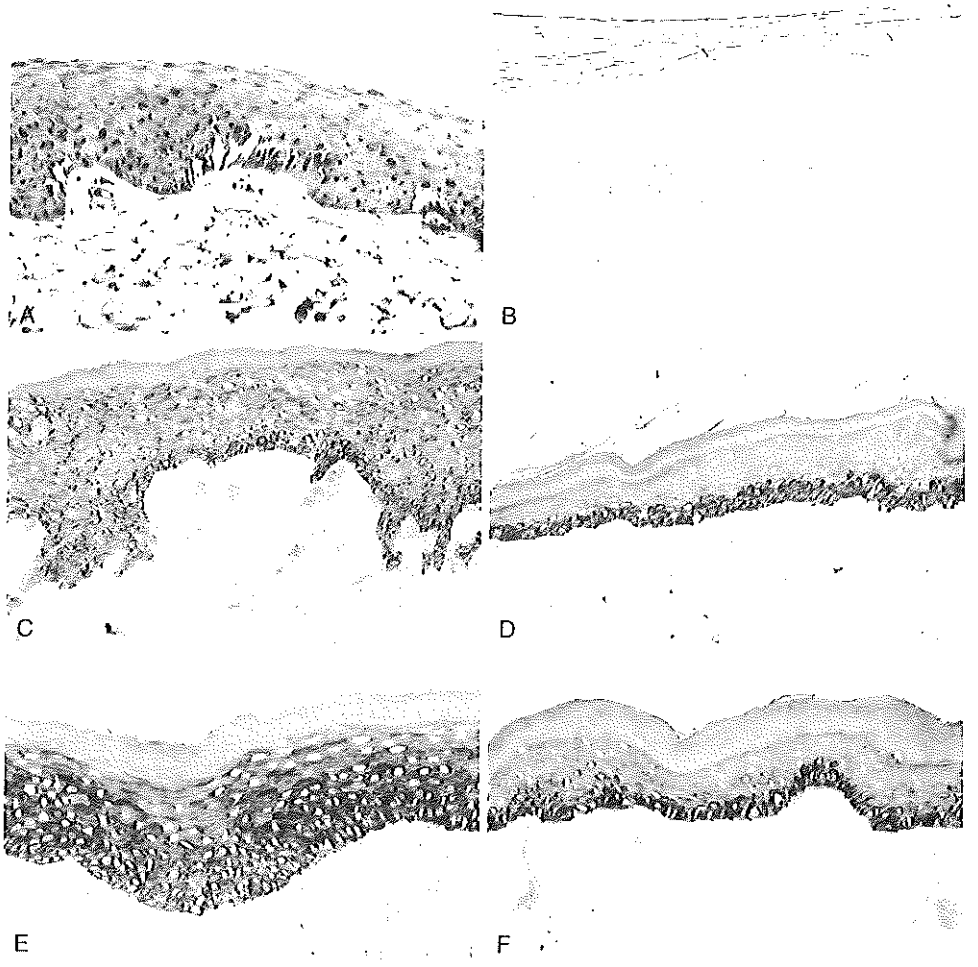

Figure 1. (**A**) In situ TGF-β1 mRNA expression in all epidermal cell layers in a burn scar at 1 month post-burn. (**B**) Negative control of figure A, stained with the TGF-β1 sense probe. (**C**) TGF-β3 protein expression in a burn scar at 1 month post-burn. (**D**) TGF-β3 protein expression in a burn scar at 7 months post-burn. (**E**) bFGF protein expression in a burn scar 1 month post-burn. (**F**) bFGF protein expression in a burn scar 7 months post-burn. The original magnification of all figures was ×250, except figure C (×160).

basal layer and slightly in the spinous and granular layers. In the dermis at 1 month post-burn a few infiltrate cells were positive, which is comparable with unburned skin. At 4 and 7 months post-burn, TGF-β2 expression was normal.

At 1 and 4 months post-burn, TGF-β3 expression was clearly higher in the burn scars (*mean score* at 1 month: 6.0; at 4 months: 4.1; unburned skin: 2.9; for both Wilcoxon signed ranks test: $P<0.01$; Figure 1C). In unburned skin, TGF-β3 was only expressed in the basal epidermal cell layer and was absent in the dermis (Figure 1D). However, in the burn scars dermal infiltrate cells showed a slight TGF-β3 expression at 1 month, which were absent at 4 and 7 months.

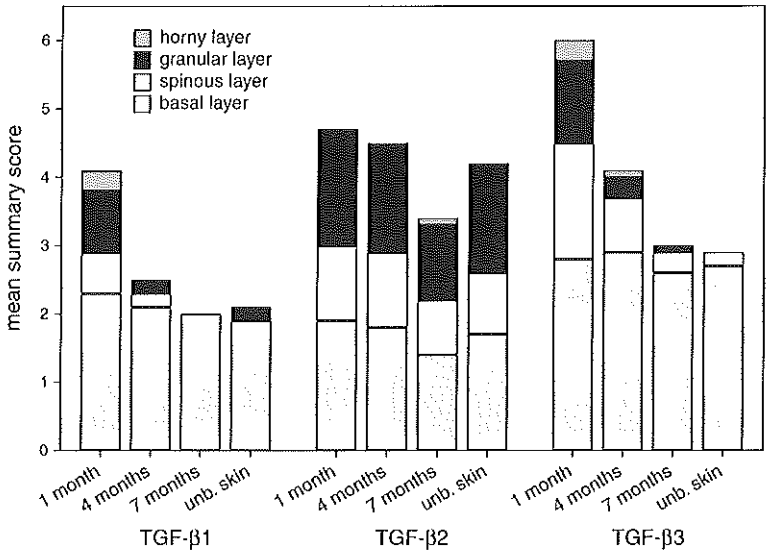

Figure 2. TGF-β1, TGF-β2 and TGF-β3 protein expression in the epidermis of burn scars at 1, 4 and 7 months post-burn, and in matched unburned skin of the same patients.

The expression of bFGF and VEGF

The expression bFGF in the burn scars at 1 month post-burn was clearly increased in the spinous and granular cell layers (Figure 1E), compared with the unburned control skin of the same patients (*mean score*: 6.8; unburned skin: 3.3; Wilcoxon signed ranks test: $P<0.01$; Figure 3). At 4 months the expression was still significantly upregulated (*mean score*: 5.3; Wilcoxon signed ranks test: $P<0.05$), but normalized at 7 months (Figure 1F). Only sporadically dermal cells of the burn scars showed bFGF expression, but less than keratinocytes.

VEGF expression was slightly higher in the basal cell layer of the 1 month old burn scar, compared with unburned skin (*mean score*: 1.9; unburned skin: 1.3; Wilcoxon signed ranks test: $P<0.01$), and had return to normal levels at 4 and 7 months. In the dermis, VEGF was expressed by a few cells in the infiltrate, declining at 4 and 7 months post-burn.

Normotrophic versus hypertrophic scars

At 4 months post-burn, TGF-β3 protein expression in the epidermis of hypertrophic scars was slightly higher than in the epidermis of normotrophic scars (*mean score* of the normotrophic scars was 3.7 and of the hypertrophic scars 4.6; Mann-Whitney U test: $P=0.08$). For TGF-β1, TGF-β2, bFGF as well as VEGF there was no difference in expression between the keratinocytes of normotrophic and of hypertrophic scars.

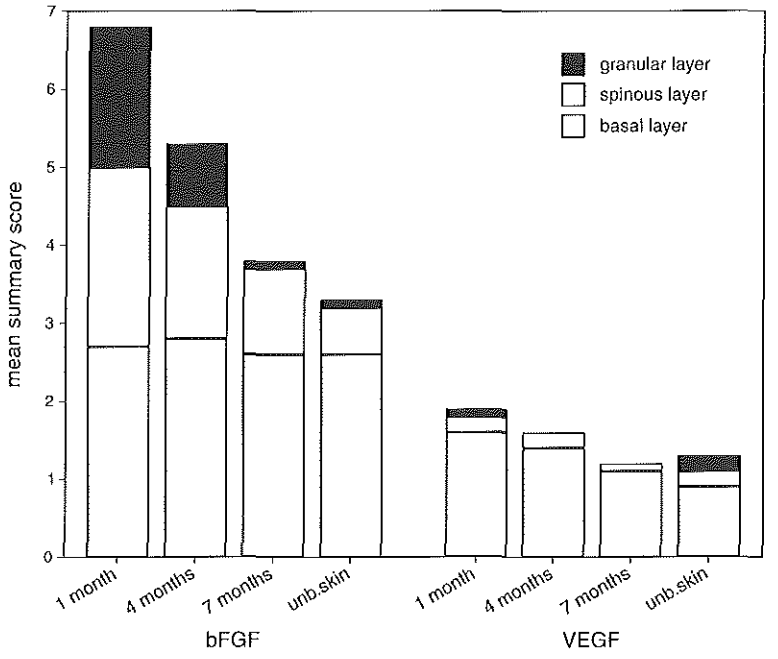

Figure 3. bFGF and VEGF protein expression in the epidermis of burn scars at 1, 4 and 7 months post-burn, and in matched unburned skin of the same patients.

DISCUSSION

It is well known that growth factors are essential for a normal wound healing process [3,20]. However, the majority of the investigations on growth factors and wound healing were performed on the acute phase of wound healing, and less is known about the chronic, maturation, phase. Macrophages, lymphocytes, platelets and fibroblasts are well recognized producers of different growth factors during the wound healing process, and thus research has been focused mainly on the role of these cells. Our study shows a high abundance of TGF-β, bFGF and VEGF in epithelial cells, and that inflammatory cells are also a potential source of growth factors, particularly at 1 month post-burn. As inflammatory cell numbers greatly decrease after 1 month, keratinocytes seem to be a major growth factor producer in the maturation phase of the wound healing process, suggesting that keratinocytes are important regulators in the scar remodeling process.

The crosstalk between keratinocytes and fibroblasts, a key effector cell during the wound healing process, has been well studied. Fibroblasts produce keratinocyte growth factor (KGF) and IL-6 in response to IL-1 produced by keratinocytes [21,22]. KGF on its turn stimulates keratinocyte proliferation and migration and promotes the re-epithelialization of the skin [23].

TGF-β has been recognized as a key growth factor in the process of scarring [3]. It

can be produced by a variety of cell types, including keratinocytes [24]. TGF-β receptors are widely distributed and found on essentially all cell types [25]. TGF-β is released as an inactive peptide bound to its propeptide and requires activation either by proteolysis or as a result of the acid environment within a wound [26]. The three isoforms of TGF-β that are present in mammals show a similar biological activity in most in vitro assays [27]. However, there are differences in their in vivo potencies and some biological activities [28]. Recent immunolocalization and in situ hybridization studies in developing mouse and human embryos have shown distinct distribution patterns of the three TGF-β isoforms. These studies suggest differential induction and regulation and consequently a distinct role of the three isoforms during embryonic growth and development [29,30].

With regard to immunohistochemical studies on TGF-β in the skin, the use of different protocols and a variety of rabbit polyclonal antibodies of different sources which detect either the latent or the active form of TGF-β may have contributed to the discordant results in the literature. The same antibody we used against TGF-β1 also stained the basal layer in frozen sections of the murine epidermis [5]. However, application of a panel of other TGF-β1 antibodies on paraffin embedded sections gave either a negative result or a suprabasal staining in normal human control skin [31-34], and a suprabasal staining in pig skin [18]. In contrast to our results, TGF-β1 mRNA was not detected in normal human epidermis in another study, possibly due to the use of different protocols [35]. We showed that keratinocytes are able to express both TGF-β1 mRNA and protein, emphasizing keratinocytes are an important source of this growth factors.

The data in the literature on TGF-β2 immunostaining are also controversial. TGF-β2 protein was detectable in all epidermal cell layers of normal human [31] and pig skin [18], but only in the basal cell layer of murine epidermis [5,36]. Another study showed no mRNA expression in normal human epidermis [37], but did show an expression in the basal layer of murine epidermis [5]. The results of immunostaining on TGF-β3 also showed variable results. TGF-β3 protein and mRNA expression were detectable in all epidermal layers of normal human skin in one study [37], but totally negative in another study [31]. Mouse epidermis was also negative [5], but in pig skin all epidermal layers showed TGF-β3 expression [18].

In our study, both TGF-β1 mRNA and protein and TGF-β2 protein were upregulated in the early burn scar, but showed a normal expression at 4 months post-burn. Both cytokines have been shown to increase the production of glycosaminoglycans, fibronectin and collagen [18], and to downregulate the expression of many matrix-degrading enzymes [38].

TGF-β3 is thought to have another role in the wound healing process than TGF-β1 and -β2, as exogenous addition of TGF-β3 or neutralizing antibodies to TGF-β1 and -β2 markedly improved the architecture of the dermis in rats after full-thickness wounds. This suggests that the balance between TGF-β1 and TGF-β-2 at the one hand and TGF-β3 at the other hand determine the outcome of the wound healing

process: TGF-β1 and -β2 are implicated in cutaneous scarring and TGF-β3 in prevention of scarring [28]. This interesting concept, however, could not be confirmed in a rabbit full-thickness wound model [39]. We showed that in human epidermis TGF-β3 expression is increased in hypertrophic scars compared with normotrophic scars. This finding also does not confirm the hypothesis that TGF-β3 is involved in the prevention of scarring, at least not in human burn wound healing. Possibly the role of the TGF-β isoforms might differ in various species.

It has previously been reported that TGF-β3 is the most abundant TGF-β isoform in hyperproliferative epithelium and might therefore play an important role in keratinocyte proliferation and differentiation [5]. And indeed, we previously showed that in the epidermis of hypertrophic scars the keratinocytes express abnormal markers of hyperproliferation and differentiation, compared with normotrophic scars [40]. This implies a relationship between TGF-β3 and keratinocyte proliferation and differentiation. However, the connection between TGF-β-isoforms and hypertrophic scarring might also be influenced by differential expression of TGF-β receptors, as it is recently shown that TGF-β receptor types I and II are overexpressed in post-burn hypertrophic scar fibroblasts [41].

Concerning the expression of bFGF, we observed it to be present in basal keratinocytes of both non-wounded and wounded skin, but also in the suprabasal layers of the latter. This upregulation of bFGF in the wounded areas was also observed in full thickness wounds in mice [10] and in psoriasis, a disease characterized by an increased pool of proliferating keratinocytes [42]. This suggests that bFGF contributes to keratinocyte proliferation. When partial-thickness burn wounds were treated with topical recombinant bovine bFGF, the healing time was shortened and the quality of the scar improved [43], whereas the healing of excisional skin wounds was delayed in bFGF knockout mice [44].

VEGF is a specific growth factor for endothelial cells [3]. The main source for VEGF in the skin appears to be keratinocytes [13]. Its expression is modulated by e.g. tissue hypoxia, as keratinocytes in the avascular epidermis are particularly dependent on the dermal vessels for oxygen. Tissue hypoxia, a characteristic feature of early healing wounds, leads to induction of VEGF expression by epithelial cells and to VEGF receptor expression on microvascular endothelial cells, and in this manner to the formation of new blood vessels [45]. In our study, VEGF was upregulated in the 1 month old burn scars, like in partial-thickness skin wounds in rat, where VEGF mRNA was present in all epidermal layers [2]. At 4 months post-burn, VEGF expression in the keratinocytes had returned to normal, which is consistent with the observation that angiogenesis has slowed down at that time.

Our results demonstrate that keratinocytes are not only passive bystanders in the burn scar repair process, but are definitely also actively involved in the scar tissue remodeling phase. They not only respond to different cytokines and growth factors present in wounds, but also produce and release such molecules to stimulate neighboring cells in the surrounding tissue.

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CHAPTER 4

Dermal aspects of normotrophic and hypertrophic burn wound healing

4.1 IMMUNE CELL INVOLVEMENT IN POST-BURN TISSUE REMODELING *

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* Submitted for publication

ABSTRACT

Although there are several reports on the composition of the cellular infiltrate in healing wounds, such data on healing burn wounds are scarce. The aim of this study is to characterize the infiltrate present in scars of spontaneously healed partial-thickness burns in order to obtain insight into the presence of immune cells involved in the remodeling phase.
Punch biopsies were taken of partial-thickness burns at 1 month post-burn and of site matched control unburned skin. After 4 and 7 months biopsies were taken of the normotrophic or hypertrophic scars developed in these burns. The biopsies were analyzed using immunostaining for markers of phagocytes (CD1a, CD14 and CD68 for monocyte-derived cells and CD16 for granulocytes), lymphocytes (CD3, CD4, CD8 and IL-2R for T lymphocytes, CD19 for B lymphocytes and CD57 for NK cells) and mast cells (tryptase).
The infiltrate in the 1 month old burn scar consisted predominantly of granulocytes, monocytes, macrophages, T helper lymphocytes and B lymphocytes.
After 4 and 7 months most immune cell numbers had turned to normal, but mast cell and Langerhans cell numbers were increased compared with normal control skin. No difference was observed in immune cell score from normotrophic and hypertrophic scars.
We conclude that phagocytes and lymphocytes likely play a role in the early phase of burn wound healing, but not in the process of hypertrophic scarring and that Langerhans cells and mast cells may be involved in the remodeling phase of the burn wound healing process.

INTRODUCTION

During the process of wound healing, a sequence of inflammation, tissue synthesis and reorganization results in the formation of mature scar tissue. Skin repair after burning is essentially similar to healing after simple trauma, yet ischaemia, hypoxia and edema slow down wound healing [1]. The end result of burn wound healing may be normotrophic healing or abnormal wound healing resulting in chronic ulcers or hypertrophic scars. Besides the disabling contractures and the disturbing cosmetic aspects, hypertrophic scars also cause a variable degree of discomfort such as severe itching [1].
Interaction between the immune and wound healing systems are suggested from studies in which the immune system is altered. Conditions known to impair the immune response, like immunosuppressive and steroid therapy, also result in delayed or defective wound healing in otherwise healthy individuals [2,3]. This may be due to a defective production of various growth factors and cytokines produced by immune cells, like TGF-β, TNF-α, PDGF and IL-1. These are capable of controlling the

growth of keratinocytes and fibroblast function and, therefore, influence quantity and quality of collagen formation [4].
The composition of the cellular infiltrate in healing wounds has been extensively studied [1,4,5]. As part of the healing process, immune regulatory cells, such as leukocytes, migrate and function at the site of the injury. Their primary task is to help cleaning the wounded area of undesirable debris and bacteria [4]. Data on the composition of the infiltrate in healing burn wounds is scarce. Characterization of the immune cells present in the healing burn wounds and particularly in the later phases of the process, might give insight into the immunological mechanisms involved in the repair process. In the present study we characterized the immune cells present in the scars of spontaneously healed partial-thickness burns and we looked for possible differences between normotrophic and hypertrophic scar formation.

MATERIALS AND METHODS

Patients and biopsies

Twenty-two patients, aged 19 to 74 years (mean age: 41 years), were treated for burns at the Burn Center of the Red Cross Hospital in Beverwijk, The Netherlands. The extent of the total burn injury in each patient varied from 5 to 62% (mean: 16%) of the total body surface area. This study was approved by the Medical Ethics Committee of the Red Cross Hospital.
From each patient, after obtaining informed consent, 3 mm punch biopsies were taken of two partial-thickness burns at three time points, 1 month post-burn (mean: 4.8 weeks, standard error of the mean (SEM): 0.3 weeks), 4 months post-burn (mean: 17.8 weeks, SEM: 0.8 weeks) and 7 months post-burn (mean: 30.6 weeks, SEM: 0.7 weeks). Punch biopsies of matched control unburned skin were also taken, but only at the start of the study. At 4 and 7 months post-burn, scars were classified as normotrophic or hypertrophic using the Vancouver Scar Scale (classification based on consistency, elevation and color) [6].

Immunostaining

Serial 5µm cryostat sections were cut from Tissue-tek (Miles, Elkhart, USA) embedded biopsies, attached to glass slides coated with poly-L-lysin and fixed in 100% acetone for 10 minutes. All incubations were performed at room temperature. An alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method was used as described by Schaumburg-Lever [7]. In brief, sections were preincubated with 10% normal rabbit serum in phosphate-buffered saline (PBS) for 10 minutes, and incubated for 1 hour with antibodies against T lymphocyte-related antigens (CD3, CD4, CD8, CD25: dilution 1:100; Becton Dickinson, San Jose, California), B lymphocytes (CD19: dilution 1:100; Immunotech, Marseille, France), NK cells (CD57: dilution 1:20; Becton Dickinson), monocyte-derived cells (CD1a: dilution 1:100; Becton Dickinson; CD14: dilution 1:300; Coulter/Immunotech, Westbrook, Maine;

CD68: dilution: 1:3000; DAKO, Glostrup, Denmark), granulocytes (CD16: dilution 1:100; CLB, Amsterdam, The Netherlands) or mast cells (tryptase: dilution 1:500; Chemicon, Temecula, California). The sections were subsequently rinsed and incubated for 30 minutes with rabbit anti-mouse-immunoglobulin antibodies (DAKO), rinsed again and incubated for 30 minutes with APAAP (DAKO). The immunoreaction was visualized by using a solution containing new fuchsin (Chroma-Gesellschaft, Köngen, Germany), sodium-nitrite, naphtol phosphate, dimethylformamide and levamisol in TRIS-HCl buffer (pH=8.0). Slides were counterstained with Mayer's hematoxylin and mounted in glycerol-gelatin (Merck, Darmstadt, Germany). The negative controls comprised concentration-matched mouse IgG (Becton Dickinson) and omission of the first and second step.

Assessment of immunostaining

Two investigators (T.E.H. and V.A.) independently assessed the density of positive cells and compared them with the expression in normal skin from healthy persons undergoing plastic surgery. If no consensus was reached, the mean value was calculated.

The density of positive cells was scored using a semi-quantitative scale ranging from 0 to 3 as described previously (0 = no positive cells, 1 = density of positive cells comparable with normal skin, 2 = increased density and 3 = markedly increased density of positive cells) [8]. As granulocytes, B lymphocytes and NK cells are normally absent, their density was scored 0 in normal skin.

Differences in score over time, differences between unburned and burned skin as well as differences between normotrophic and hypertrophic scars were statistically analyzed using the Mann-Whitney U-test (SPSS version 5.0.2, SPSS, Chicago, USA, 1993). A P-value equal to or lower than 0.05 was considered statistically significant.

RESULTS

Of the 22 burn patients that were initially included, four patients were lost to follow-up. After the second and the third biopsy a distinction was made between normotrophic and hypertrophic scars. This resulted in 38 biopsies at 1 month post-burn, 34 biopsies (23 normotrophic and 11 hypertrophic scars) at 4 months post-burn and 36 biopsies (24 normotrophic and 12 hypertrophic scars) at 7 months post-burn.

Histology

In hematoxylin-eosin stained sections, the epidermis of burn scars showed a normal architecture, except for the epidermal ridges and dermal papillae which were not as deep and as numerous as in the control skin. The epidermis at 1 month post-burn contained only sporadically inflammatory cells, which were not seen at 4 and 7 months and in the normal control skin.

The dermis of the normotrophic scars showed a normal scar architecture, whereas the hypertrophic scars showed the typical dermal collagen organization of whorls and nodules [9]. The cellular infiltrate in both scar types was mainly located perivascularly in both the papillary and the reticular dermis. At 1 month post-burn there was a dense cellular infiltrate. At 4 months this infiltrate was decreased, until only small remnants were present at 7 months, comparable with the normal control skin.

Cellular infiltrate at 1 month post-burn

Large numbers of $CD3^+$ T lymphocytes were present at 1 month post-burn, predominantly $CD4^+$ T helper lymphocytes (table 1). The number of $CD8^+$ T lymphocytes was also increased, but far less than the $CD4^+$ population (CD4/CD8 ratio = 10:1, compared to a 1:1 ratio in control skin). In contrast to the $CD4^+$ T helper lymphocytes, which were localized perivascularly, the $CD8^+$ T lymphocytes were scattered over the whole dermis. NK cells (CD57), which are normally not present in the skin, were also not observed in any burn scar. B lymphocytes (CD19), also not present in normal skin, were observed in 21 of 36 one month old scars. If present, the B lymphocytes were located in clusters.

At 1 month post-burn significantly increased numbers of monocytes (CD14), macrophages (CD68) and granulocytes (CD16) were present, mainly located perivascularly, but numbers of Langerhans cells were normal (CD1a; figure 1A).

Mast cells, characterized by tryptase, were slightly increased in the burn scars compared to normal skin. One month post-burn 23 of 35 scars showed an increased incidence of mast cells.

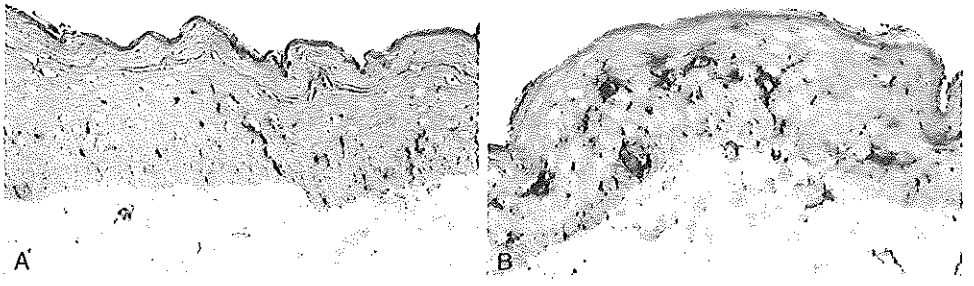

Figure 1. (A) Burn scar 1 month post-burn stained with an antibody against CD1a (specific for Langerhans cells). (B) The same burn scar as shown in A, 7 months post-burn, stained with an antibody against CD1a. Note the increased density of Langerhans cells. Original magnification ×400.

Cellular infiltrate at 4 and 7 months post-burn

At 4 and 7 months post-burn the $CD3^+$ T lymphocyte fraction had decreased to normal values, but the ratio was still not in balance (CD4:CD8 ratio after 7 months = 7:1). In normal skin few IL-$2R^+$ (CD25) T lymphocytes were present around blood vessels,

but in burn scars they were sporadic or even absent. At 4 months IL-2R$^+$ cells were absent in 16 of 34 burn scars (P-value=0.02) and at 7 months in 24 of 36 scars (P-value<0.01).
Langerhans cell numbers in the epidermis steadily increased at 4 and 7 months post-burn (figure 1B). At 4 months 23 of 33 burn scars and at 7 months 23 of 36 burn scars showed an increased number of epidermal CD1a$^+$ Langerhans cells. At 4 months, no granulocytes were observed and monocyte numbers had normalized, whereas increased numbers of macrophages were still present in 17 of 34 four month old scars. Macrophage numbers normalized at 7 months.
Mast cells were increased in 18 of 32 burn scars at 4 months and in 25 of 36 burn scars at 7 months post-burn. They were scattered over the whole dermis (figure 2A+B). For all studied cell types there was no significant difference in their numbers between normotrophic and hypertrophic scars.

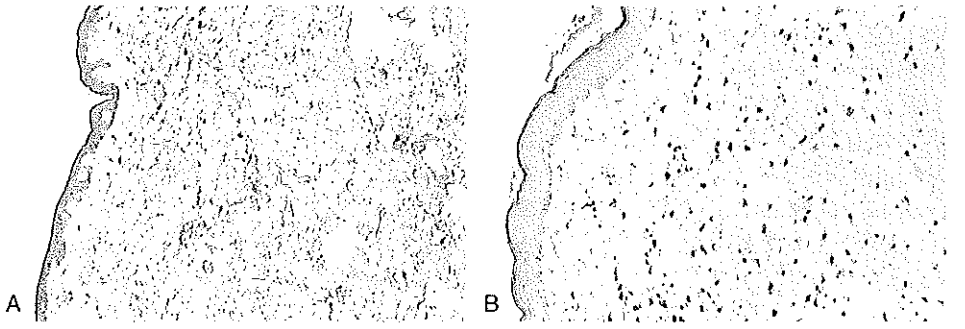

Figure 2. (**A**) Normal unburned skin stained with tryptase (specific for mast cells). (**B**) A burn scar 7 months post-burn stained with tryptase. Original magnification ×63.

DISCUSSION

This study shows that the infiltrate in the scars at 1 month post-burn consists, apart from granulocytes, monocytes and macrophages, predominantly of CD3$^+$CD4$^+$ T helper lymphocytes. CD8$^+$ lymphocyte and B lymphocyte numbers were also increased, but NK cells were absent.
It is known that T lymphocytes play an active role in wound healing, exerting many of their effects via cytokines and growth factors that have an influence on wound healing, such as proliferative and synthetic activities of fibroblasts and induction of neovascularization [4,10]. In the healing of rat flexor tendons, it was shown that predominantly CD4$^+$ T lymphocytes were present at the wound site [11] and that these activated CD4$^+$cells increased the speed of monolayer healing of microwounds in vitro [10]. The fact that more CD4$^+$cells are present in the burn wound, might be an indication that these cells play a more important role in wound healing than CD8$^+$ cells.

Concerning the infiltration of burn wounds by macrophages, we should keep in mind that macrophages are not only important in helping to clean the wounded area of undesirable debris and bacteria, but they are also essential for both fibroblast and keratinocyte proliferation and function, and for angiogenesis [4,12]. As our results show that macrophage numbers had normalized at 7 months post-burn, these cells seem to be functionally important only in the early months of tissue remodeling.
$CD1a^+$ Langerhans cells were increased at 4 months post-burn. In contrast to macrophages, their number remained increased up to 7 months, as were the mast cells. The Langerhans cells present in the epidermis of the late burn scar might have migrated from the non-injured surrounding epidermis [13]. They may also have developed from $CD14^+$ monocytes, abundantly present at 1 month post-burn, or from another bone marrow derived precursor [14]. It was observed that in the peripheral blood of burn patients in the early post-burn period, potential Langerhans cell precursors were present in significantly increased numbers, suggesting those cells are en route from the bone marrow to the epidermis [15].
The cells' most studied role within the epidermis is the immune surveillance, with a function of antigen uptake, processing and presentation to lymphocytes either in the skin or in the local lymph nodes [16]. However, on serial sections we observed that Langerhans cells present in the epidermis of the burn wound scar were only sporadically HLA-DR positive (figure 3A+B), indicating that some of the Langerhans cells repopulating the epidermis after a burn wound may have a different state of activation and consequently another function than antigen presentation. Epidermal Langerhans cells might have a regulating role comparable to the transglutaminase coagulation factor $XIIIa^+$ dermal dendrocyte, that are a source of specific cytokines.

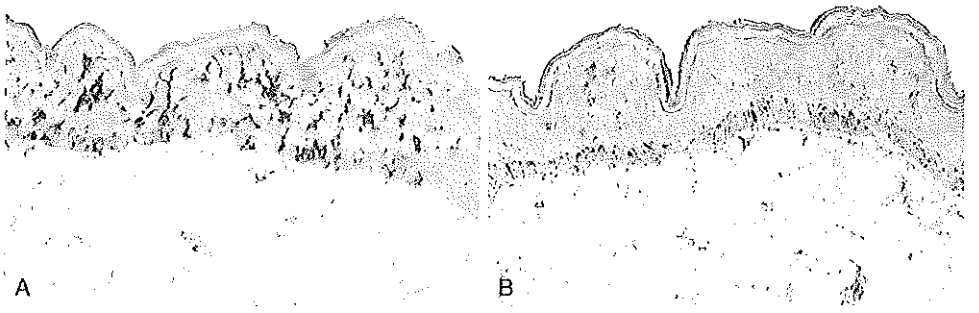

Figure 3. (A) Burn scar 7 months post-burn stained with an antibody against CD1a. (B) The same burn scar as shown in A, 7 months post-burn, stained with an antibody against HLA-DR. Note there is only sporadic positivity for $HLA\text{-}DR^+$ in the epidermis. Original magnification ×250.

In psoriasis, factor XIIIa$^+$ dermal dendrocytes stimulate keratinocytes to secrete TGF-α, a potent mitogen for keratinocytes [17]. This effect was mediated through TNF-α, a cytokine that is also produced by Langerhans cells [18].
Langerhans cells, just like factor XIIIa$^+$ dermal dendrocytes in normal skin, may interact with another important regulating cell in burn wound healing, the mast cell [19]. Like Langerhans cells, mast cells were also increased in the burn scar compared to normal control skin. The observed relationship between Langerhans cells and mast cells might be mediated through sensory nerves, as it is known [20] that neuropeptides released by sensory nerves that innervate the skin, can directly modulate the functions of Langerhans cells and mast cells. Both Langerhans cells and mast cells are able to produce an anthology of inflammatory mediators [18,21,22], that are capable of influencing fibroblast growth, collagen production and matrix remodeling activity [12,21,23]. Mast cell tryptase is a mitogen for fibroblasts [24] and mast cell chymase cleaves type I procollagen, an important constituent of the regenerating dermis, to a fibril-forming collagen molecule [25]. When mast cells are co-cultured with endothelial cells or fibroblasts, they develop a network of plasma membrane contacts that contain numerous cytoplasmic microfilament bundles [26], suggesting a close relationship with those important effector cells in wound healing. Summarizing, our data may indicate that Langerhans cells and mast cells have a regulatory function in the remodeling phase of burn wound healing rather than a function in immune surveillance.
We failed to find significant differences between normotrophic and hypertrophic scars with respect to the number of T lymphocytes, and also of the other studied immune cells. In contrast, in tattoo removal scars, more T lymphocytes were present in 4- and 5-months hypertrophic scars than in normotrophic scars [27]. The conclusion of the latter study that in hypertrophic scars the continued presence of lymphocytes and an imbalance in their cytokine secretion is the cause of the excessive fibrosis, cannot be confirmed by our results. Instead, intrinsic disturbances of fibroblasts are possibly responsible for hypertrophic scarring of burn wounds. Fibroblasts of hypertrophic burn scars demonstrated significantly increased tritium-thymidine uptake in response to EGF and an increased synthesis of collagen in response to TGF-β1, compared to fibroblasts from unaffected skin [28]. During wound healing, wound fibroblasts synthesized nitric oxide, which in turn, in an autocrine manner, increased their own collagen synthesis [29]. Similar in vitro studies, for example, of co-cultivation of fibroblasts with lymphocytes, Langerhans cells and mast cells from burn wounds are needed to elucidate the precise contribution of each cell type involved in the remodeling phase of burn wound healing.

Acknowledgements
We thank Mr. T.M. van Os for preparing the figures and Prof. H. Drexhage and Prof. R. Benner for helpful advice. This work was supported by the Dutch Burns Foundation.

Table 1. Density of positive cells or staining intensity in spontaneously healed partial-thickness burns at different time points compared with matched unburned skin of the same patient.

cell type	post-burn time (in months)	unburned control skin (mean score)	burned skin (mean score)	P-value (Mann-Whitney U-test)
Langerhans cells ($CD1a^+$)	1	1.4	1.0	*
	4		1.8	0.02
	7		1.8	0.05
monocytes ($CD14^+$)	1	1.2	1.8	<0.01
	4		1.3	*
	7		0.9	0.02
granulocytes ($CD16^+$)	1	0.1	1.1	<0.01
	4		0.2	*
	7		0.1	*
macrophages ($CD68^+$)	1	1.1	2.3	<0.01
	4		1.5	0.01
	7		1.3	*
T lymphocytes ($CD3^+$)	1	1.1	1.6	0.01
	4		1.1	*
	7		0.9	*
T helper lymphocytes ($CD4^+$)	1	1.3	2.2	<0.01
	4		1.4	*
	7		1.2	*
T cytotoxic lymphocytes ($CD8^+$)	1	1.0	1.3	0.05
	4		1.1	*
	7		0.9	0.09
B lymphocytes ($CD19^+$)	1	0.0	1.0	<0.01
	4		0.5	<0.01
	7		0.1	*
Activated T+B lymphocytes ($CD25^+$)	1	1.1	1.3	*
	4		0.6	0.02
	7		0.4	<0.01
NK cells ($CD57^+$)	1	0.0	0.1	*
	4		0.1	*
	7		0.0	*
mast cells (tryptase$^+$)	1	1.4	1.5	*
	4		1.6	*
	7		1.7	0.08

* $P \geq 0.1$

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4.2 NERVE OUTGROWTH AND NEUROPEPTIDE EXPRESSION DURING THE REMODELING OF HUMAN BURN WOUND SCARS*

A 7 MONTH FOLLOW-UP STUDY OF 22 PATIENTS AND A REVIEW OF THE LITERATURE

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* Submitted for publication

ABSTRACT

Increasing data suggest that the skin nerve system is significantly involved in wound healing. This is thought to occur mainly by the biological effects of neuropeptides released from ingrowing nerves. In order to investigate the role of the nervous system on the burn wound healing process, we monitored immunohistochemically the nerve outgrowth and the expression of the neuropeptides substance P, neurokinin A, calcitonin gene-related peptide, vasoactive intestinal peptide and neuropeptide Y in spontaneously healed partial-thickness burns and compared this with the expression in matched unburned skin.

Punch biopsies were taken at 1, 4 and 7 month post-burn. The results showed that nerve fibers regenerated in spontaneously healed partial-thickness burns, in both the dermis and the epidermis. This regeneration gradually increased in the course of time, but during the observation period did not reach the levels present in matched unburned skin. We found a significantly higher number of nerve fibers in normotrophic scars compared with hypertrophic scars. This suggests a possible regulating role for nerves in the scar tissue remodeling process. However, the number of neuropeptides containing nerves did not differ between normotrophic and hypertrophic scars, suggesting the studied neuropeptides do not have a major influence.

The data are discussed in combination with a review the literature on the biological properties of neuropeptides in skin and immune cells, especially with respect to wound healing.

INTRODUCTION

Human skin is packed with sensory nerve fibers. Their main function is to signal the presence of nociceptive stimuli to the central nervous system, where they will be interpreted as pain or itch (the orthodromic response). In case of a skin injury, signals are emitted by the sensory nerves from the injured tissue to the central nervous system. Besides the orthodromic response, the sensory nerves are able to signal efferently to the skin, the antidromic response [1]. As a result of the latter, mediators called neuropeptides are released by the nerve endings into the skin [2]. Neuropeptides are a family of extracellular messengers, which act as neurotransmitters, hormones or paracrine factors [3]. Numerous neuropeptides, including calcitonin gene-related peptide (CGRP), substance P (SP), neurokinin A (NKA) and vasoactive intestinal peptide (VIP) are expressed in sensory nerves [4]. The skin is also supplied with autonomic nerve fibers, both of the parasympathetic and sympathetic system, which innervate blood vessels and sweat glands. Neuropeptide Y (NPY) is expressed in a large population of the sympathetic neurons [4]. Protein gene product 9.5 (PGP 9.5) is a general cytoplasmic marker demonstrating all types of sensory and autonomic nerve fibers. Antibodies against PGP 9.5 consequently visualize the whole innervation of the skin [5].

An increasing amount of data suggests that the skin nerve system also significantly contributes to wound healing. This is thought to occur mainly by the biological effects of the released neuropeptides. Neuropeptides have been shown to modulate a number of important steps in normal wound healing, not only by affecting vasodilation and the inflammatory response, but also by stimulation of proliferation of epithelial, vascular and connective tissue cells [6]. Evidence of the contribution of nerves to wound healing comes from the delayed wound healing after surgical resection of cutaneous nerves in animal models [7-10], and from patients with cutaneous sensory defects due to spinal cord injury [11,12]. These trophic effects of the nervous system are possibly mediated through neuropeptides.

The beneficial clinical effects of capsaicin in diseases characterized by severe pain or itch gives more insight into the role of neuropeptides. Capsaicin is the active molecule that gives hot red peppers its hot taste. It selectively activates sensory nerves to release a group of neuropeptides, such as SP, NKA and CGRP [13]. It leads to desensitization of fibers to exogenous stimuli and even to degeneration of the sensory nerves [14]. If capsaicin is applied to burn wounds in rats, the healing process is delayed [15] and in case of grafts, the survival rate of the grafts is dramatically decreased [8]. This indicates that neuropeptides and the skin nerve innervation are important for the wound healing process and the survival of grafted tissues.

For effective signaling via neuropeptides expression of the functional receptors on the target cells is a prerequisite. In the skin, receptors for neurotransmitters are expressed by both immune, epidermal and dermal cells [6,16].

As neuropeptides are rapidly degraded, intimate contact between nerve fibers and target cells is also necessary. It has been demonstrated that cutaneous sensory fibers are in close contact not only with dermal blood vessels, mast cells and fibroblasts but extend up into the epidermis, where they are in intimate contact with both keratinocytes and Langerhans cells [5,17].

The aim of the present study was to investigate the distribution of neuropeptides during burn wound scar maturation. Insight in the sequential expression of neuropeptides during burn wound healing might lead to novel intervention strategies in problematically healing burn wounds.

MATERIALS AND METHODS

Patients and biopsies

Twenty-two patients, age 19-74 years (mean age: 40 years), were treated for burns at the Burn Center of the Red Cross Hospital in Beverwijk, The Netherlands. The extent of the total burn injury in each case varied from 5 to 78% (mean: 15%) of the total body surface area, with superficial to full-thickness burns. This study was approved by the Medical Ethics Committee of the Red Cross Hospital, Beverwijk, The Netherlands (no. 1091/95).

After obtaining informed consent from each patient, 3 mm punch biopsies were taken of two partial-thickness burns which were conservatively treated, at three time points: 1 month, i.e. after re-epithelialization had been completed (mean: 3.8 weeks, standard error (SEM): 0.4 weeks), 4 months (mean: 17.0 weeks, SEM: 0.9 weeks) and 7 months post-burn time (mean: 30.4 weeks, SEM: 0.9 weeks). Matched unburned skin of the same patient was obtained only at 1 month post-burn time. At 4 and 7 months post-burn time, the scars were classified as normotrophic or hypertrophic using the Vancouver Scar Scale, a classification system on the basis of consistency, elevation and color [18].
The biopsies were fixed in Zamboni's solution for 2 hours at 4°C and then stored in 0.1M phosphate-buffered saline (PBS) with 10% sucrose for 24 hours at 4°C. After that the biopsies were embedded in Tissue-tek (Miles, Elkhart, USA) and snap-frozen in liquid nitrogen.

Immunostaining
Serial 14µm cryostat sections were cut and attached to glass slides coated with 3-aminopropyltriethoxysilane (APES). The sections were fixed in 100% acetone for 10 minutes. Immunohistochemistry was performed using the avidin-biotin peroxidase complex (ABC) and nickel-diaminobenzidine enhancement [19]. In brief, endogenous peroxidase was blocked with 0.45% H_2O_2 and 0.1% sodium azide in PBS for 30 minutes. The sections were preincubated with 10% normal goat serum in PBS containing 0.05% Triton X-100 (rinsing buffer) for 15 minutes. The incubation with the primary antibody at the predetermined optimal dilution was performed overnight at 4°C; all other incubations were at room temperature. The antibodies applied in this study are listed in Table 1.
The sections were washed in the rinsing buffer and incubated for 1 hour with biotinylated goat-anti-mouse or goat-anti-rabbit immunoglobulins (BioGenex, San Ramon, CA, USA) containing 10% normal human serum, and finally incubated for 1 hour with ABComplex (DAKO, Glostrup, Denmark). After rinsing, the immunoreaction was visualized by using a mixture of 3'3'-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO, USA), nickelammoniumsulfate and 0.3% H_2O_2 for 5 minutes, slightly counterstained with Nuclear Fast Red (Sigma), dehydrated and embedded in Entellan (Merck, Darmstadt, Germany). The negative controls comprised concentration-matched mouse IgG (Becton Dickinson, San Jose, CA, USA), normal rabbit serum and omission of the first and second step.

Assessment of sections
Two investigators (T.E.H. and V.A.) independently examined the presence of stained nerve fibers in three linear millimeters of the biopsy, in both the epidermis and the papillary dermis. As not every biopsy showed sweat or sebaceous glands or hairfollicles, those structures were not taken into account. Initial quantification using computer-assisted image analysis was abandoned, as the available camera was not

Table 1. Specification of the antibodies used in this study.

Antibody specificity	Antibody code	Source	Dilution
PGP 9.5	13C4	UltraClone, Isle of Wight, UK	1:500
SP	PU069-UP	BioGenex, San Ramon, CA, USA	1:1750
NKA	B55-100	Euro-Diagnostica, Malmö, Sweden	1:2250
CGRP	M9013100	Locus Genex, Helsinki, Finland	1:25
VIP	PU044-UP	BioGenex, San Ramon, CA, USA	1:1000
NPY	N9528	Sigma, St. Louis, MO, USA	1:10000

able to detect small nerve fibers, especially those situated in the epidermis.
The number of nerve fibers was compared with matched unburned skin from the same patient. For all markers, the staining score was used in the statistical analysis. All statistical analyses were performed using SPSS software version 5.0.2 (SPSS, Chicago, USA, 1993). A P-value lower than 0.05 was considered statistically significant.

RESULTS

Biopsies from 22 burn patients were examined. Seven patients who initially joined the study were lost to follow-up. This resulted in 38 biopsies taken at 1 month post-burn, 28 biopsies (20 normotrophic and 8 hypertrophic scars) taken at 4 months post-burn, and 28 biopsies (18 normotrophic and 10 hypertrophic scars) taken at 7 months post-burn.
In the haematoxylin-eosin-stained sections, the epidermis of burn scars showed a normal architecture except for the epidermal ridges and dermal papillae which were either absent, or not as deep or as numerous as in matched unburned skin. The dermis of the normotrophic scars showed a normal scar architecture, whereas the hypertrophic scars showed the typical dermal collagen organization of whorls and nodules [20].

PGP 9.5 expression

Matched unburned skin showed an extensive pattern of nerve fibers in the dermis and epidermis (Table 2). In the burn scar nerve fibers were present already at 1 month post-burn. These fibers had increased after 4 and 7 months. However, within the study period the number of nerve fibers in the burn scars never reached the levels observed in the matched unburned skin (Figure 1A+B).

The nerve outgrowth in the dermis of normotrophic scars was more extensive at 7 months post-burn compared with that of hypertrophic scars, but not in the epidermis (mean number of nerves in the dermis of normotrophic scars was 8.4, in hypertrophic scars 4.4; Wilcoxon Signed Ranks test, P=0.047).

Table 2. Number of nerve fibers in the dermis or epidermis per 3 mm biopsy of matched unburned skin and in the burn scars 1, 4 and 7 months post-burn.

Marker	Matched unburned skin (mean score ± SEM)	Time point post-burn (months)	Burned skin (mean score ± SEM)	P-value (Wilcoxon Signed Ranks test)
PGP 9.5 (epidermis)	16.3 ± 3.5	1	2.0 ± 0.5	0.01
		4	4.1 ± 1.1	0.00
		7	9.0 ± 2.7	0.02
PGP 9.5 (dermis)	16.9 ± 3.6	1	3.7 ± 0.9	0.00
		4	8.2 ± 2.2	0.03
		7	7.0 ± 1.9	0.01
SP (dermis)	0.7 ± 0.2	1	1.6 ± 0.4	*
		4	1.6 ± 0.2	*
		7	0.8 ± 0.2	*
NKA (dermis)	0.7 ± 0.2	1	1.2 ± 0.3	*
		4	1.8 ± 0.5	*
		7	0.3 ± 0.1	*

*P>0.05

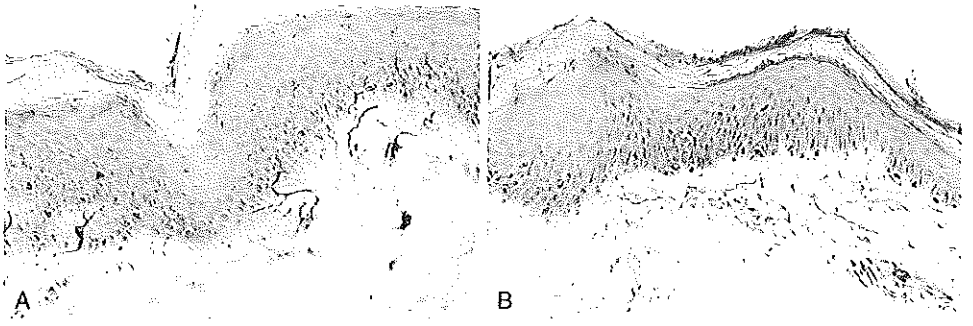

Figure 1. (**A**) Matched unburned skin stained with an antibody against PGP 9.5, showing nerves reaching into the epidermis. (**B**) A burn scar 4 months post-burn, stained with an antibody against PGP 9.5, showing regenerated nerves. Original magnification ×250.

Neuropeptide expression

In the dermis of the burn scars SP positive nerve fibers were detected in about 50% of the biopsies, but in the epidermis SP containing nerves were only observed sporadically, which was comparable to matched unburned skin. There was no significant difference in number of SP containing nerve fibers between burn scars and matched unburned skin. Also no significant difference was found between the scars at the different intervals post-burn.

The dermal infiltrate clearly contained SP positive immune cells. In the epidermis SP was also expressed by some Langerhans cells (Figure 2). Double stainings with tryptase (data not shown) showed that the SP positive dermal cells included mast cells. Due to background staining of the nickel-DAB substrate, quantification of neuropeptide-positive cells was not feasible.

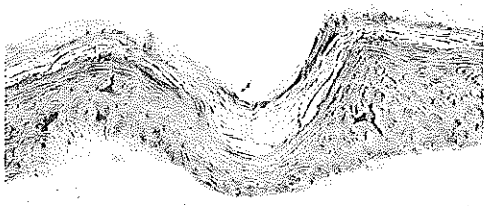

Figure 2. A burn scar 7 months post-burn, stained with an antibody against Substance P. In the epidermis two Langerhans cells show a positive staining. Original magnification ×400.

NKA containing nerve fibers were only detected in the dermis of both matched unburned skin and in the minority of the burn scars of the patients, at all 3 time points.

NKA positive nerve fibers were only sporadically detected in the epidermis of matched unburned skin, but not in the burn scars. The number of NKA positive cells in the dermis of post-burn skin, based on their histological appearance and location probably mast cells and endothelial cells, slightly increased in the course of time.
CGRP, VIP and NPY containing nerves were detected in the dermis of both unburned skin and burn scars, however, their number was too small for acceptable statistical analysis. Sweat glands, if present, were innervated by a large amount of VIP positive nerves.
In the dermis, sporadically CGRP, VIP and NPY neuropeptide positive infiltrate cells were present. In case of NPY these cells closely resembled mast cells.

DISCUSSION

Nerve outgrowth studies in wound healing

In our study we clearly showed that nerve fibers regenerate in spontaneously healed partial-thickness burns, in both the dermis and the epidermis. Their regeneration increased in the coarse of time, but within the follow-up period of 7 months did not reach the levels that were observed in matched unburned skin. A significantly higher number of nerve fibers was observed in normotrophic scars compared with hypertrophic scars. This suggests a possible regulating role for nerves in the scar tissue remodeling process.
Results of studies on the regeneration of nerve fibers in distinct kinds of healing wounds greatly differ in the literature, which might have several reasons.
First, the use of different protocols makes comparison of the results of different studies difficult. We followed the guidelines proposed by Ljungberg and Johansson in their extensive comparison of immunohistochemical techniques for detection of nerve outgrowth [21]. The thickness of the section is essential for the outcome of the study: the thicker the section, the more nerves one can evaluate in the sections. In different studies section thickness varied from 10 µm [22] to 100 µm [23].
Second, the age of the subjects also seems to be of importance. When full-thickness skin wounds were made on the foot of 1 week old rats, hyperinnervation was observed, but this reinnervation pattern was far less and only transient when similar wounds were made in adult rats [23]. In humans it was also shown that the normal intraepidermal innervation pattern is more extensive in children than in adults [24].
Third, the origin of the wound is of importance. In rats it was shown that in surgical full-thickness wounds reinnervation started as early as 3 days after wounding and resulted in hyperinnervation [23,25], whereas in burn wounds reinnervation started only after 1 week, without showing hyperinnervation [26]. In humans, superficial skin wounds showed extensive sprouting of nerves [27], whereas after deeper split-thickness skin grafts nerve fibers did not appear in the dermis or epidermis [28,29].
Fourth, the use of different species is of influence on the outcome of nerve regeneration studies. A difference in the distribution of neuropeptide containing nerves in

distinct species was found, being most abundant in pig and least extensive in man [13,30].
Fifth, we observed that the number of nerve fibers greatly differed in normal skin from various body sites.
We conclude that standardization of the nerve staining protocol is required for reliable comparison of results.

Neuropeptides studies in wound healing

In our study we observed that neuropeptide containing nerves regenerate in spontaneously healed partial-thickness burns.
Results of neuropeptide stainings meet the same kind of controversy as that of nerve outgrowth. It was shown that neuropeptide containing nerve fibers regenerate 2 weeks after wounding in rat and mice skin [31,32], but in guinea pigs neuropeptide containing nerves can be detected already 2 days after wounding [33]. These occurred in the vicinity with regenerating blood vessels in the granulation tissue.
In humans, grafted partial-thickness burns showed SP positive nerve fibers at 4 weeks post-burn, in numbers similar to that of normal skin [34]. However, another study failed to show neuropeptide containing nerves in post-burn normotrophic scars [22]. In contrast to our results, another study showed that the density of NPY, VIP, SP and CGRP containing nerves was greater in hypertrophic scars than in normal skin [22]. The investigators in the latter study did not take into account the post-burn time-point. Furthermore, they used FITC resulting in a high concomitant background fluorescence in the dermis, which complicates the quantification of nerve fibers in the dermis.
We showed that in the epidermis of a few burn scars SP positive Langerhans cells were present. It is known that Langerhans cells are in close contact with epidermal nerves, which is essential for effective signaling, as neuropeptides are rapidly degraded [17]. It has also been demonstrated in vitro that Langerhans cells express SP receptors [35], but the biological effects of SP on Langerhans cells are largely unknown.
Possibly not a particular neuropeptide, but a specific combination or a balance between different neuropeptides and their antagonists depicts the outcome of neuropeptide regulated tissue regeneration [30]. For example, it has been shown that SP can induce the release of proteolytic enzymes that degrade CGRP from mast cells [36], emphasizing the influence different neuropeptides might have on each other.
It should be considered that sensory nerves are not the only sources of neuropeptides in the skin. A variety of neuropeptides is also expressed in keratinocytes, mast cells, monocytes, macrophages, granulocytes and T-lymphocytes [37]. So not only the number of nerve fibers, but as a matter of fact also the total amount of neuropeptides in the scar must be taken into account in case of investigating their role in the wound healing process.

Mode of action of neuropeptides in the inflammatory and proliferative phase of wound healing

Neuropeptides have increasingly become recognized as mediators of the early phase of wound healing in skin. The biological properties of different neuropeptides in the skin immune system are summarized in Table 3.

Injury of the skin and concomitant disruption of blood vessels lead to extravasation of blood constituents, followed by platelet aggregation and blood clotting. In this early phase, SP, NKA and CGRP can increase vascular permeability and extravasation [38-41]. SP can induce smooth muscle contraction [42] and in this way influence the blood flow to the wounded area in the acute phase. NPY, a sympathetic neuropeptide, influences vasodilatation and vasoconstriction by either the inhibition of the presynaptic receptor or activation of the post-synaptic receptor for sympathetic stimulation of vascular muscle tone [43].

After platelet aggregation and blood coagulation, immune cells are trapped and gradually continue to migrate into the wounded area. Neuropeptides are also capable of influencing processes involved in this part of the wound healing process. SP increases the chemotaxis of granulocytes [44,45] and activates mast cells to transcribe and release TNF-α [46], an early pro-inflammatory cytokine. The released TNF-α influences vascular permeability and induces the expression of adhesion molecules on endothelial cells. SP itself can also directly increase the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on human dermal microvascular endothelial cells [47,48] and in this way facilitate transmigration of cells of the immune system across the vessel wall [49,50]. CGRP can also influence the inflammatory phase of the wound healing process. It was found to prevent macrophage activation and to inhibit the ability of macrophages to produce hydrogen peroxide [51].

In the proliferation phase of the wound healing process, cells like fibroblasts, endothelial cells and keratinocytes migrate and proliferate in order to restore the functions of the skin. SP and NKA, both member of the tachykinin family of peptides, can influence different aspects of this phase by activation of the tachykinin receptors. These receptors are expressed by cells like smooth muscle cells, endothelial cells, fibroblasts, keratinocytes and various types of immune cells, which all have a role in the wound healing process [6,16].

Several neuropeptides can influence epithelial and fibroblast migration and proliferation [52-67]. For example, VIP can inhibit epidermal growth factor (EGF)-induced proliferation [68]. On the other hand, it was also suggested that VIP is mitogenic for human keratinocytes in vitro [58,59,69,70]. Controversial effects of VIP may be due to different expression of the two receptors for VIP (VIP receptor I and II). It has been shown that these two receptors mediate distinct immunoregulatory effects [71]. Consequently not the presence of VIP itself, but of the pertinent receptor type may determine the response and outcome.

Table 3. Biological properties of neuropeptides on skin and immune cells and their potential function in wound healing.

	Granulocytes	Monocytes + Macrophages	Lymphocytes	Mast cells	Fibroblasts	Endothelial cells	Keratinocytes
SP	↑ chemotaxis [44,45,84] ↑ migration [44,45,84] ↑ activation [85] ↑ adhesion to endothelial cells [86]	↑ chemotaxis [87] ↑ IL-1, IL-6, TNF-α and IFN-γ production [88,89] ↑ proliferation [90]	↑ proliferation [90,91] ↑ IL-2, IL-4, IL-10 and IFN-γ production [88,92]	↑ histamine release [46,93] ↑ TNF-α release [46,93]	↑ migration [60,61,63,66] ↑ proliferation [60,61,63,66] ↑ matrix metalloproteinase-2 activity [79]	↑ ICAM-1, VCAM-1, E- and P-selectin expression [47,48,84] ↑ proliferation [63,74] ↑ differentiation [63,74]	↑ migration [52,53,57] ↑ proliferation [52,53,57] ↑ IL-1α, IL-1β, IL-8 and GM-CSF production [37,94]
NKA	↑ activation [85] ↑ adhesion to endothelial cells [86]	-	-	↑ histamine release [95]	↑ migration [61,63] ↑ proliferation [63] ↑ chemotaxis [61]	↑ proliferation [63]	-
CGRP	↑ adhesion to endothelial cells [96,97] ↑ activation [98]	↓ activation [51] ↓ H_2O_2 production [51] ↑ phagocytosis [99]	↓ proliferation [100] ↑ IL-2, IL-4, IL-10 and IFN-γ production [92]	↑ histamine release [101,102] ↑ TNF-α release [101,102]	↑ IL-6 production [103]	↑ proliferation [73]	↑ proliferation [59]
VIP	↓ cytotoxicity [104]	↑ IL-10 production [105] ↓ IL-12 production [106]	*VIPrec I:* ↓ chemotaxis [71] *VIPrec II:* ↓ proliferation [107]	-	-	-	↑ migration [54]
NPY	↑ adhesion to endothelial cells [108]	↑ chemotaxis [109] ↑ activation [109]	↑ IL-2, IL-4, IL-10 and IFN-γ production [92]	↑ histamine release [110]	-	↑ proliferation [111,112] ↑ differentiation [111]	↓ proliferation [59]

Substance P, NKA and CGRP influence angiogenesis by stimulating the proliferation of arterial smooth muscle cells and endothelial cells [62,63,72,73], while SP also enhances angiogenesis by stimulating endothelial cell differentiation, to form new capillaries [74-76].

Mode of action of neuropeptides in the maturation and remodeling phase of wound healing

Relatively little is known about the role of neuropeptides in the tissue remodeling phase of wound healing. The main feature of the maturation phase is the deposition and remodeling of collagen in the wound. The degradation of wound collagen is controlled by a variety of collagenases derived from granulocytes, macrophages, keratinocytes and fibroblasts [77]. These specific enzymes are able to degrade the triple helical structure of collagen at specific sites [78]. It has been shown that SP can influence this process by increasing the overall matrix metalloproteinase-2 activity in fibroblasts [79].

Despite earlier suggestions [22], the role of SP in inducing hypertrophic scarring might be minor, as SP induces fibroblast proliferation, which is not upregulated in hypertrophic scars. The higher numbers of fibroblasts in hypertrophic scars compared with normotrophic scars [80] is not the consequence of excessive fibroblast proliferation, but due to a decreased level of apoptosis-inducing proteins in fibroblasts of hypertrophic scars [81-83].

Final remarks

Studies on the role of neuropeptides in the wound healing process need to take into account not only the presence of neuropeptides, but also the presence of receptors and enzymes involved in the degradation process. Recent developments in neuropeptide research, like the availability of specific receptor antagonists, allow functional studies which will give more insight into the role of specific neuropeptides and the nervous system in wound healing and will provide tools to interfere in case of a disturbed wound healing process.

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4.3 REGENERATION OF (PRO)COLLAGEN I AND ELASTIC FIBERS DURING BURN SCAR MATURATION

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ABSTRACT

Although it is known that collagen and elastic fibers regenerate in scars, no prospective study has yet been performed to investigate the dynamics of procollagen I and elastic fiber regeneration in burn wound scars. The aim of this study was to obtain insight into the regeneration and structure of collagen I and elastic fibers in spontaneously healed partial-thickness burns. Especially the difference in the regeneration of collagen and elastic fibers in normotrophic and hypertrophic burn scars was investigated. As TGF-β1 is a potent enhancer of both collagen I and elastin synthesis, we also monitored the expression of TGF-β1 mRNA in fibroblasts in the burn scars.
Punch biopsies were taken of partial-thickness burns at 1 month post-burn and of site matched control unburned skin. At 4 and 7 months post-burn biopsies were taken of the normotrophic or hypertrophic scars developed in these burns. The biopsies were analyzed for procollagen I and elastic fibers on ultrastructural and light microscopic level, and with in situ hybridization for the detection of TGF-β1 mRNA.
We showed that fibroblasts in the burn scars expressed a higher level of TGF-β1 mRNA compared with control unburned skin.
The expression of procollagen I was increased in post-burn scars, and it remained elevated even until 7 months post-burn. Procollagen I was significantly more abundant in hypertrophic scars than in normotrophic scars, both at 4 and 7 months post-burn.
Elastic fibers were detected in the newly formed papillary dermis of burn scars already at 1 month post-burn, suggesting that elastogenesis by fibroblasts starts in an early post-burn phase.
We conclude that the synthesis of collagen and elastic fibers in partial-thickness burn scars is a rapidly starting process and continues for several months.

INTRODUCTION

During wound healing, a sequence of inflammation, tissue synthesis and reorganization results in the formation of mature scar tissue. Skin repair after burning is essentially similar to healing after simple trauma, yet burn-related ischaemia, hypoxia and edema slow down wound healing [1]. The outcome of burn wound healing can be normotrophic healing or abnormal wound healing resulting, for example, in chronic ulcers or hypertrophic scars [1].
The connective tissue matrix of the skin consists of interwoven, mainly collagen, fibers, which are packed in bundles. When the skin is stretched, collagen, with its high tensile strength, prevents tearing, and the elastic fibers return it to its original state. Collagen makes up to 70-80% of the dry weight of the dermis, whereas elastic fibers only account for about 2% [2]. Skin contains primarily type I collagen, with smaller amounts of collagens type III, type IV and type V [3]. Procollagen I is synthesized by fibroblasts. After excretion from the cell, procollagen peptidases cleave the

propeptides. The collagen I molecules, with a molecular weight of 285 kDa, can now form fibrils and cross link to each other to form a stable structure [4]. Elastic fibers, on the other hand, consist of two distinct protein components: the amorphous elastin core and a surrounding elastic microfibril. Mature elastin fibers are made of individual polypeptide chains, called tropoelastin (molecular weight 72 kDa), which are covalently linked to the microfibrillar component [4].
TGF-β1 stimulates collagen I transcription [5-7] and stabilizes elastin mRNA [8]. In this way TGF-β1 is a potent enhancer of both collagen I and elastin synthesis.
It is known that collagen and elastic fibers regenerate in scars [9,10], but no prospective study has yet been done to demonstrate the regeneration of these fibers in spontaneously healed partial-thickness burn wounds. We therefore monitored the regeneration of both procollagen I and elastic fibers using transmission electron microscopy and immunohistochemistry in normotrophic and hypertrophic burn wound scars during a 6-months follow-up.

MATERIALS AND METHODS

Twenty-two patients aged 19-74 years (mean age: 41 years) were treated for burns at the Burn Center of the Red Cross Hospital in Beverwijk, The Netherlands. The extent of the total burn injury in individual cases varied from 5% to 62% (mean: 16%) of the total body surface area. The protocol of the present study has been approved by the Medical Ethics Committee of the Red Cross Hospital in Beverwijk. After informed consent had been obtained from each patient, two 3-mm punch biopsies were taken of two spontaneously healed partial-thickness burns at three time points: 1 month (after re-epithelialization has been completed; mean: 4.8 weeks, standard error (SEM): 0.3 weeks), 4 months (mean: 17.8 weeks, SEM: 0.8 weeks) and 7 months (mean: 30.6 weeks, SEM: 0.7 weeks) post-burn. Control unburned skin of the same patients was also obtained at 1 month post-burn. At 4 and 7 months post-burn, the scars were classified as normotrophic or hypertrophic using the Vancouver Scar Scale, a classification system based on consistency, elevation and color [11].
One biopsy of each burn was embedded in Tissue-tek (Miles, Elkhart, USA) and frozen in liquid nitrogen for in situ hybridization and immunohistochemistry and the other was immediately fixed in glutaraldehyde-formaldehyde for electron microscopy.

Electron microscopy

After glutaraldehyde-formaldehyde fixation, the biopsies were post-fixed in 1% (w/v) osmium tetroxide at 4°C and dehydrated in acetone. The specimens were embedded in LX 112 (Epon). Ultrathin sections were cut and mounted on copper grids (300 mesh) and contrasted with uranyl acetate (10 minutes at 45°C) and lead citrate. They were examined with a Zeiss 902 electron microscope.

In situ hybridization

In situ hybridization was performed on 5 µm frozen sections. We used a 500 bp *Sma*I-*Bam*HI fragment of TGF-β1 cDNA cloned into pBluescript KS (Stratagene, La Jolla, CA, USA) [12]. The specific cRNA probes were labeled with digoxigenin following the manufacturer's protocol (Boehringer, Mannheim, Germany). The in situ hybridization was performed as described previously [12,13]. Briefly, after pretreatment the sections were hybridized with 50 ng of the probe per slide during 16 hours at 62°C. Subsequently, sections were washed in 2x standard saline citrate (SSC) with 50% formamide at 50°C, then in 0.1x SSC with 20 mM β-mercaptoethanol at 62°C, and finally treated with 2U/ml RNAse T1 (Boehringer) in 2x SSC plus 1 mM EDTA at 37°C. The immunodetection of digoxigenin-labeled hybrids was done using nitro blue tetrazolium (NBT) as chromogen and bicholylindolyl phosphate (BCIP) as coupling agent (Boehringer). The sense riboprobes were included as negative controls and did not show any staining.

Immunohistochemistry

Serial 5-µm cryostat sections were cut and attached to glass slides coated with poly-L-lysin and fixed in 100% acetone for 10 minutes. The slides were stained by resorcin fuchsin to detect elastic fibers in light microscopy. An alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method was used for detection of procollagen type I as described previously [14]. In brief, sections were preincubated with 10% normal rabbit serum in phosphate-buffered saline (PBS) followed by incubation with mouse monoclonal antibodies against human procollagen type I. This antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, USA, and the Department of Biological Sciences, University of Iowa, Iowa City, USA (contract N01-HD-6-2915 from the NICHD).

All incubations were performed at room temperature. The sections were rinsed, incubated for 30 minutes with rabbit anti-mouse-immunoglobulin antibodies (DAKO, Glostrup, Denmark) and incubated for 30 minutes with APAAP (DAKO). The immunoreaction was visualized by using a solution containing new fuchsin (Chroma-Gesellschaft, Köngen, Germany), sodium-nitrite, naphtol phosphate, dimethylformamide and levamisol in TRIS-HCl buffer (pH=8.0). Slides were counterstained with Mayer's haematoxylin and mounted in glycerol-gelatin (Merck, Darmstadt, Germany). The negative controls involved concentration-matched mouse IgG (Becton Dickinson, San Jose, CA, USA) and omission of the first and second step.

Two investigators (T.E.H. and V.A.) independently assessed the staining intensity and compared it with the expression in control unburned skin. The staining intensity was scored, using a semi-quantitative scale ranging from 0 to 3 as described previously (0 = not detectable, 1 = light staining, 2 = moderate staining and 3 = strong staining) [14]. In most cases there was consensus, but in case of any discrepancy the mean value was calculated.

Differences in the score between control unburned skin with burned skin, differences over time, and differences between normotrophic and hypertrophic scars were statistically analyzed using the Mann-Whitney U-test (SPSS version 5.0.2, SPSS, Chicago, USA, 1993). A P-value equal to or lower than 0.05 was considered statistically significant.

RESULTS

Biopsies from 22 burn patients were examined. This resulted in 38 biopsies taken at 1 month post-burn, 34 biopsies (23 normotrophic and 11 hypertrophic scars) taken at 4 months post-burn, and 36 biopsies (24 normotrophic and 12 hypertrophic scars) taken at 7 months post-burn.

Histopathology

In the haematoxylin-eosin-stained sections, the epidermis of burn scars showed a normal architecture except for the epidermal ridges and dermal papillae which were not as deep or as numerous as in control unburned skin.
The dermis of the normotrophic scars showed a normal scar architecture, whereas the hypertrophic scars showed the typical dermal collagen organization of whorls and nodules [15].

The expression of TGF-β1

TGF-β1 mRNA was expressed by keratinocytes in the epidermis and by fibroblasts, endothelial cells and immune cells in the dermis of the burn scars at 1 month post-burn. The expression decreased at 4 and 7 months, but even at 7 months post-burn the expression in fibroblasts of the burn scars was clearly higher compared with matched unburned skin (figures 1A+B). In matched unburned skin TGF-β1 mRNA was expressed predominantly by basal keratinocytes and by some immune cells, endothelial cells and fibroblasts in the dermis.

Procollagen I

Procollagen I, only sporadically present in control unburned skin, was clearly upregulated throughout both the papillary and the reticular dermis in the burn scars at 1 month post-burn (figure 2). It was mainly present in the cytoplasm of fibroblasts. The semi-quantitative procollagen I expression was significantly increased in 31 of the 36 scars compared with unburned control skin (Mann-Whitney U test; $P<0.0005$). At 4 months and even at 7 months post-burn the procollagen I expression was still higher as unburned control skin ($P<0.0005$; figures 3A+B). At 7 months post-burn the procollagen was mainly located as fibers in the extracellular matrix of the dermis. The procollagen expression was significantly higher in the whole dermis in hypertrophic scars compared with normotrophic scars (figure 4), both at 4 months post-burn ($P<0.0005$) and at 7 months post-burn ($P=0.003$).

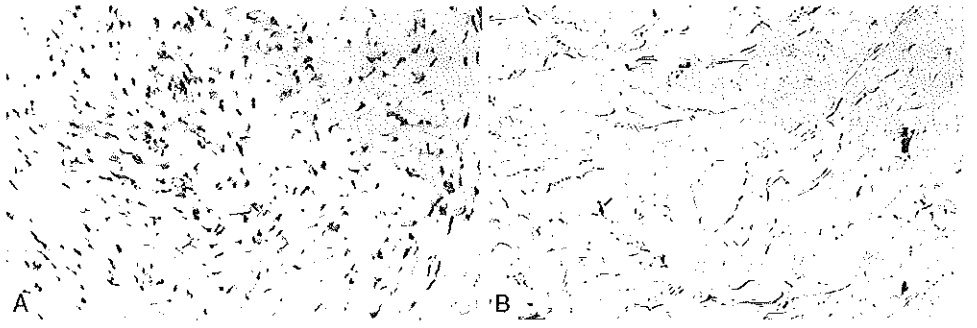

Figure 1 (**A**) In situ TGF-β1 mRNA expression in the dermis at 7 months post-burn, showing abundantly positive fibroblasts. (**B**) In situ TGF-β1 mRNA expression in the dermis of a matched unburned skin, showing only sporadically positive fibroblasts. Original magnification ×250.

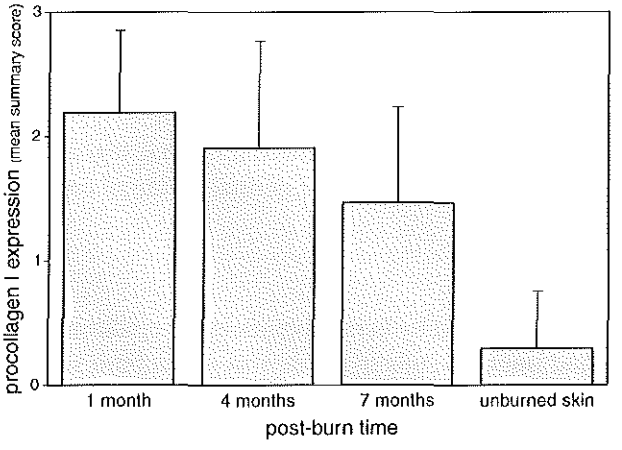

Figure 2. Semiquantitative score of the expression of procollagen I in burn scars at 1, 4 and 7 months post-burn, and in unburned control skin (± standard deviation).

Elastic fibers

In the unburned control skin, the resorcin fuchsin staining showed thin elastic fibers in the papillary dermis. Deeper in the reticular dermis the fibers were thicker and were running parallel to the skin surface.

At 1 month post-burn the elastic fibers were sporadically present in the papillary dermis of the burn scars. In the upper part of the reticular dermis elastic fibers were mostly absent, but in the deeper part they were present, although sometimes fragmented. At 4 and 7 months post-burn the amount of elastic fibers increased first in the reticular dermis and then in the papillary dermis. Their structure seemed to be different, i.e. thinner and shorter compared to unburned control skin. Within the observation period of 7 months the elastic fibers did not reach the levels present in the normal unburned control skin.

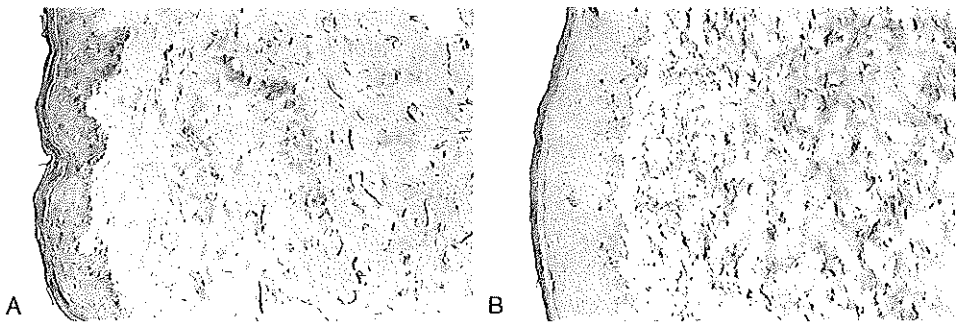

Figure 3. Immunostaining of procollagen I in (**A**) unburned control skin, showing no procollagen I expression, and (**B**) a burn scar at 7 months post-burn, showing procollagen I expression. Original magnification ×160.

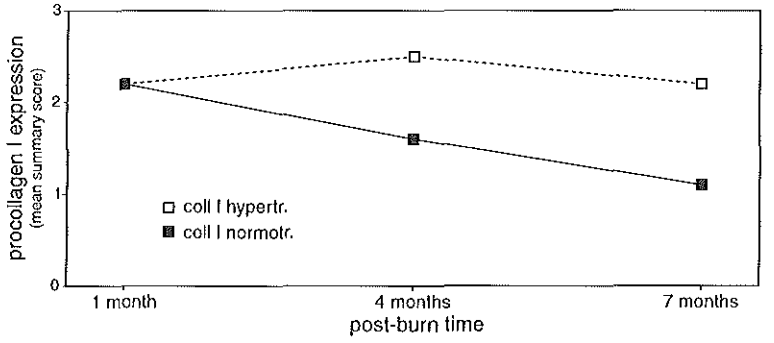

Figure 4. Comparison of the relative expression of procollagen I in normotrophic and hypertrophic burn scars.

Transmission electron microscopy showed that the elastic fibers in the dermis were fragmented at 1 month post-burn (figure 5). The elastic fibers showed a normal ultrastructural architecture on electron microscopic level at 4 months post-burn.

DISCUSSION

The wound strength is related to the deposition of collagen and other extracellular elements by fibroblasts. The net quantity of collagen in the skin at a certain timepoint is the result of a balance between biosynthesis and breakdown. Collagen deposition and matrix remodeling after wounding is a continuous process aimed to achieve maximal wound strength. During the remodeling phase collagen type III, the first collagen to be deposited in the granulation tissue, is gradually replaced by type I [16]. We showed the amount of procollagen I is increased in post-burn scars, and remains elevated even until 7 months post-burn. This is in agreement with another study which showed that collagen I mRNA levels were elevated in scars, whereas in normal skin

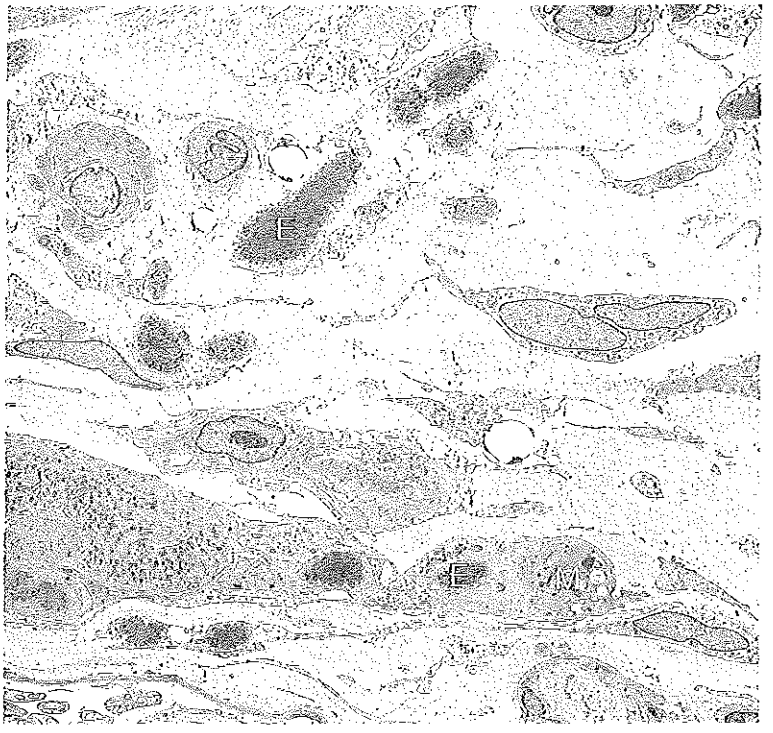

Figure 5. Burn scar at 1 month post-burn, showing fragmented elastic fibers (E) phagocytosed by a macrophage (M). Original magnification ×1100.

no collagen I mRNA expression was detected [17]. After binding of TGF-β1 to its receptor, a rapid induction of the transcription of collagen I takes place [5-7]. In situ hybridization showed that fibroblasts within the burn scars expressing a higher level of TGF-β1 mRNA compared with control unburned skin, supporting the idea that TGF-β1 induces collagen I transcription.

Matrix remodeling not only involves collagen synthesis, but also collagen degradation. Matrix metalloproteinases (MMP) form a family of zinc-dependent endopeptidases that play an important role in the breakdown of the extracellular matrix [18]. Their expression is under the control of a variety of cytokines, hormones and oncogene products [19], including TGF-β which is inhibitory for MMP expression [20].

Mast cell chymase can act as a procollagen peptidase that cleaves the procollagen I to the fibrilforming collagen I [21]. Furthermore, TNF-α produced by activated mast cells is able to stimulate MMP expression [22,23]. Since mast cell numbers are higher in the burn scars at 7 months post-burn compared with normal skin (see chapter 4.1), they may also be important regulatory cells in the metabolism of the extracellular matrix.

The amount of procollagen I is significantly higher in hypertrophic scars than in normotrophic scars, both at 4 and 7 months post-burn. A similar difference was pre-

viously reported at the mRNA level by Zhang et al. [17]. However, Ghahary et al. [24] showed that the accumulation of collagen I in hypertrophic scars is not only the result of a higher production, but also of a reduced matrix degradation by MMP.
Our results suggest that elastic fibers regenerate at an early stage in human partial-thickness burn wound scars. This confirms previous studies on human surgical wound scars [9,25,26]. The fragmented elastic fibers present in the burned area are phagocytosed by macrophages. Elastogenesis by fibroblasts starts early post-burn, as we detected elastic fibers in the newly formed papillary dermis of burn scars, although sporadically, already at 1 month post-burn. But even after 7 months, the appearance and quantity of the elastic fibers was not normal as the fibers were thinner and shorter than in control unburned skin. With confocal laser scanning we observed that the elastic fibers lengthen in the course of time. However, during the observation period of 7 months the three-dimensional network of the elastic fibers and collagen fibers did not return to normal, neither in normotrophic nor in hypertrophic scars (data not shown).
Elastin expression can be modulated by TGF-β1, which expression is increased in fibroblasts of the healing burn wound. TGF-β1 is a potent enhancer of elastin synthesis, largely via stabilization of its mRNA [8].
In summary, in this study we show that the synthesis of collagen and elastic fibers in partial-thickness burn scars is a rapidly starting process, which continues for several months.

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CHAPTER 5

General discussion

5 GENERAL DISCUSSION

Successful burn wound healing is the result of a complex crosstalk between fibroblasts, epithelial, endothelial and immune cells resulting in restoration of the integrity and function of the skin. This crosstalk includes direct cell-to-cell contact, cytokines and growth factors. The pathophysiology of the transition, whereby normal wound healing after burn injuries is modified in such a way that excess scar tissue is formed, is largely unknown.

In scar formation and particularly in hypertrophic scar formation the balance between collagen production and breakdown is the central aspect. Since fibroblasts are the producers of collagen, research has been mainly focused on the role of fibroblasts in the pathophysiology of scar formation. However, several other cell types are now increasingly recognized as key regulators of wound healing via crosstalk with fibroblasts and endothelial cells. These cells possibly are also involved in hypertrophic scar formation. The next paragraphs discuss the results described in this thesis aimed to answer the questions presented in chapter 2.1. Much of the data collected point to keratinocytes as one of the central regulating cell types involved in wound healing and hypertrophic scar formation.

Is epidermal activation and maturation altered during the development of hypertrophic scarring?

In chapter 3.1 we showed that keratinocytes express an altered phenotype in the wound healing process, even long after re-epithelialization has been completed. We observed a higher expression of markers for proliferation, differentiation and activation in the epidermis of scars than in normal control skin at 1 month post-burn. The keratinocytes showed an increased expression of keratins 5, 16 and 17, filaggrin, transglutaminase and CD36. All keratinocyte proliferation and differentiation markers showed normal expression at 7 months post-burn, with the exception of the activation marker CD36 that remained upregulated. This indicates that keratinocytes of burn scars are still in an altered state at 7 months post-burn. Furthermore, we demonstrated a striking difference in keratinocyte phenotype between normotrophic and hypertrophic scars at 4 months post-burn. Keratinocytes in hypertrophic scars expressed higher levels of proliferation, differentiation and activation markers than did normotrophic scars, as was demonstrated by the higher expression of keratin 16 and CD36. The keratinocytes in hypertrophic scars might be activated by factors originating from the dermis. Alternatively, keratinocytes in the hyperactivated state might also be the initiators of the hypertrophic process.

Can keratinocytes influence the process of hypertrophic scarring by producing growth factors with pronounced effects on the dermal compartment?

In chapter 3.2 we demonstrated in the epidermis of burn scars an increased production and altered distribution of TGF-β1 protein and mRNA, and of TGF-β2, TGF-β3, bFGF and VEGF. These growth factors have potential growth regulatory effects on other effector cells in the wound healing process, namely fibroblasts, immune cells and endothelial cells. TGF-β1 can increase the production of extracellular matrix proteins by fibroblasts [1]. So fibroblasts, the main source of the extracellular matrix, can be influenced by keratinocytes, which thereby influence the amount and composition of the extracellular matrix.

Keratinocytes can influence the production and the remodeling of the extracellular matrix in several ways. Recently, it was shown that when conditioned media from human keratinocyte cultures were added to human fibroblast cultures, a significant decrease in collagen synthesis was measured compared to fibroblast cultures grown without conditioned media [2]. Co-culture experiments with keratinocytes and fibroblasts showed that this effect was also present in case the cells were kept physically separated by a cell-impermeable membrane [2]. This suggests the effect is mediated by a soluble factor and not necessarily by cell-to-cell contact.

One of the potential factors in the crosstalk between keratinocytes and fibroblasts is PDGF. It has been shown that keratinocytes are a major source of cutaneous PDGF, but they do not express receptors for PDGF [3,4]. PDGF receptors do occur on mesenchymal cells. PDGF stimulates fibroblast proliferation and their production of extracellular matrix proteins [3,5]. This shows that PDGF is produced by keratinocytes not as an autocrine, but as a paracrine factor. Studies using recombinant human PDGF have shown that PDGF can improve wound healing [6].

Vice versa, keratinocytes can be influenced by fibroblasts, clearly illustrating the intimate crosstalk between these two cell types. Fibroblasts stimulate keratinocyte proliferation and in this way influence the re-epithelialization of wounds. It has been demonstrated that in cultures of keratinocytes grown on collagen gels containing viable fibroblasts, a virtually normal epidermal architecture was formed within 7-10 days [7]. The extensive cooperation between these cell types is further supported by the observation that the presence of keratinocytes as well as fibroblasts is necessary for the formation of the basement membrane of the skin [8].

Further evidence of the crosstalk between keratinocytes and fibroblasts is: (1) keratinocyte-derived IL-1α synergistically increases KGF and IL-6 production in fibroblasts [7,9]; (2) fibroblast-derived KGF and IL-6 stimulate keratinocyte proliferation [10-12]; and (3) fibroblasts on their turn decrease the IL-1 activity via negative feedback signaling [9], by increasing the production of IL-1 receptor antagonist [13].

Keratinocytes can also influence angiogenesis. In chapter 3.2 we showed that keratinocytes in burn scars express bFGF and VEGF, potent angiogenic factors. On their turn, vascular smooth muscle cells can produce KGF, an important mediator of

epithelial growth and differentiation. The vascular smooth muscle cells themselves do not express KGF receptors [14], indicating that these cells produce KGF for paracrine purposes, e.g. to influence keratinocyte proliferation. Keratinocytes can even upregulate the production of KGF in vascular smooth muscle cells, just like it does in fibroblasts, by producing IL-1, which increases their mRNA and protein level of KGF [14].

What is the contribution of immune cells in the healing process of normotrophic and hypertrophic scars?

Besides fibroblasts, other cells in the wound bed, like macrophages, lymphocytes, mast cells and endothelial cells, are also involved in the regulation of wound healing [15-17]. In chapter 4.1 we demonstrated that the infiltrate in burn scars 1 month post-burn consisted predominantly of granulocytes, monocytes, macrophages, T helper lymphocytes and B lymphocytes. Epidermal-dermal crosstalk also involves these immune cells, as it was previously shown that keratinocytes can influence the proliferation and maturation of cells of the immune system and that keratinocytes themselves have important immunologic functions [18-20]. The crosstalk between keratinocytes and T lymphocytes includes the promotion of T lymphocyte maturation by keratinocytes [21,22] and vice versa the influence of keratinocyte proliferation by T lymphocytes. When the supernatant of different T cell clones is added to keratinocyte cultures derived from normal skin, the proliferation decreases [23], but when added to keratinocyte cultures derived from pathological conditions, such as psoriasis, proliferation increases [24,25].

At 4 and 7 months post-burn the immune cell infiltrate had returned to normal levels, but mast cell and Langerhans cell numbers were still increased compared with normal control skin (chapter 4.1). Possibly these cell types are involved in the long-term remodeling of the healing burn wound. Mast cells can influence all phases of the wound healing process [26], by producing mediators like histamine, chymase, tryptase and TNF-α that influence fibroblasts, immune cells and endothelial cells in vitro [17,27]. As mast cell numbers increase in experimentally induced wounds, it is suggested that they also have a role in vivo [17].The number and function of mast cells is influenced by keratinocytes. Keratinocytes produce stem cell factor, a mast cell growth factor, and mast cell differentiation factors [28]. On their turn, mast cells can activate keratinocytes [27]. Trypsin, produced by skin mast cells, is able to upregulate mRNA levels of both granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 in keratinocytes. Furthermore it can increase the release of both cytokines [29].

We observed that Langerhans cell numbers were upregulated in the tissue remodeling phase of burn wound healing. As HLA-DR was not upregulated on the Langerhans cells, it is conceivable that these Langerhans cell are in a different stage of differentiation and thus probably exert another function than antigen presentation. Langerhans cells are also potent producers of several cytokines and growth factors [30] and

in this way can modulate the tissue remodeling. The close anatomical relationship between Langerhans cells and keratinocytes, whereby dendrites from Langerhans cells surround several keratinocytes, suggests an active signaling between these two cell types. Furthermore, Langerhans cells can in vitro only be cultured for a prolonged time when these cells are seeded into a skin equivalent, which is consistent with an important role of keratinocytes on the growth and differentiation of Langerhans cells [31,32].
Taken together, epidermal-dermal crosstalk, and in particular crosstalk between keratinocytes and immune cells, is possibly an important factor in the regulation of wound healing.

Are neuropeptides involved in the process of post-burn tissue remodeling?
The expression of neuropeptides and their roles are extensively discussed in chapter 4.2. Neuropeptides can regulate different aspects of the wound healing process, both in the early and in the late phase. We observed that the number of neuropeptide containing nerves was very limited in the burn scars, suggesting that neuropeptides derived from nerves do not play a major role in the burn wound healing process. However, we have to keep in mind that neuropeptides can also be produced by several other cell types. Substance P, which can influence several cell types in the wound healing process, can also be produced by mast cells for example [33], a cell type which occurs in increased numbers in burn scars.

Are elastic fibers regenerated in human burn wounds, and at which pace? Does the production of collagen normalize in the course of time?
In chapter 4.3 we showed that fibroblasts in partial-thickness burn wound scars regenerate elastic fibers. The procollagen I production was higher in hypertrophic scars than in normotrophic scars, demonstrating that this effector function of fibroblasts is dysregulated under these conditions. In chapter 3.2 we showed that keratinocytes produce TGF-β1, which is an important stimulatory factor for both elastogenesis and collagenesis [34-37].
Besides the increased production of procollagen I, the production of collagenases, the enzymes mediating collagen degradation, is impaired in fibroblasts from hypertrophic scars [38,39]. Together this leads to the accumulation of excessive extracellular matrix in hypertrophic scars.
Keratinocytes are able to produce collagenases themselves. This is particularly important during re-epithelialization when collagenases are necessary for facilitation of the movement of keratinocytes over the collagen-rich dermis [40]. Keratinocytes can also upregulate the production of collagenases in fibroblasts [41], emphasizing the balancing role keratinocytes may play in the net amount of collagen present in human skin.

Conclusions

We conclude that keratinocytes fulfil important regulatory functions in the epidermal-dermal crosstalk during the burn wound healing process (figure 1), and propose that the absence of these regulatory activities in the epidermis during the early phase of the wound healing process contributes to hypertrophic scarring. This is supported by the observations made when a burn wound is covered with a mesh graft. A mesh graft is a skin graft used to increase the surface of a split skin graft by making a wire-netting structure in the grafted skin. In a part of the patients treated with a mesh graft, hypertrophy will occur in the places where the epidermis did not cover the woundbed. Sites where epidermis was present from the beginning, generally heal in level with the surrounding skin (figure 2). The excessive amount of collagen synthesis in the absence of an epidermis suggests that epidermal cells normally prevent excessive matrix synthesis via interactive signaling.

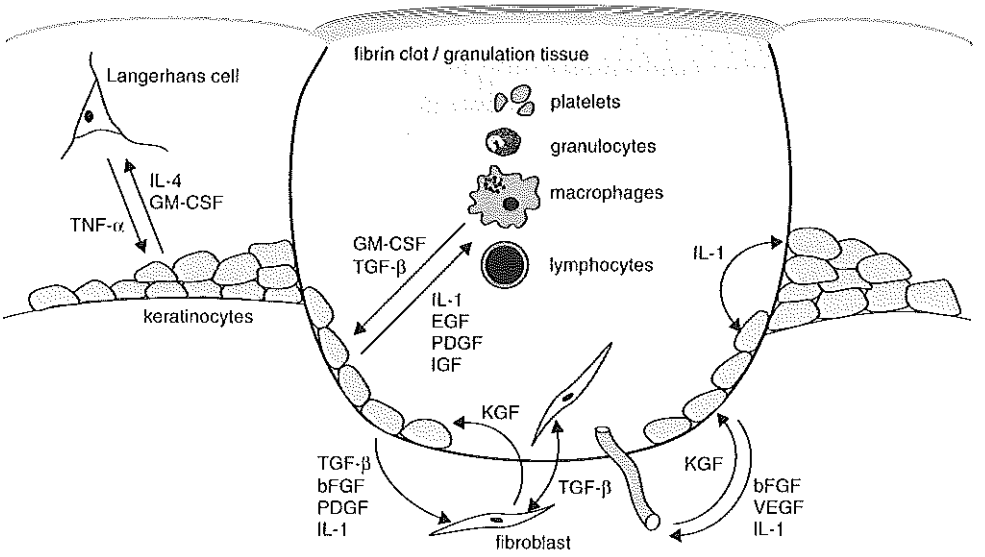

Figure 1. Cartoon illustrating the key players in the wound healing process.
In the early inflammatory phase platelets produce an anthology of vasoactive mediators, proteases, growth factors and chemotactic factors to recruit granulocytes, macrophages and lymphocytes.
Macrophages play a critical role in the transition between inflammation and repair, because they provide a second wave of growth factors and chemotactic factors necessary to promote the formation of granulation tissue. Granulation tissue supports the new epidermis.
Keratinocytes will then migrate over the wounded area and proliferate. By production of several cytokines and growth factors, they become important regulators of neovascularization, matrix formation, re-epithelialization and remodeling.

The pattern of the healed mesh graft shows two interesting points. First, the influence of the epidermis is strictly limited to the transplanted area, as only the places where the original epidermis of the graft was present, show normotrophic healing. This

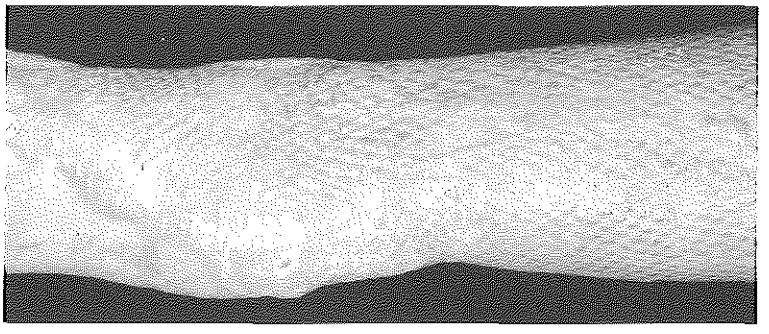

Figure 2. Burn wound scar on the leg of an 18-year-old man, treated with a mesh graft. Note the mild hypertrophy at the places where the graft did not cover the burn wound.

suggests the regulating factors produced by the epidermal cells act only locally. Second, the epidermal cells need to downregulate the fibroblast activity at a certain time point in the healing process. When epidermal cells are missing in the first weeks of the burn wound healing process, fibroblast collagen synthesis proceeds and hypertrophic scarring is the result. This is supported by the clinical observation that burn wounds that take longer than 3 weeks to re-epithelialize have a higher rate of hypertrophy [42]. Once the process of hypertrophy has started, it is probably difficult to stop, as delayed re-epithelialization will not prevent hypertrophic scar formation.
In a study where keratinocytes were seeded on the surface of fibroblast-populated type I collagen gels (lattices), it was observed that a 50% decrease in the amount of procollagen type I and type III mRNA was found in the fibroblasts of the lattices after 2 and 4 days, compared with lattices without keratinocytes. No significant modulation was found of other extracellular matrix proteins, like collagen types IV and VI, elastin and laminin [41]. This suggests a direct relationship between the absence of keratinocytes and hypertrophic scar formation, and that other cells in the epidermis, like Langerhans cells, seem to be less relevant for the regulation of extracellular matrix formation. Comparison of fibroblasts of hypertrophic scars with normal fibroblasts demonstrated that hypertrophic scar fibroblasts showed a higher collagen type I and type III mRNA expression and that they produced more collagen than fibroblasts from normal skin [43,44].
These were exactly the same extracellular matrix proteins of which the amount of mRNA was reduced in the fibroblasts of the lattices seeded with keratinocytes as mentioned above. These two studies together with our own in vivo observations, suggest not only a direct relationship between the keratinocytes and the fibroblasts in vitro, but also in hypertrophic scar formation in vivo.

Not all patients in similar circumstances will develop hypertrophy, so other factors influence this process as well. It is known that black and Asian people are 10 times more susceptible to keloid and hypertrophic scar formation than Caucasians, suggesting a genetic predisposition [45].

Future research

Further research is needed to unravel the precise molecular interactions between keratinocytes, fibroblasts and the other effector cells in wound healing. In vitro studies on the influence of different cytokines and growth factors, produced by keratinocytes, on the proliferation and function of fibroblasts should give more insight into the factors involved in the crosstalk between these cell types. As fibroblasts in scars are in a potentially activated state, it is important that in studies on hypertrophic scar fibroblasts the comparison is made with fibroblasts from normotrophic scars and not with fibroblasts from normal skin, as often has been done so far [46-51]. In vivo studies are crucial to increase the insight into the interaction of the different cell types, as the complex interactions in the skin can never be fully reproduced in vitro. For example, TGF-β1, a potent inhibitor of keratinocyte proliferation in vitro, can act stimulatory as well as inhibitory on keratinocyte proliferation in mouse skin in vivo [52].

In situ hybridization studies whereby different cytokines and growth factors in keratinocytes, fibroblasts and endothelial cells are closely monitored at different time points, should also give more insight into the factors influencing the wound healing process. In vivo studies using antagonists against these factors or neutralizing antibodies, can give important additional, functional information. As hypertrophic scarring is a process unique to man [53], research in other species is unlikely to give answers to the etiopathology of this disorder.

Left-right comparison studies with skin biopsies taken from burn patients whereby one part of the burn is treated with particular growth factors or inhibitors, and another part not, should be extremely valuable. However, such studies are likely difficult to perform from both the patient's and a medical ethical point of view. Already in the present study we encountered quite some difficulties in collecting biopsies from burn patients.

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SUMMARY

During wound healing, a sequence of inflammation, tissue regeneration and reorganization results in the formation of mature scar tissue. Skin repair after burning is essentially similar to healing after simple trauma, yet burn-related ischaemia, hypoxia and edema slow down wound healing. The process of burn wound healing can result in a normally healed wound or in an abnormally healed wound, for example a chronic ulcer or a hypertrophic scar. Besides disabling contractures and disturbing cosmetic aspects, hypertrophic scars also cause a variable degree of discomfort, such as severe itching.

The aim of this study was to search for factors in the burn scar which can be responsible for the formation of a hypertrophic scar and which may explain the clinical complaints such as pain and itch.

In our study, 29 burn patients were included which were treated at the Burn Center of the Red Cross Hospital in Beverwijk, The Netherlands. One month post-burn, i.e. when re-epithelialization was usually completed, 3 mm skin biopsies were taken of two partial-thickness burn-sites and of a control unburned skin-site. Three and six months later, biopsies were taken from the same burn-site as the 1 month post-burn biopsy. To investigate the involvement of keratinocytes, immune cells, neuropeptides and fibroblasts in burn wound healing and in particular in hypertrophic burn wound healing, we evaluated their presence by immunohistochemistry, electron microscopy and in situ hybridization in biopsy sections.

In chapter 3.1 we describe the analysis of the biopsies for markers of keratinocyte proliferation, differentiation and activation (keratins 5, 10, 16 and 17, filaggrin, transglutaminase and CD36). We observed a higher expression of these markers in the epidermis of scars at 1 month post-burn, compared with normal control skin of healthy persons. There was a striking difference between normotrophic and hypertrophic scars at 4 months post-burn. Keratinocytes in hypertrophic scars displayed a higher level of proliferation, differentiation and activation than did normotrophic scars. At 7 months post-burn all keratinocyte proliferation and differentiation markers showed a normal expression, but the epithelial activation marker CD36 remained upregulated in both normotrophic and hypertrophic scars. We hypothesized that the development of hypertrophic scarring is not an isolated dermal defect, but rather the result of a defect in the crosstalk between dermal and epidermal cells.

Chapter 3.2 describes the expression of the growth factors TGF-β1, -β2, -β3, bFGF and VEGF in the epidermis, which was analyzed by using both immunohistochemistry and in situ hybridization. We observed a higher expression of all growth factors studied in the epidermis of scars at 1 month post-burn than in control unburned skin. At 4 months the keratinocytes still displayed a higher expression of TGF-β3 and

bFGF, but the expression of TGF-β1, TGF-β2 and VEGF in the epidermis had normalized. The expression of TGF-β3 in the epidermis of hypertrophic scars was slightly higher than in normotrophic scars. At 7 months post-burn all growth factors studied showed a normal expression in the keratinocytes. Our results suggest that keratinocytes are not "bystanders" in the repair process, mainly important for re-epithelialization, but also regulate the scar maturation.

The role of the dermal compartment in the spontaneous healing of partial-thickness burns is discussed in chapter 4. In chapter 4.1 we investigated the participation of immune cells in burn wound healing by using immunohistochemistry and markers characteristic for Langerhans cells, monocytes, macrophages, granulocytes, T helper and T cytotoxic lymphocytes, B lymphocytes, NK cells and mast cells. The infiltrate in the 1 month old burn scar consisted predominantly of granulocytes, monocytes, macrophages, T helper lymphocytes and B lymphocytes. After 4 and 7 months, most immune cell numbers had turned to normal, but mast cell and Langerhans cell numbers were still increased compared with normal control skin. No difference was observed in immune cell numbers from normotrophic and hypertrophic scars. We conclude that phagocytes and lymphocytes likely play a role in the early phase of the burn wound healing process, but not in the process of hypertrophic scarring. Furthermore, Langerhans cells and mast cells may be regulatory cells in the remodeling phase of burn wound healing.

In chapter 4.2 we studied the outgrowth of neuropeptide containing nerves in the burn wound scars by using immunohistochemistry and Protein gene product 9.5 (PGP 9.5) for the detection of nerves, and substance P, neurokinin A, calcitonin gene-related-peptide, vasoactive intestinal peptide and neuropeptide Y for the detection of neuropeptides. We showed that nerve fibers regenerate in spontaneously healed partial-thickness burns, in both the dermis and the epidermis. The regeneration increased in the coarse of time, but never reached the levels present in control unburned skin. We found a significantly higher number of nerve fibers in normotrophic scars compared with hypertrophic scars. This suggests a possible regulating role for nerves in the scar tissue remodeling phase and hypertrophic scar formation. However, the number of neuropeptide containing nerves did not differ between normotrophic and hypertrophic scars, suggesting that the studied neuropeptides do not have a major influence.

Finally, in chapter 4.3 we describe the regeneration of elastic fibers and the production of collagen by fibroblasts by using both electron microscopy and immunohistochemistry. Furthermore, we detected TGF-β1 mRNA by in situ hybridization. We showed that the expression of procollagen I was increased in post-burn scars and that it remained elevated at least 7 months post-burn. Procollagen I was significantly more abundant in hypertrophic scars than in normotrophic scars, both at 4 and 7 months post-burn.

Elastic fibers were detected in the newly formed papillary dermis of burn scars already at 1 month post-burn, suggesting that elastogenesis by fibroblasts starts early

in the burn wound healing process. However, the quantity and architecture of normal, healthy elastin was not reached within the observation period of 7 months of this study.

The fibroblasts in the burn scars expressed a higher level of TGF-β1 mRNA at all intervals post-burn, compared with fibroblasts in control unburned skin. This is in agreement with other studies that showed that TGF-β1 contributes to increased collagen and elastin synthesis. We conclude that the synthesis of collagen and elastic fibers in partial-thickness burn scars is a rapidly starting process which continues for several months.

Until recently it was thought that both the cause and the consequences of hypertrophic scars were situated in the dermis. The reason for this is that microscopically the difference between normotrophic and hypertrophic scars lies in the dermis. In the hypertrophic scar there is an extensive collagen deposition, and the collagen fibers are not arranged regularly. From our studies we conclude that the development of hypertrophic scarring is not an isolated dermal defect, but rather the result of a defect in the crosstalk between dermis and epidermis. The keratinocytes exhibit important local regulatory functions in the burn wound healing process. The hypertrophic healing, which is often observed after severe burn injuries, may well be due to the absence of these regulatory activities of the epidermis in the early phase of the wound healing process.

Future research on burn wound healing and the etiopathology of hypertrophic scar formation should include molecular interactions between keratinocytes, fibroblasts and the other effector cells in wound healing. In vitro studies on the influence of different cytokines and growth factors, produced by keratinocytes, on proliferation and function of fibroblasts should give more insight into the factors involved in the crosstalk between these cell types. In situ hybridization studies whereby different cytokines and growth factors in keratinocytes, fibroblasts and endothelial cells are closely monitored at different time points, should give additional insight. In vivo studies using antagonists against these factors or neutralizing antibodies can supplement these data with functional information. Such studies are crucial as the complex interactions in the skin can never be reproduced in vitro.

As hypertrophic scarring is a process unique to man, research should focus on humans, because research in other species is highly unlikely to lead to relevant answers to questions on the etiopathology of this problem.

SAMENVATTING

De genezing van een wond verloopt schematisch in drie fasen:

1) de ontstekingsfase, waarbij het lichaam op de verwonding hetzelfde reageert als bij een ontsteking: er ontstaat roodheid, warmte, een zwelling en pijn. Granulocyten, lymfocyten en macrofagen voorkomen infectie van het wondgebied en produceren diverse groeifactoren en cytokinen die nodig zijn voor een goede wondgenezing;
2) de regeneratiefase, waarbij de opperhuid, de bloedvaten en het bindweefsel opnieuw worden aangemaakt;
3) de remodelleringsfase of maturatiefase, waarbij er een herstructurering van het bindweefsel plaatsvindt als gevolg waarvan het weefsel van de littekens sterker wordt.

Brandwonden genezen op dezelfde manier als andere wonden, zoals snijwonden, alleen gaat het proces langzamer. Dit komt doordat bij verbrandingen vaak een groot oppervlak is aangedaan en er in de wond een tijdelijk gebrek aan zuurstof en voedingsstoffen is.

Als tijdens de remodelleringsfase een overmaat aan bindweefsel in de lederhuid wordt gemaakt, en/of dit onvoldoende wordt afgebroken, kan er een zogenaamd hypertrofisch litteken ontstaan, in tegenstelling tot een normaal, normotroof litteken. Zo'n hypertrofisch litteken is niet alleen cosmetisch storend, maar kan ook aanleiding tot klachten geven, zoals jeuk en pijn. Dit komt relatief veel voor bij kinderen. Hiervoor is tot dusver geen afdoende behandeling, omdat inzicht in de oorzaak van het ontstaan van deze hypertrofe littekenvorming grotendeels ontbreekt. De meest gebruikte behandeling is de toepassing van druk door middel van strakke elastische kleding, zodat het litteken als het ware geen ruimte heeft om uit te groeien.

Het doel van het onderzoek dat in dit proefschrift is beschreven, was het vergroten van het inzicht in de genezing van tweedegraads brandwonden en met name in de achtergrond van de vorming van hypertrofische littekens en de daarmee gepaard gaande klachten.

Hiertoe werden bij 29 patiënten van het Brandwondencentrum van het Rode Kruis Ziekenhuis in Beverwijk huidbiopten afgenomen. De biopten werden afgenomen van een conservatief behandelde tweedegraads brandwond, en van onverbrande huid als controle, één maand na het ontstaan van de brandwond. Na 4 en 7 maanden werden opnieuw huidbiopten afgenomen. De biopten werden genomen van zowel de normotrofe als hypertrofe littekens die zich in deze brandwonden hadden ontwikkeld. De huidbiopten werden vervolgens met behulp van immunohistochemie, in situ hybridisatie en elektronenmicroscopie onderzocht.

In hoofdstuk 3.1 wordt het herstel van de opperhuid (epidermis) na verbranding beschreven. Dit werd bestudeerd met behulp van markers die karakteristiek zijn voor de proliferatie, differentiatie en activatie van de opperhuidcellen, de keratinocyten (keratine 5, 10, 16 en 17, filaggrine, transglutaminase en CD36). Een maand na de verbranding komen deze markers in verbrande huid verhoogd tot expressie ten opzichte van normale huid. Na 4 maanden is er een verschil te zien tussen hypertrofe en normotrofe littekens. In de hypertrofe littekens is niet alleen de activatie, maar ook de proliferatie en differentiatie van de keratinocyten nog steeds verhoogd, terwijl de keratinocyten van de normotrofe littekens alleen nog tekenen van verhoogde activiteit vertonen. Na 7 maanden is dit verschil verdwenen. De keratinocyten van beide soorten littekens brengen dan wel nog steeds de activatiemarker CD36 tot expressie. Geconcludeerd kan worden dat bij een hypertroof litteken de afwijkingen niet alleen te vinden zijn in de lederhuid (dermis), maar ook in de epidermis. De vorming van een hypertroof litteken is waarschijnlijk niet een geïsoleerd dermaal defect, maar meer het gevolg van een defecte interactie (crosstalk) tussen de dermis en de epidermis.
In hoofdstuk 3.2 is de productie van de groeifactoren TGF-β1, -β2, -β3, bFGF and VEGF door de keratinocyten onderzocht met behulp van zowel immunohistochemie als in situ hybridisatie. Na 1 maand produceerden de keratinocyten deze groeifactoren in verhoogde mate. Na 4 maanden kwam TGF-β3 in keratinocyten van hypertrofe littekens verhoogd tot expressie in vergelijking met normotrofe littekens. De productie van alle groeifactoren nam vervolgens af en was na 7 maanden in de littekens vergelijkbaar met normale huid.
Onze resultaten geven aan dat de keratinocyten tijdens het wondgenezingsproces niet alleen betrokken zijn bij de vorming van nieuw epitheel ter bedekking van het onderliggend weefsel, maar ook direct of indirect invloed uitoefenen op andere cellen die betrokken zijn bij het wondgenezingsproces.
In hoofdstuk 4.1 wordt de aanwezigheid van diverse typen immuuncellen tijdens het wondgenezingsproces beschreven. Na 1 maand zijn de volgende typen witte bloedcellen in de zich herstellende wond aanwezig: granulocyten, monocyten, macrofagen, helper T lymfocyten en B lymfocyten. Deze cellen zouden dus van invloed kunnen zijn op de vroege fase van het wondgenezingsproces. Na 4 en 7 maanden zijn hun aantallen genormaliseerd; wel komen Langerhanscellen en mestcellen dan in verhoogde aantallen voor. Wij vonden geen verschil in het aantal immuuncellen tussen normotrofe en hypertrofe littekens. Het is mogelijk dat de Langerhanscellen, samen met de mestcellen in de dermis, in brandwondlittekens een regulerende functie hebben in de remodelleringsfase van de wondgenezing.
Hoofdstuk 4.2 beschrijft de uitgroei van neuropeptide-bevattende zenuwen in de brandwondlittekens. We onderzochten dit met behulp van immunohistochemie van PGP 9.5 voor de detectie van zenuwen, en substance P, neurokinine A, calcitonine gene-related-peptide, vasoactief intestinaal peptide en neuropeptide Y als neuropeptiden. Reeds na 1 maand waren er zowel in de dermis als in de epidermis zenuwen aanwezig. Het aantal zenuwen nam progressief toe, maar na 7 maanden was

het aantal nog steeds niet gelijk aan dat in normale huid. In hypertrofe littekens werd minder uitgroei van zenuwen waargenomen dan in normotrofe, hetgeen erop zou kunnen wijzen dat zenuwen mogelijk een regulerende functie in de remodelleringsfase hebben. Neuropeptiden-bevattende zenuwen kwamen echter nauwelijks voor in de brandwondlittekens, wat suggereert dat zij geen belangrijke invloed uitoefenen op het brandwondgenezingsproces.

Hoofdstuk 4.3 beschrijft de uitgroei van elastische en collagene vezels in het bindweefsel van brandwondlittekens met behulp van immunohistochemie, elektronen microscopie en in situ hybridisatie. We namen waar dat elastische vezels al vrij snel na de verbranding weer worden aangemaakt in brandwondlittekens. De hoeveelheid en de morfologie van de elastische vezels was na 7 maanden echter nog steeds afwijkend in vergelijking met normale huid.

De aanmaak van collageen type I was verhoogd tijdens het brandwondherstelproces. In hypertrofe brandwondlittekens was er een grotere aanmaak van collageen type I dan in normotrofe, wat de grotere hoeveelheid bindweefsel in de hypertrofe littekens verklaart. TGF-β1 mRNA bleek in verhoogde mate tot expressie te komen in de fibroblasten van brandwondlittekens. Dit is een belangrijke waarneming, omdat TGF-β1 een regulerende groeifactor is voor de aanmaak van zowel collageen als elastine door fibroblasten.

Tot nog toe werd gedacht dat de hoofdoorzaak en het gevolg van hypertrofe littekens in de dermis gelegen was. Het onderzoek dat in dit proefschrift wordt beschreven, toont aan dat de epidermis hier ook bij betrokken is. Waarschijnlijk is de interactie tussen de keratinocyten en de fibroblasten belangrijk voor de functie van zowel de fibroblasten als de keratinocyten. Indien de keratinocyten ontbreken in de vroege fase van het brandwondgenezingsproces, is de kans groter dat de fibroblasten teveel bindweefsel gaan vormen.

Verder onderzoek is nodig om de precieze oorzaak van de vorming van hypertrofe littekens de achterhalen. Daarbij moet met name aandacht worden geschonken aan de groeifactoren en cytokinen die door keratinocyten kunnen worden geproduceerd. Aangezien hypertrofe littekens niet voorkomen bij dieren, zijn studies met mensen onontbeerlijk.

LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
APAAP	alkaline phosphatase anti-alkaline phosphatase
APES	aminopropyltriethoxysilane
BCIP	bicholylindolyl phosphate
bFGF	basic fibroblast growth factor
CGRP	calcitonin gene-related-peptide
EGF	epidermal growth factor
FGF	fibroblast growth factor
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IL-1	interleukin-1
IL-6	interleukin-6
KGF	keratinocyte growth factor
MMP	matrix metalloproteinase
NBT	nitro blue tetrazolium
NKA	neurokinine A
NPY	neuropeptide Y
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PGP 9.5	protein gene product 9.5
SSC	standard saline citrate
SEM	standard error of the mean
SP	substance P
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

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"En overigens, wees gewaarschuwd; er is geen einde aan het maken van veel boeken en veel doorvorsen is afmatting voor het lichaam. Van al het gehoorde is het slotwoord: Vrees God en onderhoud Zijn geboden, want dit geldt voor alle mensen." Prediker 12: 12-13

Eveline.

CURRICULUM VITAE

Tineke Eveline Hakvoort werd geboren op 11 december 1969 te Delft. Aldaar doorliep zij het VWO aan het Christelijk Lyceum Delft. In 1987 startte zij haar studie Geneeskunde aan de Erasmus Universiteit Rotterdam, waar ze op 17 september 1992 voor haar doctoraal examen en op 16 december 1994 voor haar artsexamen slaagde. Van 1995 tot 1999 was zij als assistent-in-opleiding werkzaam op de afdeling Immunologie van de Erasmus Universiteit, waar zij, in samenwerking met de Brandwondencentra in Nederland, het onderzoek deed dat in dit proefschrift beschreven wordt. Op 3 november 1998 kreeg zij tijdens het 10e congres van de International Society for Burn Injuries, dat in Jeruzalem, Israel, werd gehouden, voor haar onderzoek een internationale prijs, de "William's Award for Outstanding Burn Research". Sedert 1 mei 1999 is zij als arts-assistent werkzaam op de afdeling Interne Geneeskunde van het IJsselland Ziekenhuis in Capelle aan den IJssel.