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Delayed wound healing in aged skin rat models after thermal injury is associated with an increased MMP-9, K6 and CD44 expression

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ABSTRACT

Age-related differences in wound healing have been documented but little is known about the wound healing mechanism after burns. Our aim was to compare histological features and immunohistochemical expression of matrix metalloproteinase-9 (MMP-9), collagen IV, K6 and CD44 in the burn wound healing process in aged and young rats.

Following burns the appearance of the wound bed in aged rats had progressed but slowly, resulting in a delayed healing process compared to the young rats.

At 21 days after injury, epithelial K6, MMP-9 and CD44 expression was significantly increased in aged rats with respect to young rats; moreover, in the aged rat group we observed a not fully reconstituted basement membrane. K6, MMP-9 and CD44 expression was significantly increased in wounded skin compared to unwounded skin both in young and aged rats.

We hypothesise that delayed burn skin wound healing process in the aged rats may represent an age dependent response to injury where K6, MMP-9 and CD44 play a key role. It is therefore possible to suggest that these factors contribute to the delayed wound healing in aged skin and that modulation could lead to a better and faster recovery of skin damage in elderly.

1. Introduction

Thermal injury is one of the most severe forms of trauma that affects an organism [1]. Even with the development of improved burn patient care, many problems which arise for burn patients are associated with the cutaneous wound healing process. Burn wound healing is highly dynamic with complex interactions between numerous cell types, cytokines and proteins. Pathogenic abnormalities, ranging

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from disease-specific intrinsic flaws in blood supply, angiogenesis and matrix turnover to extrinsic factors due to infection and trauma, contribute to interfere in the mechanisms underlined the healing of the wounds [2]. In addition, it is well known that ageing is associated with a delay in the rate of the healing process [3,4]. The cutaneous wound healing process goes through several sequential phases including cell migration and proliferation, and extracellular matrix (ECM) deposition and remodeling. Initiation of wound repair is associated to the release of signalling molecules, such as cytokines, chemokines and growth factors [5]. Furthermore, the ability of keratinocytes to migrate into the wound area is extremely crucial during this process [6] and several hours following an injury to the skin, re-epithelisation is initiated, with keratinocytes migration from the wound edge into the wound clot [7]. In healthy epidermis, keratinocytes are not activated and they slowly proliferate in the basal layer and differentiate in the suprabasal layers. When an injury occurs, keratinocytes are prepared to respond very quickly by producing sentinel molecules ready to signal promptly that the tissue needs to become activated. Keratinocytes are capable of following an alternative differentiation route, known as regenerative differentiation, as a physiological adaptation of epidermal homeostasis. Regenerative differentiation is characterised by an overexpression of several proteins and, in particular, K6 is tightly associated with the keratinocyte phenotype of regenerative differentiation *in vivo* [8].

Metalloproteinases (MPs) are indispensable for the completion of the wound healing process, and increased levels of MMP-9 have been identified in many chronic wound types [9]. It has also been observed that MMP-9 (gelatinase B), a zinc-dependent endopeptidase, is up-regulated in burn wounds [9,10]. Interestingly, one of the major substrates of MMP-9 is collagen IV, an essential component of basement membranes (BMs). Studies in mice suggest that type IV collagen is indispensable for structural integrity and functions of the BM [11] and for the interaction of BMs with cells [12].

CD44 molecules are a family of transmembrane cell adhesion glycoproteins that are involved in cell–cell and cell–matrix interactions, leucocyte homing and activation, cellular migration, ECM assembly and cytokine binding and activation [13,14]. It has been observed that CD44 showed the highest expression levels on the plasma membranes of the hyperplastic cells [15]. In addition, it has been suggested that CD44 may have functions in the epidermis other than those related to hyaluronan (HA), such as binding growth factors [16] or MPs [17]. HA has been shown to influence keratinocyte proliferation and migration, and epidermal wound healing through its cell surface receptors such as CD44 [18].

Animal models make it possible to study the wound healing process in great detail and allow to us overcome several ethical considerations that limit the use of human volunteers. Considering that full-thickness wounds, like third degree burn wounds, heal leading to excessive scarring.

Since age-related differences in wound healing have been clearly documented [19] but little is known about wound healing mechanisms after burns, our aim was to investigate the burn wound healing process in the aged and young rats. We compared the histological features and the immunohistochemical expression of markers for proliferation, differentiation, re-epithelialisation and dermal repair (MMP-9, collagen IV, K6 and CD44).

2. Materials and methods

2.1. *Animals*

Twenty young (7–10 months, weighting 220–320 g) and 20 old (19–28 months, weighting 340–420 g) male Wistar rats were selected for this study. All animals were housed in individual cages under constant temperature (22 °C) and humidity with 12-h light/dark cycle, and had access to chow and water as much as desired throughout the study. The study was approved by the animal research Ethics Committee of the I.N.R.C.A. – I.R.R.C.S., Ancona, Italy.

2.2. *Experimental protocol*

Twelve young and 12 old rats were used as models of wound healing burned skin and eight young and eight old animals were used as controls of unwounded skin of each age.

Rats were anaesthetised by intra-muscular injection of ketamine/xylazine (40 and 13 mg kg^{-1}, respectively) and the back was shaved and washed with povidon iodide propanolol solution. A copper bar (12 mm × 12 mm) heated in boiling water (100 °C for 10 min) was placed on the paraspinal site of each animal for 40 s without pressure. Only the weight of the block was used to create the burns. No pressure was added. After reheating the probe in boiling water, a second burn was made symmetrically on the other side of the back. Bar application resulted in two full-thickness burns (zone of coagulation). In order to avoid variations, one person (FO) created all the burns. The rats were then resuscitated with 1 ml of Ringer's lactate solution administered by an intraperitoneal injection and returned to their cages. In order to avoid infections and/or damages, the wounds were protected by a band of cloth.

To mimic the clinical situation in burned patients, 48 h after the injury surgical debridement was performed in all the animals with isoflurane general anaesthesia to reduce the high risk of wound colonisation. During the surgery, the animals were maintained under body controlled temperature (36/37 °C) using a homeothermic blanket (Harvard apparatus). The wounds were left to heal by secondary intention (*i.e.*, the wound edges were not closed by sutures). To protect the wounds from outside contamination and infection, a sterile hydrated gauze was used. The animals were returned to individual cages and examined daily.

Each dressing change was made during general anaesthesia with isoflurane. After each measurement, the dressing is removed and the wound is flushed with sterile bandages physiology. At 7, 14 and 21 days after burns, tissue specimens were collected from each treated area. Moreover, specimens were collected from the unwounded rat group. All the samples were processed for morphological analysis. All the