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Immunohistochemical detection of CD14 and combined assessment with CD32B and CD68 for wound age estimation

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ABSTRACT

Estimation of wound age is a major topic of study for forensic pathologists, but few markers exist that can indicate a specific period 1–5 days postinfliction, and a method to estimate wound age with high accuracy has not yet been established. This study examined CD14 as such a marker in mouse skin wounds of different ages (0 min and 1, 2, 3, 5, 7, and 9 days) and in human subjects (group 1, 0–1 day; group 2, 1–5 days; group 3, >7 days) using Western blot analysis and/or immunohistochemical staining. In addition, we evaluated a combination of immunohistochemical markers in human skin wounds using transmembrane proteins, CD14, CD32B, and CD68, expressed on inflammatory cells. The expression of CD14 was detected only during 1–5 days postinfliction and, thus, the evaluation of CD14-expressing cells could specify wound age during 1–5 days postinfliction in mouse skin wounds. The ratio of samples assessed to be $CD14^+$ was significantly high in human skin wounds in group 2. Combined assessment using the three markers increased the specificity of diagnosis and shortened the range of wound age, compared with the assessment using a single marker. Our results indicate that CD14 may be a useful marker of wound age, 1–5 days postinfliction, and that combined assessment with CD14, CD32B, and CD68 may be a good method for the accurate estimation of wound age.

1. Introduction

Examination of wounds is one of the most important aspects in forensic practice. Forensic pathologists routinely estimate wound age based on appearance, such as the color of subcutaneous hemorrhage and scab formation, of the wound [1]. In addition, Berlin blue staining, which detects hemosiderin in subcutaneous tissues until approximately 7 days postinfliction, is conducted using conventional methods for wound age estimation [2]. However, it is difficult to accurately and objectively diagnose wound age at a stage as early as <7 days, particularly in 1–5 days postinfliction, using this method.

Several studies have conducted wound age estimation using biological substances [3–11]; however, only a few markers and methods were effective for high-accuracy wound age estimation in 1–5 days postinfliction. Therefore, identifying a marker and establishing a method for estimating wound age in 1–5 days postinfliction is important in forensic practice.

Cooper et al. [12] reported a portfolio of >1000 genes expressing across repair responses in neonatal mice using microarray analysis. Previously, we revealed that the mRNA expression of CD14, which is one of the genes exhibiting drastically changed time-course expression, reached peak levels at 12–24 h postinfliction in mouse skin [13]. In that study, our results indicated that the CD14 protein may be a useful marker in the 1–5 days postinfliction because there may be delayed protein expression following mRNA increase; this could be because of the additional time required to translate mRNA into protein, and protein expression may last much longer than that of the mRNA because of its high stability against degradation after death.

However, simultaneous detection of plural markers may be effective as a method to increase the accuracy of wound age estimation [3,10,11]. CD32B and CD68 are transmembrane proteins expressed on inflammatory cells similar to CD14. Therefore, we hypothesized that CD32B and CD68 may exhibit expression patterns similar to CD14.

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The objectives of this study were as follows: (1) to examine the expression of CD14 in mouse and human skin wounds with Western blotting and/or immunohistochemistry to evaluate the efficacy of CD14 as a marker for wound age estimation in 1–5 days postinfliction; (2) to conduct a preliminary study of whether the addition of markers, such as CD14-expressing cells, yielded more accurate estimation of wound age in mouse skin wounds; and (3) to investigate the time-course expression of CD32B and CD68 as well as CD14 to evaluate the effectiveness of combined assessment of wound age with these three markers by immunohistochemistry.

2. Materials and methods

2.1. Animal experiments

Pathogen-free 6-week-old male BALB/c mice purchased from SLC, Inc. (Shizuoka, Japan) were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and their dorsa were shaved. Furthermore, six full-thickness wounds 4 mm in diameter were resected from the dorsum of each mouse using biopsy punch. Each mouse was housed in a sterilized cage and given autoclaved food and redistilled water to prevent bacterial infection. The mice in the study groups ($n = 5$ for all groups except the 5–days group; $n = 4$ in the 5–days group) were euthanized at different time points of 0 min, 1, 2, 3, 5, 7, and 9 days after wound infliction. The entire wound area was harvested and stored at −80 °C until analysis. Skin tissues at time point of 0 min were examined as the control. The Animal Care Committee of Nagasaki University approved this research protocol (Approval No. 1410211181).

2.2. Human skin wound specimens

Human skin wound specimens [$n = 97$ from 44 individuals, age 16 days to 86 years (mean, 56.2 years)] of different postinfliction intervals, ranging from a few minutes to 30 days, were collected at forensic autopsies, and the postmortem interval till specimen collection was <3 days for all specimens. The wound specimens were classified into three groups according to wound age as follows: group 1 (0–1 day; $n = 38$), group 2 (1–5 days; $n = 50$), and group 3 (>7 days; $n = 9$). Samples of uninjured skin from the same individuals were collected as the control. The Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences (Medical Course) approved this research protocol (Approval No. 15011660).

2.3. Antibodies

The following monoclonal or polyclonal antibodies (mAbs or pAbs) were used in this study: rabbit anti-CD14 pAbs (ab106285, Abcam, Cambridge, UK), rat anti-Ly6G6C mAbs (ab2557, Abcam), rat anti-F4/80 mAbs (ab6640, Abcam), rabbit anti-CD32B mAbs (ab45143, Abcam), mouse anti-CD68 mAbs (ab955, Abcam), goat anti-mouse CD14 pAbs (AF982, R&D Systems, Inc., Minneapolis, USA), rabbit anti-beta Tubulin pAbs (ab6046, Abcam), Histofine® Simple Stain Mouse Max-PO (R), Histofine® Simple Stain Max-PO (R), Histofine® Simple Stain Max-PO (M) (Nichirei Biosciences Inc., Tokyo, Japan), Alexa Fluor® 488 goat anti-rabbit IgG (H + L) antibody, Alexa Fluor® 568 goat anti-rat IgG (H + L) antibody (Thermo Fisher Scientific Inc., MA, USA), Secondary Antibody Solutions Alk-Phos. Conjugated (Anti-Rabbit), and Secondary Antibody Solutions Alk-Phos. Conjugated (Anti-Goat) (Thermo Fisher Scientific Inc.).

2.4. Western Blot analysis in mouse skin wounds

Skin tissues of mice were homogenized with a lysis buffer (50 mM Tris–HCl, pH 8.0, 2% SDS, 10 mM DTT) containing Phosphatase Inhibitor Cocktail and Protease Inhibitor Cocktail (Nacalai Tesque, Inc., Kyoto, Japan). After the protein concentration of all of the fractions was determined with a Bio-Rad protein assay (Bio-Rad Laboratories, California, USA), equal amount of proteins were electrophoresed on NuPAGE® Novex 4–12% Bis–Tris Gel (Thermo Fisher Scientific Inc.). Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane under 20 V for 7 min with iBlot™ (Thermo Fisher Scientific Inc.), and the membranes were treated with a PVDF Blocking Reagent from Can Get Signal® (TOYOBO CO., LTD., Osaka, Japan) for 30 min at room temperature and then incubated overnight at 4 °C with goat anti-mouse CD14 pAbs diluted to a 1:1000 concentration. β-Tubulin was used as an internal control. For detection, the membranes were incubated with a second antibody-solution – conjugated alkaline phosphatase at a 1:2000 dilution, and the blots were allowed to react with Novex® AP Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). After imaging with LAS-3000 mini (FUJIFILM, Tokyo, Japan), Quantity One (Bio-Rad Laboratories) was used to analyze the average density values.

2.5. Immunohistochemical analysis in mouse skin wounds

The wound tissue specimens of mouse were fixed in 4% paraformaldehyde with phosphate-buffered saline (PBS), embedded in paraffin, and cut into 3-μm sections with a microtome. Deparaffinized sections were irradiated with microwaves two times for 5 min in Dako Target Retrieval Solution, pH 9 (Agilent Technologies, California, USA). The sections were immersed in an endogenous peroxidase blocking reagent (0.3% H_2O_2, 0.2% phenylhydrazine, 1 mM PBS) for 30 min at room temperature, treated with Dako Protein Block Serum-Free Ready-to-use (Agilent Technologies) for 1 h at room temperature, and incubated overnight with rabbit anti-CD14 pAbs (dilution, 1:500) at 4 °C. Subsequently, the sections were reacted with Histofine® Simple Stain Mouse Max-PO (R) for 30 min and stained with a coloring reagent (50 mM Tris–HCl buffer, pH 7.6, 0.02% 3, 3′-diaminobenzidine, 0.006% H_2O_2) for 3 min at room temperature. Nuclear staining was performed by incubation with hematoxylin for 10 min at room temperature.

2.6. Double-color immunofluorescence analysis in mouse skin wounds

Double-color immunofluorescence analysis was conducted to determine the types of CD14-expressing cells during skin wound healing in the mouse. Deparaffinized sections were treated as described earlier with irradiation and endogenous peroxidase blocking. The sections were further incubated in a mixture of rabbit anti-CD14 pAbs (dilution 1:500) and rat anti-F4/80 mAbs (macrophage marker; dilution 1:500) or rabbit anti-CD14 pAbs (dilution 1:500) and rat anti-Ly6G6C mAbs (neutrophil marker; dilution 1:500) at 4 °C overnight. After incubation with a mixture of Alexa Fluor® 488 goat anti-rabbit IgG (H + L) antibody for rabbit anti-CD14 pAbs and Alexa Fluor® 568 goat anti-rat IgG (H + L) antibody for rat anti-F4/80 mAbs or rat anti-Ly6G6C mAbs for 1 h at room temperature, the sections were mounted with a VECTASHIELD Mounting Medium with DAPI (VECTOR LABORATORIES, INC. California, USA) and observed under a fluorescence microscope.

2.7. Immunohistochemical analysis in human skin wounds

Human wound specimens were fixed in 10% formaldehyde with PBS, embedded in paraffin, and then sliced into 3-μm sections with a microtome. Sections deparaffinized for the detection of CD14 were irradiated with microwaves two times for 5 min in Dako