The Heme-Heme Oxygenase System in Wound Healing; Implications for Scar Formation

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Abstract: Wound healing is an intricate process requiring the concerted action of keratinocytes, fibroblasts, endothelial cells, and macrophages. Here, we review the literature on normal wound healing and the pathological forms of wound healing, such as hypertrophic or excessive scar formation, with special emphasis on the heme-heme oxygenase (HO) system and the versatile effector molecules that are formed after HO-mediated heme degradation. Excessive scar formation following wounding is thought to relate to prolonged oxidative and inflammatory stress in the skin. Evidence is accumulating that the heme-HO system forms a novel and important target in the control of wound healing. Heme-protein derived heme can act as a potent oxidative and inflammatory stress inducer, and excess levels of heme may thus contribute to delayed resolution of oxidative and inflammatory insults in the skin. This emphasizes the need for a timely reduction of the levels of heme. Heme-binding proteins, heme transporters, and the heme degrading protein, HO, form therefore a necessary defense. Deficiencies in these defense proteins or a disturbed redox status, as in diabetic patients, may render individuals more prone to heme-induced deleterious effects. A better understanding of the heme-heme oxygenase system as target during wound healing may result in novel strategies to reduce scar formation.

Keywords: Heme, wound healing, scar formation, inflammation, heme oxygenase, (myo)-fibroblasts.

INTRODUCTION

Wounds and other tissue injuries can be caused by a great variety of circumstances, including surgery, burns, and ischemia-reperfusion. The healing process varies depending on the severity of the causative agent. In this review we describe the physiological changes during the normal healing in the skin. Attention is also paid to pathological healing with special reference to excessive and hypertrophic scar formation and the role of heme as a possible pathological factor in this process. Special situations in this respect regarding diabetes and the role of angiogenesis are reviewed. Subsequently the possibility to counteract the process of excessive scarring by influencing the heme-heme-oxygenase (HO) system is considered.

PHYSIOLOGY OF WOUND HEALING

The skin consists of an epidermal and a dermal layer separated by the lamina basalis, and forms a versatile organ with highly adaptive properties. In addition to the various homeostatic mechanisms, the skin has to cope continuously with changes in the external environment, such as heat, cold, UV-radiation, oxidative stress, injury and wounds [1, 2]. Wounds are associated with hemorrhage, hemolysis and cell injury, and result in a local accumulation of free heme proteins and heme [3-5]. Wounding induces a complex cascade of events to stop and prevent blood loss, to kill possible intruding pathogens, which eventually results in restoration of the site of injury and homeostasis [6, 7].

Following injury the repair takes place in different, but overlapping phases: the inflammatory, proliferative and remodeling phase.

The inflammatory phase is primarily intended to provide protection for the host and is characterized by classical clinical features, such as redness, swelling, heat, pain and loss of function. The skin microenvironment changes dramatically after injury [7-9]. One of the earliest changes in the skin after wounding is the release of modified, denatured tissue proteins. In the wounded area a pro-oxidant microenvironment is created providing signals to generate an inflammatory process.

Inflammation protects from pathogenic invaders, gets rid of damaged cells after injury, prevents further damage and prepares for tissue repair (reviewed in [3]). The inflammatory response involves several stages: 1) dilation of capillaries to increase blood flow; 2) microvascular structural changes and escape of plasma proteins from the bloodstream; 3) the so-called “leukocyte-adhesion cascade”; 4) elimination of possible pathogens; and 5) the resolution of inflammation.

The injury will firstly lead to disruption of blood vessels, thereby activating the coagulation cascade. Besides clot formation, coagulation induces the production of vasoactive, pro-inflammatory agents and activation of the complement cascade. Activation of the different cascade systems results
in the generation of bradykinin, C3a and C5a, and the activation of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-8. Subsequently, there is increased blood vessel permeability, chemokine expression, adhesion molecule expression and recruitment of cells (granulocytes, macrophages and lymphocytes) to the site of inflammation.

The end of the inflammatory phase is marked by reduced levels of pro-inflammatory cytokines and decreased number of inflammatory cells, which in turn is the sign for the next stage in the wound healing process: the proliferative phase. This is characterized by processes such as re-epithelialization, neovascularization, and wound contraction. Keratinocytes, endothelial cells and fibroblasts start to migrate into the provisional extracellular matrix in the wound area, and start proliferating. Especially fibroblast activity is responsible for the build-up of new extracellular matrix (ECM) to restore lost tissue.

Finally, the remodeling phase takes place when the wound area is fully re-epithelialized. Fibroblasts will differentiate into myofibroblasts and generate more ECM proteins. These cells are largely responsible for wound contraction. Wound contraction causes a rapid reduction of wound size, thereby reducing the chance of wound infection by infiltrating pathogens. This is the result of both myofibroblast contraction and reorganization of ECM. The subsequent reorganization and synthesis of new ECM results in stronger fibers and increased tensile strength but this newly formed tissue never reaches the quality of normal uninjured skin.

During this phase, myofibroblasts in the highly cellular granulation tissue disappear via programmed cell death (apoptosis), which ultimately results in a relatively acellular scar. However, during hypertrophic scar formation the myofibroblasts tend to persist, which lies at the base of the mechanism of excessive ECM production and wound contraction as observed in this disorder [10-12]. Hypertrophic scars are not only disfiguring but are a well-known risk factor for scar contraction, which can lead to lifelong morbidity because of significant impairment of function [13].

**PATHOLOGICAL WOUND HEALING: EXCESSIVE SCARRING**

Wound contraction is important for wound closure, but under certain conditions such as following burns, deep dermal injuries and surgery related to cleft lip and palate, it may lead to excessive scar formation and pathological scar contracture [14, 15] (Fig. 1). Cleft lip and/or palate is a developmental disorder of the face that is characterized by a cleft in the upper lip and/or palate and alveolar bone.

![Fig. (1). A. Patients with cleft lip and palate need to undergo various operations, which as adverse effect can result in the formation of scarring [187]. This can restrict maxillary growth and may lead to midfacial deficiency. B. and C. Also burn wounds can result in excessive or hypertrophic scars that are not only cosmetically disfiguring, but are also clearly hampering function. (Pictures of burn wounds were kindly provided by Prof. Dr. Esther Middelkoop, Association of Dutch Burn Centres, Beverwijk, The Netherlands).](image-url)
Affected patients need multiple surgeries, which are often resulting in excessive scarring that can compromise normal growth of the maxilla and the development of the dentition (Fig. 1) [16]. Also burn patients often demonstrate large excessive scars following wound healing (Fig. 1) [13]. Thus, after major trauma to the skin, the formation of hypertrophic or excessive scars can lead to extensive functional and cosmetic morbidity, which is a major source of discomfort. Since (myo)fibroblasts are major producers of ECM, produce less matrix metalloproteinase (MMPs), and are over-represented in hypertrophic scars compared to normal skin and normotrophic scars, they are considered to be responsible for the production of hypertrophic scars [13]. Nedelec and colleagues indicated that the development of hypertrophic scars and scar contractures may be the result of delayed or defective myofibroblast apoptosis [12]. The prolonged presence of fibroblasts and myofibroblasts results in abnormal ECM production and may lead to an imbalance of ECM turn-over, resulting in an excessive and rigid scar [14]. Accelerating the rate of apoptotic myofibroblast cell death was demonstrated to reduce the cell number, and may be a means for providing effective treatment of scar contracture [12].

Although a substantial amount of molecular and cellular data have been generated in order to better understand the process of wound contraction and scar contracture, the decisive and controlling mechanisms still remain to be elucidated. A major determinant of excessive scar formation is wound healing time; if a wound takes more than 21 days to heal, 78% of wound sites develop into excessive or hypertrophic scars [17]. The prolonged healing time, for example in an open or infected burn wound, is caused by overproduction of reactive oxygen species (ROS) and inflammatory mediators, which extend the proliferative phase via increased concentrations of growth factors known to stimulate fibroblast proliferation [18]. Moreover, excess amounts of ECM are produced, which interferes with normal tissue remodeling, and causes hypertrophic scar formation [19]. A tight control of inflammatory cell-derived ROS is important since excess of ROS, or a decreased expression of ROS-detoxifying proteins, corresponds with aberrant wound healing [20, 21]. Induction of ROS or inflammation can disrupt otherwise scarless fetal wound healing by increasing fibroblast proliferation and fibrosis [20, 22]. In contrast, overexpression of cytoprotective genes has demonstrated to restore delayed wound healing in diabetic mice [23].

Evidence is accumulating that the heme-HO system is involved in the oxidative and inflammatory processes leading to excessive scarring. Because this heme-HO system may be an important target to counteract hypertrophic scar formation the state of art in this field will be discussed in detail in the next part of this review.

HEME AS A PATHOLOGICAL FACTOR THAT MEDIATES OXIDATIVE AND INFLAMMATORY STRESS AND FIBROSIS

Heme (iron protoporphyrin IX) is a complex of an iron atom linked to the four ligand groups of porphyrin that is made in every mammalian nucleated cell via a series of enzymatic reactions. In response to excess levels of heme, δ-aminoluvinate synthase (ALA-S), the rate-limiting enzyme for heme synthesis is, as a feedback, inhibited in non-erythroid cells [24]. The heme molecule provides a multitude of crucial biological functions and possesses several essential signaling properties as reviewed in Wagener et al. 2003 [3].

Heme can also interact with various inactive apo-heme proteins giving rise to functional heme proteins. For example, in hemoglobin and myoglobin it plays a critical role in oxygen transport and storage, respectively, while in cytochromes it is involved in electron transport, energy generation, and chemical transformation [3]. Furthermore, heme is indispensable for a wide array of other enzyme systems, such as cytochromes, cyclo-oxygenase (COX), and nitric oxide synthase (NOS) [25].

However, heme exerts a dual role: in small amounts it acts by itself or as the functional group of heme proteins providing diverse and indispensable cellular functions, whereas, in excessive amounts, free heme can cause severe tissue damage [26-28].

Cell damage results in a local accumulation of free heme proteins and heme. Previous data support a contribution of heme to wound healing since it can activate a wide array of processes, such as vasoconstriction, pro-coagulating effects of mononuclear-cells, activation of complement factors, platelet aggregation, and differentiation and proliferation of various cell types [29]. Heme has been shown to promote the formation of ROS via the Fenton reaction, and the (per)oxidation of biomolecules, like low density lipoprotein, which can result in cellular injury [28, 30]. This is further exemplified by studies of Nath and colleagues, who demonstrated that large amounts of heme (proteins) can cause severe kidney injury [31].

We and others demonstrated previously that free heme not only promotes oxidative stress, but also possesses pro-inflammatory properties both in vitro and in vivo [3]. Heme enhances vascular adhesion molecules ICAM-1, VCAM-1 and E-selectin, causes vascular permeability, and promotes leukocyte recruitment, and the induction of inflammatory cytokines [3, 4, 30, 32-39].

Moreover, strongly increased levels of ECM are found following exposure of mice to large amounts of heme, which may contribute to the development of fibrosis [35, 40]. It is therefore likely that free heme plays a role in the etiology of many inflammatory and pathological conditions and may function as a “molecular switch” [3, 4, 41, 42]. The important inflammatory mechanisms of heme have already been shown to affect a wide variety of conditions, including hemolytic diseases [43] and sickle cell disease [36, 41], and it is thought to be involved in many more pathological processes. In recent studies, it was elegantly demonstrated that heme is the major culprit in the pathogenesis of malaria by promoting progression to cerebral malaria [42, 44, 45]. In addition, we and others also showed that heme is important in the process of wound healing [4, 5]. The sheer presence of heme is enough to recruit both macrophages and granulocytes [4]. Free heme activates granulocytes, causes granulocyte migration, and delays granulocyte apoptosis, by activating pro-survival signaling pathways (PI3K/AKT, ERK, and NF-κB), which results in prolonged presence of...
inflammatory cells in the wound area [46-48]. The presence of large amounts of heme induces the recruitment of leukocytes, which is indispensable for the elimination of pathogens and for the wound healing process [4, 35]. Macrophage-derived cytokines and growth factors are important in the transition from the inflammatory phase to tissue repair. However, since heme can also potentiate the injurious effects of agents such as hydrogen peroxide [49], and TNF [50], it is likely that heme-induced recruitment of leukocytes will create an environment in which these injurious agents can interact with each other, thereby postponing resolution of inflammatory and oxidative stress in the skin.

Our results demonstrated that the local release of heme functions as an early trigger to start inflammatory processes following injury, and we therefore postulated that heme could act as a danger signal [3, 4]. Moreover, it has recently been demonstrated that heme can indeed activate Toll-like receptor molecule-4 (TLR-4), and can promote TNF production in macrophages [51]. Also hemozoin, a crystallized aggregate of heme molecules formed by the malaria parasite, may activate an alternative TLR ligand, suggesting a differential response [52].

Inflammation is normally a beneficial response to tissue injury, protects against invading pathogens, and prepares for the restoration of tissue. In the pathogenesis of a wide variety of diseases including rheumatoid arthritis and atherosclerosis, inflammation-mediated tissue injury plays an important role [53]. The prolonged presence of heme may be an important factor that skews normal wound healing via chronic inflammatory and oxidative stress in the skin towards pathological scarring (Fig. 2).

Thus, heme-induced ROS and pro-inflammatory insults likely contribute to cellular damage when heme is present for too long. The recent advances in the study of heme as a pro-oxidative, pro-inflammatory and cytotoxic molecule, and its possible role in the etiology of pathological conditions underscores the need for a tight control of the levels of free heme [3].

**MECHANISMS OF PROTECTION AGAINST HEME-INDUCED OXIDATIVE AND INFLAMMATORY STRESS AND FIBROSIS**

**Heme Binding Proteins**

The heme- and hemoglobin (Hb)-scavenging molecules hemopexin and haptoglobin are thought to act as a first line of defense against the injurious actions of heme [3, 4, 54]. These scavengers have been demonstrated to protect against heme- and Hb-mediated insults such as lipid peroxidation [55]. Interestingly, reduction of heme-, and Hb-scavenging molecules like hemopexin and haptoglobin has demonstrated to also aggravate several of the pro-inflammatory charac-

![Fig. (2)](image)

*Following wounding large amounts of heme are released locally that can initiate a wide range of pro-oxidative, pro-inflammatory and deleterious actions, including cytokine/chemokine production, adhesion molecule expression (inflammation), activation of the innate immune system, leukocyte recruitment, and fibrosis. We postulate that these properties of heme strongly contribute to excessive scar formation. Heme-binding proteins, heme transporters, heme-degrading proteins, and anti-oxidant systems protect us against these heme-induced insults. Since the levels of these protective mechanisms and/or effector molecules are differentially regulated in individuals, induction hereof in people with too little defense could possibly lead to amelioration of chronic wounds and prevent the formation of excessive scarring (See text for further details).*
characteristics of heme. For example, the group of Tolosano demonstrated elegantly that vaso-occlusion in the spleen, resulting in splenomegaly, was strongly enhanced in haptoglobin-hemopexin (HpHx) double knock-out mice following acute hemolysis, suggesting the involvement of plasma hemoglobin and heme [40]. Also, the single haptoglobin or hemopexin knock-out animals had increased levels of vaso-occlusion compared to wild-type animals, although less than in the double knock-out. This vaso-occlusion was likely the result of heme-induced vascular adhesion molecule expression, since ICAM-1 and VCAM-1 expression was even further enhanced following exposure to heme in hemopexin knock-out mice compared to wild-type mice [37]. The proinflammatory effects of Hb and heme were further substantiated by the analysis of acute hemolysis in splenectomized HpHx double knock-out mice. In these mice the liver took on the role of scavenging free Hb and heme from the circulation, and showed both strong inflammation and fibrosis [40]. This makes it tempting to speculate that hemoglobin and heme do not only contribute to oxidative stress and inflammation, but also to fibrosis (see Fig. 2) [35].

Also in patients with chronic venous ulcers large amounts of heme that are thought to contribute to injury, were found in ulcer fluid, plasma, and skin sections [5]. α1-Microglobulin, another heme scavenger, was found to protect against these heme-induced oxidative and inflammatory insults during skin wound healing [5]. Immenschuh and colleagues demonstrated that heme also induces the expression of the heme-binding protein 23 (HBP23; peroxiredoxin), an intracellular heme scavenger that is likely involved in protecting the intracellular environment from heme-induced ROS formation [56].

Heme Transporters

It was long thought that lipophilic heme can easily diffuse through the plasma membrane, however, recent studies cast doubt on this because of the anionic carboxylate side chains of the heme molecule, supporting a more important role for heme transporters in heme homeostasis [57]. Recently, a number of heme/porphyrin transport proteins have been identified, such as heme carrier protein 1 (HCP1), feline leukemia virus receptor C (FLVCR), breast cancer resistance protein (ABCG2/BCRP), and ABCB6. These proteins could be important to protect the cell from heme-mediated oxidative or inflammatory insults (thoroughly reviewed in Krishnamrthy 2007) [57]. FLVCR and BCRP can protect cells from heme-mediated insults by effluxing heme from the cells [58, 59], whereas HCP1 can shuttle heme into duodenum cells. BCRP, a member of the ABC transporter family, is a half transporter, and is believed to function as a homodimer [60]. BCRP contributes to the excretory processes of a diverse range of drugs and toxic substances, including porphyrins (e.g. heme) and flavonoids [59, 61, 62]. Moreover, heme accumulates in tissues of Bcrp knockout mice [58, 61, 62]. ABCB6 is important in mitochondrial heme influx and could therefore also play a major role in apoptotic processes important in wound healing.

It will therefore be of great interest to study the role of these heme transporters during wound healing in more detail.

Heme Oxygenase

During the resolution phase of inflammation, the pro-oxidative and pro-inflammatory properties of heme and cytokines need to be counteracted. Heme is degraded by the microsomal enzyme HO into the versatile effector molecules carbon monoxide (CO), biliverdin, and iron (Fig. 2) [63]. Biliverdin is directly converted by biliverdin reductase (BVR) into the antioxidant bilirubin. It has been demonstrated that HO-1 induction is accompanied by increased ferritin synthesis, whereas inhibition of HO activity causes a decrease in ferritin levels [64, 65]. Ferritin is a protein that can scavenge the pro-oxidant iron, rendering it inactive [66]. This protective effect may be very important at sites with increased heme and iron, as in skin ulcers or after UV radiation [5, 65, 66].

HO expression represents an important mechanism of protective response in a wide variety of cellular stresses [67]. An overwhelming body of evidence indicates that the heme-HO-1 system is tightly involved in diverse (patho)physiological processes. HO-activity provides cytoprotection, inhibits apoptosis, and reduces oxidative stress and inflammation in a multitude of models, and is e.g. important for successful organ transplantation [3, 68-71]. These actions are probably mediated via its down-stream effector molecules [66]. Furthermore, biliverdin/bilirubin functions as a potent antioxidant, whereas CO induces cGMP-mediated vasodilatation and possesses several signaling properties [72].

At present, two HO-isoforms are known in humans. HO-1 is highly inducible by a variety of stimuli including oxidative stress, cytokines and its substrate heme, whereas HO-2 is mainly constitutively expressed [3]. HO-2 iso-enzymes probably function in normal heme capturing and metabolism [73, 74]. It is important to take into account that the heme-HO system has regulatory functions in a wide variety of processes that could be important in the resolution phase of wound healing, such as amelioration of inflammation, protection against apoptosis, and proliferation [70, 75, 76].

We and others previously demonstrated that HO-1 overexpression can counteract the various cytotoxic, pro-oxidative and pro-inflammatory actions of heme [3, 43, 77]. HO-1 down-modulates inflammatory adhesion molecules and attenuates leukocyte adhesion to the vascular endothelium upon exposure to heme or other pro-inflammatory stimuli, whereas inhibition of HO-activity exacerbates heme-mediated oxidative and inflammatory injury both in vitro and in vivo [34, 35, 78-80].

Exposure of the skin to oxidative stress or to UV radiation promotes the release of the non-covalently bound prosthetic heme group from heme-proteins [81]. Recently, a simple but elegant mechanism by which CO protects against diseases was revealed: it prevents the release of the injurious heme from cell-free heme-proteins, by binding to the ferrous iron of the protoporphyrin IX ring [45]. This insight has already been shown to be important in diseases as malaria and atherosclerosis, but likely also plays a role in many more conditions, including wound healing. Moreover, it adds to the understanding of the protective actions of HO-activity.

The important cytoprotective properties of HO-1 are further exemplified by a human case of HO-1 deficiency
we have now shown that silencing of HO-2 expression allows a strong basal and UVA-induced expression of HO-1 in epidermal keratinocytes [98]. However, it is clear that Bach1 itself is the central factor suppressing HO-1 expression in fibroblasts since knock-down of Bach1 leads to a strong constitutive expression of HO-1 which is not further enhanced by irradiation with UVA. These studies also strongly support the concepts that 1) it is the level of free heme that plays a key role in regulation of HO-1 expression and 2) levels of HO-2 expression strongly influence HO-1 expression (but not vice-versa) by determining the levels of heme that can interact with Bach1.

Differential Levels of Protection Against Heme

When large amounts of free heme proteins or heme (locally) accumulate, like in a blood clot, in a wound, or after extravascular deposition, their scavengers may get overwhelmed or are unable to reach them [3]. When the protective proteins against excess levels of heme in the skin are depleted, the skin may be more prone to heme-induced injurious effects, the formation of inflammatory and oxidative stress, and subsequently excessive scars. This is exemplified by the following:

For haptoglobin, three major polymorphisms with different hemoglobin-scavenging capacity have been described that are associated with different prevalence of many inflammatory diseases [99]. The polymorphism with decreased hemoglobin binding is associated with a higher prevalence for cardiovascular diseases [100]. This is in line with our findings of heme-mediated oxidative and inflammatory effects [3].

Although genetic polymorphism of hemopexin has been reported for rabbits and pigs, possible human polymorphisms of hemopexin need further exploration [101]. Since albumin can bind heme to a lesser extent, it is possible that in case of such a deficiency in scavengers this plasma protein can also provide some protection [3].

Furthermore, the length of a guanine-thymidine (GT)_n-repeat polymorphism in the promoter region of the HO-1 gene determines the level of HO induction, and corresponds clinically with differential susceptibility to various conditions [102, 103]. Since HO-activity protects against inflammatory and oxidative injury, we expect that people carrying a HO1 promoter polymorphism leading to less HO1 induction, have increased chance of developing heme-mediated excessive scarring.

Thus, functional relevant polymorphisms for heme scavengers, like haptoglobin and hemopexin, and antioxidant enzymes like the heme-degrading enzyme HO could therefore determine the level of protection against heme, and possibly, the severity of scar formation. This is especially important for people with increased risk of heme release or people with an already compromised redox balance, such as diabetes patients or people with glutathione deficiency [104].

In pathophysiological conditions, particularly those associated with diabetes, wound healing is also significantly disturbed [105, 106]. Interestingly, Quan and colleagues elegantly demonstrated that hyperglycemia decreases HO-
activity in the endothelium and increases the production of ROS [104, 107], suggesting that patients with hyperglycemia are deprived from the protecting effects of the HO-effector molecules CO, bilirubin and ferritin.

Both investigations on animal models and clinical studies demonstrated that diabetic wounds are characterized by a dramatic reduction in the production of growth factors and their receptors playing an integral role in the process of skin repair [105, 106, 108]. Among those mediators the pivotal role of VEGF has been shown. Accordingly, growing evidence indicates that chronic wounds have decreased expression of VEGF and fewer blood vessels in the granulation tissue when compared with physiologic wounds [109]. Particularly, in genetically diabetic mice the level of VEGF is severely reduced during the healing process [110]. Also the synthesis of stromal cell derived factor-1 (SDF-1) is dramatically attenuated in diabetic conditions [111].

We have determined the course of HO-1 induction in the injured skin of diabetic mice 1 day after wounding. Interestingly, the highest induction of HO-1 within the wound bed is observed during 1-3 days after insult, but in diabetic db/db mice it could be significantly delayed up to 8 days [112]. In order to determine whether this is associated with the impaired skin regeneration observed in those animals we have performed gene transfer of HO-1 using adenoviral vectors. Indeed, these experiments revealed that attenuation of the healing process can be partially reversed by HO-1 overexpression [112].

Additionally, attenuating the oxidative stress and the exaggerated inflammation in the wounds by overexpression of such concomitantly anti-inflammatory gene(s) can moderate the overall healing response driving it into the direction of proper tissue repair [113]. Noteworthy, the role of HO-1 may be particularly important, as in chronic wounds the expressions of other antioxidant genes, such as superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, or catalase, can be significantly decreased [114]. The same concerns the concentrations of antioxidants such as ascorbic acid, vitamin D, and glutathione [114].

Genetic variation and redox balance thus determine the differential protection against the injurious effects of heme between individuals.

Wound Healing and Modulation of Heme-Enzymes by the Heme-Heme Oxygenase System

Heme is the crucial part of many major enzymatic systems, such as nitric oxide synthase (NOS), and cyclooxygenase (COX) [87]. HO controls the activity of these heme-proteins in two ways: directly, by regulating the availability of heme for the synthesis of heme-proteins (apo-enzyme vs holo-enzyme), or indirectly, via the generation of CO, which can bind to the heme moiety of heme-proteins and thereby affecting their enzymatic activity [115]. Moreover, CO binding would inhibit the release of heme from heme proteins [3, 45].

COX, NOS and arginase are important enzymes during the inflammatory phase of wound healing, but also in later processes such as wound contraction [116]. They exist in several isoforms that can be constitutively expressed or inducible. Their products, COX-derived prostaglandins, NOS-derived NO, and arginase-derived ornithine and polyamines have been described as important mediators in skin repair [117, 118]. COX can convert arachidonic acid (AA) into PGE2, which is an important inflammatory mediator. Recent work with inducible NOS (iNOS)-deficient mice identified iNOS-derived NO as pivotal for wound healing [119]. This is further supported by the finding that inhibition of iNOS during repair leads to a potent effect of iNOS-derived NO on epithelial gene expression, which severely impaired re-epithelialization. L-arginine represents the only substrate for NOS, which results in the generation of citrulline and NO [120]. These observations may explain the observation that L-arginine improves wound healing in animal models and in humans, because it is the only substrate for NOS enzymatic activity.

Alternatively, arginases, which convert L-arginine into ornithine and urea, may compete with NOS for their substrate at the wound site. Arginine levels in wound fluids remain low during healing [121]. Ornithine is the precursor for polyamines and proline. Polyamines are essential for cell proliferation whereas proline is necessary for collagen formation.

Kampfer and colleagues [122] demonstrated that the arginase system is induced upon skin injury in mice, which paralleled the well-established expressional and activity kinetics of iNOS after injury. Furthermore, impaired healing was associated with a significantly increased arginase activity, whereas improved healing showed to be characterized by a re-adjustment of iNOS and arginases at the wound site. Thus, a balanced presence of the iNOS and arginase activities are crucial to drive an efficient skin repair.

It is likely that the heme-heme oxygenase system is pivotal in modulating COX activity and the balance between NOS and arginase activity in fibroblasts and macrophages, and subsequently in the wound healing process.

Myofibroblast Apoptosis and the Heme-Heme Oxygenase System

The induction of myofibroblast apoptosis in hypertrophic scars has received considerable attention because of its obvious therapeutic significance. Fibroblasts derived from normal scars had a higher rate of apoptosis than cells from hypertrophic scars as was displayed by low levels of anti-apoptotic factors such as Bcl-2 and Bcl-xL and high levels of the pro-apoptotic factor Bax [123]. In a murine model of hypertrophic scar formation decreased fibroblast apoptosis was also detected, which was accompanied by enhanced levels of the pro-survival factor Akt [124]. This study also demonstrated that in p53-knock-out mice, with down-regulated apoptosis, hypertrophic scar formation was increased, whereas less scar hypertrophy was seen in pro-apoptotic Bcl-2 knock-out mice.

Apoptosis can generally occur in either a caspase-dependent or -independent fashion. For caspase-dependent apoptosis two major pathways have been identified. The first is the extrinsic pathway, which involves activation of death receptors like tumor necrosis factor (TNF) receptor, FAS and...
TRAIL. Fas-associated death domain and TNF-receptor-associated death domain can recruit and activate caspase-8 to form a death-inducing signaling complex (DISC) [125]. DISC can generally propagate the death signal either by splicing Bid, which upon translocation to the mitochondria can cause mitochondrial outer membrane permeabilization (MOMP) or it can lead to the release of pro-apoptotic proteins, such as cytochrome c [126]. Cytochrome c in turn can form an apoptosome by interacting with apoptotic protease activating factor-1 (Apaf-1) and procaspase 9. This complex is able to activate caspase 9, which in turn activates the effector caspases 3, 6 and 7, leading to apoptosis [127]. Alternatively, DISC can also directly activate these effector caspases [128]. Secondly, caspase-dependent apoptosis can occur via the intrinsic pathway, which is regulated by the mitochondrion, and characterized by MOMP [129], that is controlled by the family of Bcl-2 proteins [130]. These Bcl-2 proteins are either anti- or pro-apoptotic and a shift in balance between these two groups ultimately determines cell survival or cell death. The pro-apoptotic Bcl-2 family members Bax and Bak probably contribute to MOMP by facilitating the release of cytochrome c from the mitochondrial intermembrane space [131, 132]. Caspase-independent cell death relies on the Bax-mediated release of pro-apoptogenic factors like apoptosis inducing factor (AIF) and EndoG from the mitochondria, which subsequently translocate to the nucleus and contribute to chromatin condensation and DNA damage [133].

Heme oxygenase is considered to be a cytoprotective enzyme and numerous publications show that it affords protection against apoptosis (refs. in [134, 135]). Since oxidative stress is clearly involved in apoptosis, the protective effect of HO-activity most likely depends on the antioxidant properties of HO-derived reaction products. For instance, the antioxidant actions of bilirubin can protect cultured cells from cell death caused by oxidative stress [136]. Iron released from heme-break down by HO can have pro-oxidant actions via the Fenton reaction and could therefore be considered to be deleterious. However, as mentioned previously, iron can also upregulate the expression of the sequestering protein ferritin, which protect cells from iron released from HO-activity following induction of oxidative stress by ultraviolet A radiation [65]. The exposure of cells to exogenous CO potently inhibited TNFα-induced apoptosis in mouse fibroblasts, indicating the anti-apoptotic potential of CO [137]. In addition, HO-1-derived CO protected endothelial cells from TNFα-induced apoptosis [138]. This effect was shown to depend on the activation of p38 MAPK and the expression of NF-xB-responsive anti-apoptotic genes. On the contrary, HO and CO can induce Fas-mediated apoptosis in Jurkat T-cells [139, 140], and CO can also induce apoptosis in endothelial cells [141].

We previously showed that the polyphenol curcumin is a potent inducer of dermal fibroblast apoptosis and that the concomitant induction of HO-1 protects against curcumin-induced apoptosis [142]. Curcumin-induced apoptosis of human dermal fibroblasts turned out to be completely caspase-independent but relies on the mitochondrial generation of ROS and subsequent mitochondrial release of apoptosis inducing factor through translocation of the pro-apoptotic factors p53 and Bax [143]. The enhanced levels of HO-1 induced by curcumin protects fibroblasts against the deleterious effects of curcumin and, therefore, HO or its effector molecules could enable the fine-tuning of the balance between acellular and hypertrophic wound healing.

**FIBROSIS AND THE HEME-HEME OXYGENASE SYSTEM**

Fibroblasts are responsible for the production and deposition of several ECM proteins such as collagens and fibronectin, proteins which are essential for tissue repair after injury. These products give the tissue strength, integrity and flexibility. However, sometimes, especially after burns, and sometimes following surgery related to cleft lip and palate, this healing process derails and a pathological situation is formed (Fig. 1) were fibroblasts number dramatically increases by enhanced proliferation and/or failure to go into apoptosis, resulting in fibrosis/hypertrophy. Moreover, the increased number of myofibroblasts produce excessive amounts of ECM proteins, leading to an inferior repair tissue.

As described above, increased heme levels have been associated with the formation of fibrosis [40]. The possible protective role of HO in skin has been the subject of only few studies, and evidence for a role for HO in dermal fibrosis is therefore scarce. In systemic sclerosis patients (SSC) it was found that fibroblasts are extra vulnerable to apoptosis induced by curcumin. It was concluded that, in these patients, the expression of phase 2 detoxification enzymes HO-1 and glutathione S-transferase P1 could not be upregulated [144]. In a psoriasis model, upregulation of HO-1 by CoPP dramatically reduced psoriasis skin lesions by preventing keratinocyte hyperproliferation [145]. However, in several other organ systems such as lung, heart, kidney, and liver, a clear relation between HO, its effector molecules, and fibrosis has been described.

In the bleomycin-induced model of pulmonary fibrosis the systemic inhibition of HO-activity by Zn-deuteroporphyrin-IX,2,4-bisethyleneglycol caused reduced levels of transforming growth factor-β (TGFβ) accompanied by significantly less collagen accumulation, which ultimately resulted in less fibrosis [146]. Additionally, viral overexpression of HO-1 in the lung prevented bleomycin-induced fibrosis [147]. Besides HO, also its effector molecule CO has been shown to prevent bleomycin-induced lung fibrosis as was shown by decreased fibroblast proliferation and decreased expression of the ECM molecules collagen-1 and fibronectin [148]. Very recently, the same group showed that CO protects against fibrosis, and that it suppresses alpha-smooth muscle actin (αSMA) expression, which was dependent on ERK signaling [149]. Also for the HO effector molecule bilirubin, evidence exists in support of an antifibrotic effect. Wang and colleagues showed in rats that the development of bleomycin-induced pulmonary fibrosis was attenuated in hyperbilirubinemic animals as shown by improved lung histology and reduced levels of TGFβ [150].

The antifibrotic properties of 15-d-PGJ2 in liver fibrosis, such as growth inhibition, inhibition of collagen synthesis, and induction of apoptosis could be attributed to the activity of HO, and more specifically, to the HO-effector molecule bilirubin and not CO [151]. Recombinant adenovirus-mediated in vivo overexpression of HO-1 in hepatic
stellate cells resulted in a reduced expression of type 1 collagen, impaired proliferation, and reduced levels of profibrotic TGFβ [152]. Recently, it was shown that increased endogenous HO-1 by administration of the HO-inducer CoPP could reduce hepatic fibrosis in an immune liver fibrosis model of rat [153].

Upregulation of the HO-system by systemic treatment with hemin resulted in decreased cardiac fibrosis/hyptrophy, whereas inhibition of HO activity by CrMP exacerbated fibrosis in spontaneously hypertensive rats [154]. Interestingly, this reduction in fibrosis was accompanied by HO-mediated upregulation of cGMP levels, which could explain the reduced levels of NF-kB and AP-1, transcription factors well known to be involved in the fibrotic process. The same group recently reported that the beneficial effects of hemin therapy on several fibrotic parameters could be abolished by the HO-activity inhibitor CrMP [155]. Furthermore, hemin-induced HO-1 attenuated TGFβ and ECM proteins such as collagen and fibronectin. Post myocardial infarction was inhibited by viral overexpression of HO-1 in the left ventricle [156]. Although extensive myocardial scarring and fibrosis, associated with elevated levels of collagen-1 and -3 and MMP-2 activity, was observed in the LacZ-control group, these effects were strongly reduced in HO-1-treated animals. Moreover, virally overexpressed HO-1 caused a reduction in proliferation of isolated cardiac fibroblasts.

Curcumin is a well-known antifibrotic agent and a known upregulator of HO-1. In a glomerular model of fibrosis, curcumin-induced HO-1 was able to reduce several markers of fibrosis, such as expression of fibronectin, TGFβ, PAS and PAI-1, and these effects could be blocked by co-administration of curcumin with the HO-activity inhibitor ZnPP [157]. Further evidence of an antifibrotic effect of HO is given by the study of Kie et al. in which mice deficient in HO-1 demonstrated more fibrosis after kidney obstruction compared to wild type mice [158]. Obstructed kidneys of HO-1 deficient mice also presented with greater infiltration of inflammatory cells, tubular TGFβ expression and aSMA expression.

In a murine model of arthritis HO-1 was strongly induced by the systemic treatment with CoPP, which aside from inhibiting cartilage erosion, unexpectedly resulted in extensive fibrosis of the joint. Treatment with the HO-inhibitor SnPP also reduced arthritis severity but did not result in fibrosis [159].

These data clearly demonstrate that HO can act as an anti-fibrotic agent in several organs and, therefore, modulation of HO-activity could also be beneficial to treat pathological scar formation in the skin and oral mucosa.

MMPs are the natural enzymes responsible for the degradation of the ECM and since HO seems to be a protective factor against fibrosis, a likely target of HO-activity could be the expression/activity of MMPs. Indeed, several studies show that overexpression or induction of HO affects expression and activity of several MMPs. Hemin-induced HO-1 expression was able to inhibit breast cancer invasion through the suppression of MMP-9 expression [160]. This effect could be blocked by the HO-activity inhibitor SnPP and mimicked by addition of CO but not by BV/BR, indicating that this effect was mediated by HO. In contrast, it was shown that in breast cancer cells 15d-PGJ2, in addition to CoPP and hemin, induces HO-1 expression, which subsequently resulted in the induction of MMP-1 [161]. Addition of iron chelators and antioxidants abrogated HO-mediated MMP-1 induction, indicating the involvement of free iron and ROS in this process. In osteoarthritis, the pharmacological induction of HO-1 by CoPP resulted in inhibition of MMP-1 and MMP-13 expression [162], whereas HO-induction decreased MMP-9 activity in a model of acute arthritis [163]. In lung epithelial cells, CO was able to block expression and activity of MMP-1 and -2, indicating that also the products of HO-activity can affect MMPs [164]. So, targeting HO-activity could be a novel approach to modulate MMP-activity and thereby improve the outcome of pathological wound healing.

WOUNDS, ANGIogenesis and hEme oXYGENase

Vascularization of the wound, achieved by angiogenesis (formation of new blood vessels from preexisting capillaries) or by vasculogenesis (formation of new blood vessels from endothelial progenitor cells), is necessary for proper tissue repair. Factors stimulating the formation of new blood vessels are mainly secreted by keratinocytes and macrophages in the inflammatory phase, and include vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), keratinocyte growth factor (KGF) and insulin-like growth factor (IGF) (for review see: Gartner and colleagues [106]).

Numerous conditions affect the process of wound healing. Several genetic models have demonstrated the significance of various genes for the proper regeneration of the skin [165, 166]. Among them are crucial genes influencing inflammation and the formation of blood vessels such as HO-1. Indeed, involvement of HO-1 in blood vessel formation has been previously revealed, and so far numerous studies demonstrated its significance for the synthesis of VEGF-A in vascular smooth muscle cells [167], endothelial cells [168, 169], keratinocytes [170] and cardiomyocytes [171]. Also the production of SDF-1 was upregulated by HO-1 overexpression in myocardium [171]. Moreover, HO-1 works also downstream of both VEGF [168, 172] and SDF-1 [173] in endothelial cells and endothelial progenitor cells stimulated with those growth factors. In contrast, prostate cancer cells overexpressing HO-1 reduced VEGF-A levels [174]. Thus, under certain conditions HO-1 does not promote but inhibits angiogenesis, suggesting a more complex relationship between HO-1 and angiogenesis. This is described in more detail in the excellent review of Was et al. in this issue of Current Drug Targets [175]. Hence, the role of HO-1 in vascularization during the regenerative processes can be important for the healing of either skin or other injured tissues, but may be more complex than initially thought.

Of particular importance was the demonstration that the level of HO-1 expression in the skin significantly influenced the tissue vascularization. Accordingly, the number of CD31-positive capillaries was similar in wild type mice and in animals lacking one Hmox-1 allele. However, in homozygous Hmox-1 knockout individuals the number of
blood vessels was significantly lower, by about two-fold, while in the skin of transgenic mice, having additionally the human Hmox-1 gene expressed in keratinocytes, the number of capillaries was doubled in comparison to the wild type counterparts. It is well known that the skin of hyperglycemic db/db mice, which are used as a model of human type 2 diabetes, have a less dense network of vasculature in the injured skin [176]. The same was observed in our studies. However, adenoviral gene transfer of HO-1 increased the number of capillaries in the skin of diabetic mice while this did not occur in animals injected with control vector [112].

Various experimental models have demonstrated the improvement of wound healing after topical application of proangiogenic proteins or transfer of their genes. Indeed, delivery of VEGF protein [177, 178] or overexpression of VEGF-A or VEGF-C [179, 180] accelerated blood vessel formation and reduced the time of wound closure in various models. Similar results have been achieved in mice undergoing PDGF-BB [181], FGF-1 [182], FGF-2 [181], SDF-1 [183], IGF and KGF [184] or angiopoietin-1 [185] protein treatment or gene transfer. However, despite some beneficial effects of therapy with growth factors, most of the animal experiments and clinical trials have reported only modest improvements in wound closure [186, 187]. As wound healing is a complicated process, requiring the orchestration of actions of several cell types and numerous mediators, one can therefore presume that upregulation of gene(s) working upstream the angiogenic cytokines, and regulating the expression of several of them, can be better than augmenting the production of only one factor.

**WOUND HEALING TIME AND HEME OXYGENASE**

Because of the reduced risk of infection, a fast wound closure and a short healing time have always been thought of as beneficial. However, accelerated wound contraction can exacerbate scar formation [12]. Nowadays, antibiotics/antimycotics are very efficient in combating infections and consequently allow for slower wound closure. In an in vitro study we showed that curcumin can block fibroblast-mediated collagen gel contraction, and that restoration of HO-activity can protect against this effect [142].

The first approach to demonstrate the role of HO-1 in wound healing time was performed in an excisional wound healing model in mice devoid of the Hmox-1 gene [173]. We revealed that lack of both Hmox-1 alleles in 3-month old animals significantly attenuated skin closure. Moreover, while in wild type mice application of SDF-1 accelerated wound healing, the effect of this growth factor was impaired in HO-1 knockout mice [173]. In our recent study [112] a comprehensive approach was undertaken to investigate the significance of HO-1 for effects on wound healing time. To this end we have employed several animal models, investigating the effect of pharmacological inhibition of HO-1 expression, genetic knockout of either one or two Hmox-1 alleles as well as transgenic overexpression of HO-1 in keratinocytes [112].

Our study has revealed that pharmacological inhibition by local or systemic treatment of mice with SnPPIX or knockout of both Hmox-1 alleles significantly impaired closure of cutaneous wounds in 3 month old animals. Importantly, in the aging, 6 month old mice the lack of even one Hmox-1 allele significantly affected skin closure and in such older specimens the total absence of HO-1 could result in complete abrogation of repair [112]. Further, the absence of HO-1-activity reduced angiogenesis and had an inhibitory effect on keratinocyte migration and survival. Importantly, our observations are supported by other recent studies which demonstrated that pharmacological induction of HO-1 with tin chloride significantly accelerated wound healing time and decreased inflammation in injured corneal epithelium [177].

Additionally, we showed that in HO-2 knock-out mice wound closure was also delayed compared to WT animals and that this was related to increased inflammation [86]. Interestingly, in hyperbilirubinemic rats, wound closure time was shorter than in wild-type controls, indicating that high levels of bilirubin accelerate wound healing [86]. Of note, clinical data from sickle cell anemia patients, which are characterized by elevated serum bilirubin, as a result of a higher rate of heme degradation, indicate that they are better protected against leg ulcers, again suggesting a role for HO-1 in wound healing [178].

Combined, these results clearly show that HO-activity is intimately involved in the later stages of wound healing and is not only important in the resolution of inflammation. However, more research is required to assess the effects of HO-mediated acceleration of wound healing on scar formation.

**CONCLUSIONS AND FUTURE THERAPEUTIC POSSIBILITIES**

Summarizing, we expect that heme can play a central role in the pathogenesis of excessive scarring. The prolonged presence of heme-induced pro-oxidative, pro-inflammatory, and deleterious insults in the skin may initiate the formation of fibrosis and excessive scar formation. Heme-binding proteins, heme transporters, heme-degrading proteins, and anti-oxidant systems protect us against these heme-induced insults. Since the levels of these protective mechanisms or effector molecules are differentially regulated in individuals, induction hereof in patients with an impaired defense could possibly lead to amelioration of chronic wounds and prevent the formation of excessive scarring. Small concentrations of heme act cytoprotective by the swift up-regulation of HO-1 via the release of Bach1 from the HO-1 promoter, whereas large amounts of heme present for prolonged periods of time may act deleterious on tissue via its pro-oxidative and pro-inflammatory functions [3, 35, 179], which cannot be neutralized anymore by the anti-oxidative and anti-inflammatory properties of the HO-1 end-products.

HO-1, working both as a factor limiting the extent of uncontrolled inflammation-driven angiogenesis while inducing the non-inflammatory vascularization [172, 180] may be a good candidate for therapeutic approaches based on either gene transfer or augmentation of the endogenous expression of HO-1. Various small molecular compounds, activating the NrF2 transcription factor and inducing the expression of not only HO-1 but also other anti-oxidant and protective genes (for review see [104, 181]) can be tested for this purpose.
The possibility of HO-1 augmentation can also be considered in those clinical approaches which involve the application of cultured keratinocytes for treatment of severe burns. Such isolated keratinocytes can be relatively easily modified with gene transfer or endogenous HO-1 can be enhanced by pharmacological treatment. Our data [112] indicate that HO-1 may enhance the migration of keratinocytes, improving also their survival in stressful conditions and augmenting their proliferation, as has also been suggested by previous studies [182]. The application of the end-products of HO-1 activity can be additionally tested, as both CO and biliverdin can enhance VEGF synthesis in endothelial cells [183] and keratinocytes [170, 184], respectively.

Importantly, it is also possible that compounds already known to play a beneficial role in wound healing act via HO-1 induction. Regranex™, the recombinant PDGF-BB protein is the only one growth factor registered for the clinical treatment of diabetic ulcers [185]. Interestingly, PDGF-BB is a potent inducer of HO-1 expression [186]. Therefore, it is tempting to suggest that the beneficial effects of this biological, as demonstrated in some diabetic patients involve HO-1 induction.

Further research to the deleterious effects of heme and the protecting effects of heme-neutralizing mechanisms in wound healing and scarring is warranted, and is likely to result in novel strategies to prevent or attenuate excessive scarring.

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