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Original communication

A fundamental study on the dynamics of multiple biomarkers in mouse excisional wounds for wound age estimation

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A B S T R A C T

Wound age estimation is a classic but still modern theme in forensic practice. More experiments on different types of wound are needed to further improve its accuracy. In this study, mouse skin excisional wounds were created to simulate dermal defective injury. The neutrophil and macrophage infiltration, fibroblast and fibrocyte accumulation as well as their myofibroblastic transformation were examined. In addition, some wound healing-related molecules, including IL-1β, IL-6, TNF-α, IFN-γ, MCP-1, CXCL12, VEGF-A, EGF, KGF, pro-col Iα2 and pro-col IIIα1, were quantified by Western blotting and real-time quantitative PCR. Neutrophils and macrophages profoundly infiltrated in the wound at 12 h–1 d and 3 d–10 d respectively. Fibroblasts and fibrocytes accumulated in the wound from 3 d, and transformed into contractile myofibroblasts from 5 d post injury. The transformation ratios of fibroblasts and fibrocytes were highest at 7 d–10 d and 10 d respectively (over 50%). MCP-1 and CXCL12 increased from 12 h to 5 d, and IL-1β, TNF-α and pro-col IIIα1 up to 7 d. IL-6 and VEGF-A increased from 12 h to 1 d–10 d. Pro-col Iα2 increased from 7 d to 21 d. IFN-γ decreased from 12 h to 10 d. By comprehensive analysis of these factors from the perspective of morphometrics, protein and gene expressions, this study provided us with fundamental information for wound age estimation, especially in the wounds with full-thickness defection.

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1. Introduction

Wound age estimation is a classic but still modern theme in forensic pathology, which provides useful information for crime scene reconstruction.[1,2] Skin wound healing is a complicated process that involves coordinated interactions among diverse cells and regulatory mediators in a series of overlapping but distinct stages, including inflammation, new tissue formation, and remodeling.[3–5] Based on this cognition, forensic pathologists carried out extensive experiments to search chronological markers for wound age estimation. The acquired information referring to time-dependent appearances of effector cells[6–11] and expressions of regulatory mediators (cytokines,[1,12–15] chemokines,[14,16,17] growth factors,[17–19] proteases,[17,20] members of extracellular matrix,[17,21–23] etc.), provides valuable experiences for wound age estimation.

Wound age estimation should take into consideration the local factors, e.g. the size and type of lesions.[24] In forensic practice, we often encounter some severely injured wounds with full-thickness defection. For these cases, the existing experiences mostly derived from incisional wound studies might not provide accurately chronological information. Therefore, some specific studies in defective wounds should be carried out to improve the accuracy of wound age estimation. Wound contraction is an important characteristic of skin wound healing, which is especially obvious in defective injury. It is well accepted that under mechanical and cytokine stimuli, some fibroblasts in the wound cavity would transform into α-SMA-expressing myofibroblasts, which possess contractile property and contribute to wound contraction.[5] Meanwhile, bone marrow-derived mesenchymal progenitor fibrocytes, which co-express leukocyte and mesenchymocyte markers (CD45, CD34, collagen I,

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pro-collagen I, etc.), also contribute to the fibroblast populations and have potential to differentiate into myofibroblasts.[25–27] Previous studies have testified the appearances of fibroblasts, fibrocytes, and myofibroblasts are good candidates for wound age estimation.[7,10] Therefore, we hypothesize the time-dependent transformation of fibroblast-to-myofibroblast and fibrocyte-to-myofibroblast might also be usefully chronological biomarkers.

On wound age estimation, there is a consensus that various parameters with different, but complementary characteristics should be examined, so that the combination of results minimizes error margin in time calculation.[24] Therefore, besides the fibroblast and fibrocyte accumulation and their myofibroblastic transformation, some other biomarkers that have been well accepted being critical for skin wound healing, including the neutrophil and macrophage infiltration, as well as the expressions of chemokines (MCP-1, CXCL12), inflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ), growth factors (EGF, KGF, VEGF-A), and matrix proteins (pro-col Iα2, pro-col IIIα1) were measured and analyzed comprehensively, aiming to provide information for chronological estimation in defective wounds.

2. Materials and methods

2.1. Animal model of skin wound

A total of 60 male 8-week-old healthy BALB/c mice, each weighing 25–30 g, were used in this experiment. Excisional wounds were created according to the instruction of Birch et al.[28] The mice were anesthetized by sodium pentobarbital (i.p. injection). Two full-thickness dermal excisional wounds were created symmetrically over the midline on the dorsal skin with a 6-mm-diameter sterile biopsy punch. After surgery, mice were housed individually in a temperature-controlled sterile environment with a 12-h light/dark cycle, and were allowed commercial mouse chow and distilled water *ad libertum*. Mice were euthanized at 12 h, 1 d, 3 d, 5 d, 7 d, 10 d, 13 d, 17 d and 21 d post-injury (six mice at each time point). Six mice without surgery were used as control. Two 1 cm × 1 cm skin specimens centered on the wounds were collected from each mouse. One specimen was used for immunostaining procedure, and the other for Western blotting and qRT-PCR.

2.2. Tissue preparation and immunostaining

Skin specimens were fixed in 4% paraformaldehyde buffered with PBS (pH 7.4) and embedded in paraffin. 4 μm-thickness consecutive sections were prepared. Immunohistochemical staining was performed using the streptavidin–peroxidase method. Briefly, deparaffinized sections were heated 5 min in 0.01 mol/L sodium citrate buffer (pH 6.0) for antigen retrieve. Endogenous peroxides activity was quenched in 3% hydrogen peroxide and nonspecific binding were blocked with 10% non-immune goat serum. Afterward, the sections were incubated with rabbit anti-MPO polyclonal antibody (pAb) (1:100, ab9535, Abcam, Cambridge, UK) and rat anti-F4/80 monoclonal antibody (mAb) (1:100, ab6640, Abcam) overnight at 4 °C respectively, followed by incubation with corresponding Histostain-Plus Kit (Zymed Laboratories, CA, USA) according to the manufacturer's instructions. Sections were routinely counterstained with hematoxylin. Cell numbers of neutrophils (MPO^+) and macrophages ($F4/80^+$) were analyzed in five sections of each wound and five randomly selected microscope fields per section close and parallel to wound edges at 400-fold magnification (0.07223 mm^2).

Triple immunofluorescent procedures were performed with rat anti-CD45 mAb (550539, BD Biosciences, CA, USA), goat anti-pro-col I pAb (sc-25974, Santa Cruz Biotechnology, CA, USA), and rabbit anti-α-SMA pAb (ab5694, Abcam). Briefly, deparaffinized and antigen-retrieved (as aforementioned) sections were blocked with 5% normal donkey serum (Jackson ImmunoResearch, PA, USA), followed by incubation with CD45 (1:50), pro-col I (1:200), and α-SMA (1:200) antibodies overnight at 4 °C. After washing, the sections were further incubated with Alexa Fluor® 488 donkey anti-rat IgG (1:200, A21208, Invitrogen, CA, USA), Alexa Fluor® 594 donkey anti-goat IgG (1:200, A11058, Invitrogen), and Alexa Fluor® 350 donkey anti-rabbit IgG (1:200, A10039, Invitrogen) at room temperature (RT) for 2 h. The immunofiuorescent images were taken by fluorescence microscopy (DM4000 B, Leica Microsystems, Wetzlar, Germany). As negative controls, sections were incubated with normal rat, goat and rabbit non-immune IgG or PBS instead of the primary antibodies, and no positive signal was detected. Cell numbers of general fibroblasts (pro-Col I^+), general myofibroblasts (pro-Col I^+/α-SMA^+), fibrocytes ($CD45^+$/pro-Col I^+), and fibrocyte-derived myofibroblast ($CD45^+$/pro-Col I^+/α-SMA^+) were analyzed in five sections of each wound and five randomly selected microscope fields *per* section close and parallel to wound edges at 400-fold magnification (0.07223 mm^2). The general fibroblast-to-myofibroblast transformation was reflected by the ratio of myofibroblast number to fibroblast number. The fibrocyte-to-myofibroblast transformation was reflected by the ratio of fibrocyte-derived myofibroblast number to fibrocyte number.

Table 1
Primer sequences of biomarkers detected in the present study.

Biomarker	Primer sequence (5′–3′)		Product size (bp)	GenBank ID
IL-1β	Forward	ACGGACCCCAAAAGATGAAG	139	NM_008361
	Reverse	TTCTCCACAGCCACAATGAG		
TNF-α	Forward	CTTCTGTCTACTGAACTTCGGG	134	NM_013693
	Reverse	CAGGCTTGTCACTCGAATTTTG		
MCP-1	Forward	GTCCCTGTCATGCTTCTGG	144	NM_011333
	Reverse	GCTCTCCAGCCTACTCATTG		
CXCL12	Forward	TCCTCAACACTCCAAACTGTG	149	NM_013655
	Reverse	GACTCACACCTCTCACATCTTG		
KGF	Forward	AAGACTGTTCTGTCGCACC	146	NM_008008
	Reverse	CACTTTCCACCCCTTTGATTG		
EGF	Forward	AGAAACACCAAGACCCCAAG	149	NM_010113
	Reverse	TGTGCCCATTCCATCTATGTG		
β-actin	Forward	ACCTTCTACAATGAGCTGCG	147	NM_007393
	Reverse	CTGGATGGCTACGTACATGG		

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