Clinical Immunology 164 (2016) 78–84

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Neutrophils contribute to fracture healing by synthesizing fibronectin^{+} extracellular matrix rapidly after injury

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ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 2 February 2016
accepted with revision 3 February 2016
Available online 12 February 2016

Keywords:
Osteoimmunology
Bone
Regeneration
Repair
Inflammation
Leukocytes

ABSTRACT

The role of inflammatory cells in bone regeneration remains unclear. We hypothesize that leukocytes contribute to fracture healing by rapidly synthesizing an "emergency extracellular matrix (ECM)" before stromal cells infiltrate the fracture hematoma (FH) and synthesize the eventual collagenous bone tissue.
53 human FHs were isolated at different time points after injury, ranging from day 0 until day 23 after trauma and stained using (immuno)histochemistry.
FHs isolated within 48 h after injury contained fibronectin^{+} ECM, which increased over time. Neutrophils within the early FHs stained positive for cellular fibronectin and neutrophil derived particles were visible within the fibronectin^{+} ECM. Stromal cells appeared at day 5 after injury or later and collagen type I birefringent fibrils could be identified during the second week after injury.
Our study suggests that neutrophils contribute to bone regeneration by synthesizing an "emergency ECM" before stromal cells infiltrate the FH and synthesize the eventual bone tissue.

1. Introduction

Leukocytes are well known for their role in innate immune responses to invading microorganisms, but only little is known about their role in bone tissue regeneration. After bone injury, a blood collection forms around the fracture site, which is generally referred to as fracture hematoma (FH). Leukocytes rapidly infiltrate this FH, which marks the inflammatory phase of fracture healing [1]. The current literature suggests that the inflammatory phase of fracture healing affects downstream processes of bone repair [1, 2]. This is illustrated by the finding that removal [3] or repetitive irrigation [4] of the early FH impairs bone healing in animals. Moreover, transplantation of the early FH into muscle tissue induces ectopic bone formation within muscle tissue [5].

These findings imply that inflammatory processes, which occur during early bone repair, are essential for adequate bone regeneration. However, other studies suggest that certain inflammatory conditions can negatively affect the outcome of fracture healing [1, 2]. For instance, open fractures, severe soft-tissue injury and presence of multiple injuries, which are considered to induce local and systemic inflammation, have been associated with impaired fracture healing [6]. Moreover, local injection of beta glucan into the FH [7] or intraperitoneal injection of lipopolysaccharides [8], which are experimental models of local and systemic inflammation, negatively affect the outcome of bone regeneration in rats.

In summary, the currently available evidence implies that inflammation has a significant impact on the outcome of bone tissue regeneration. Clarifying the role of inflammatory cells during bone healing may contribute to the development of therapies that augment tissue regeneration and/or prevent impairment of bone healing after local and systemic hyper-inflammatory conditions.

We hypothesize that inflammatory cells contribute to bone healing by synthesizing some sort of emergency extracellular matrix (ECM) before stromal cells infiltrate the FH and synthesize the eventual bone ECM, which consists mainly of mineralized collagen type 1 fibrils [9, 10].

To test this hypothesis, we analyzed the temporal changes in the composition of the human fracture hematoma during early bone

Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; FH, fracture hematoma; H&E, hematoxylin and eosin; IgG, immunoglobulin G; LPR, liquid permanent red; PBS, phosphate buffered saline; RA, rheumatoid arthritis; TGF, transforming growth factor; TMA, tissue microarray.

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http://dx.doi.org/10.1016/j.clim.2016.02.001

healing. We determined whether ECM was present within FHs that were isolated before stromal cells infiltrated the FH and collagen type I positive fibrils could be identified.

2. Materials and methods

2.1. Isolation of fracture hematomas

53 fracture hematomas (FH) of closed fractures were isolated from trauma patients during an Open Reduction Internal Fixation procedure between 01-03-2011 and 01-04-2013. Four time-groups were defined based on the time between injury and isolation of the FH: 1) within 2 days, 2) 3–5 days, 3) 6–10 days and 4) >10 days. As a control, peripheral blood was drawn from healthy donors into a coagulation tube using a Vacutainer® system. After 1 h, the coagulated blood was removed from the coagulation tube and treated similar to the freshly isolated fracture hematomas.

All FHs were fixed directly after isolation in 3.7% buffered formaldehyde solution (pH 4.0) for at least one week. The FHs were then dehydrated and embedded in paraffin with a Leica Embedding Center (EG1160, Leica Microsystems). Sections of 4 μm were cut with a Microm microtome (HM 335E, Thermo Scientific) and incubated at 54 °C overnight to allow firm adherence of the tissue to the microscopy slides (Superfrost Ultra Plus, Thermo Scientific). Fracture hematomas were residual samples and therefore collected without informed consent, unless the patient refused explicitly (opt-out method). Our local medical–ethical review committee has approved this study.

2.2. Tissue microarray

A tissue microarray (TMA) was built to allow simultaneous and thereby comparable staining of all fracture hematomas in a single procedure. Two 1 mm cylindrical biopsy cores of each fracture hematoma were transferred to a single TMA paraffin block with a TMA GRAND Master microarrayer (3DHISTECH). The biopsy points were identified based on a hematoxylin and eosin (H&E) stained section of the entire fracture hematoma. A Dako CoverStainer was used to stain the sections with H&E. Two criteria were used to determine the biopsy locations: 1) when cells with a fibroblast-like morphology [11, 12] could be identified, that area was biopsied and 2) when these cells could not be identified within the H&E sections, a representative area of the FH was biopsied. Subsequently, multiple sections of 4 μm were cut and incubated at 54 °C overnight. The slides were deparaffinized, rehydrated and stained as follows 1) Hematoxylin and Eosin, 2) Picrosirius Red, 3) immunohistochemistry of CD45 (leukocytes), CD66b (neutrophils), CD68 (monocytes/macrophages), collagen type I and insoluble cell-derived fibronectin (the antibodies used do not recognize soluble plasma derived fibronectin).

2.3. Analysis of collagen fibers

Collagen fibers are anisotropic and therefore birefringent. Picrosirius Red binds to collagen and enhances its natural birefringence. Placing the FH section stained with PicroSirius Red between two polarization filters with 90 degrees of rotational difference between both filters allows clear identification of birefringent fibrils. In brief, TMA sections were deparaffinized, washed in dH_2O and stained with PicroSirius Red solution (Sirius red F3B (80115, Klinipath) diluted 1 g/L in saturated aqueous picric acid (36011, Riedel-deHaën) 1 h at room temperature. The sections were rinsed twice with 0.01 M HCl. The cell nuclei were stained with Hoechst 33258 (861405-100MG, Sigma-Aldrich) 10 μg/mL in dH_2O for 10 min in the dark at room temperature and rinsed in 3 changes with dH_2O. The sections were dehydrated, embedded with a ClearVue coverslipper (Thermo Scientific) and stored in the dark until further analysis.

2.4. Immunohistochemistry

Five TMA sections were stained using immunohistochemistry.

In the first section, leukocytes were stained red and collagen type I was stained blue. In the second section, neutrophils were stained red and insoluble cell-derived fibronectin was stained blue (the antibodies used do not recognize soluble plasma derived fibronectin). In the third section, macrophages were stained red and insoluble cell-derived fibronectin was stained blue. In the fourth section, neutrophils were stained red and monocytes/macrophages were stained blue. In the fifth section, two isotype-matched control antibodies were applied. The nuclei of all cells were fluorescently labeled with Hoechst.

TMA sections were deparaffinized and rinsed in dH_2O. The sequential alkaline phosphatase (ALP) double immunostaining was carried out as described previously [13, 14] with Liquid Permanent Red Substrate Chromagen (K0640, Dako) and the Vector Blue Alkaline Phosphatase Substrate Kit III (SK-5300, Vector Laboratories).

As first primary antibodies, we used mouse anti-human Leukocyte Common Antigen CD45 (Clones 2B11 + PD7/26, Dako, 3,75 μg/mL), mouse anti-human CD66b (MCA216, AbD Serotec, 10 μg/mL), mouse anti-human CD68 (NCL-CD68-KP1, Novocastra, Leica Biosystems, stock diluted 1:100) and mouse IgG1 as negative isotype control (X0931, Dako, 10 μg/mL). The second primary antibodies were mouse anti-human CD68 (NCL-CD68-KP1, Novocastra, Leica Biosystems, stock diluted 1:100), mouse anti-human cellular fibronectin (IST-9/ab6328, Abcam, 2 μg/mL), mouse anti-human collagen type I (COL-1/ab90395, Abcam, stock diluted 1:100) and mouse IgG1 as second negative isotype control (X0931, Dako, 10 μg/mL). Secondary antibodies were BrightVision polyclonal ALP-Anti-Mouse IgG (DPVM110AP, ImmunoLogic, undiluted).

2.5. Imaging and analysis

Each stained TMA core was imaged with a Leica DFC425C camera (Leica Microsystems) mounted to a Leica microscope (Leitz DMRXE, Leica Microsystems), using a point revisiting automated stage. A custom built algorithm was used to count cell numbers and the amount of Vector Blue Stained ECM or birefringent fibrils. The number of cells and the amount of extracellular matrix was normalized for the amount of tissue that was present on the image by subtracting all white pixels from the image. Representative images were taken with an Olympus DP70 camera, connected to an Olympus BX51 microscope using Cell^F software version 3.4 (Olympus Soft Imaging Solutions). All bright field images were exported as TIFF from Cell^F. Each bright field images was imported into Adobe Photoshop CS6 version 13 and arranged without manipulation of the original images. Fluorescent images of each filter were exported from Cell^F as TIFF files and also imported into Adobe Photoshop. In Photoshop, the blue channel of Hoechst images were merged with the red channel of Liquid Permanent Red (LPR) images without further modifying the images. The scale bar of the merged Hoechst/LPR image was not white, since it only contained a blue and red channel image of the scale bar. The white scale bar from the original Hoechst was therefore copied and pasted onto the merged Hoechst/LPR image. The merged images were subsequently arranged in Photoshop and exported as TIFF images. Graphs were made using GraphPad Prism version 5.03 (GraphPad Software, Inc.). Fracture hematoma characteristics were analyzed using SPSS version 20.0.0 (IBM Corporation).

2.6. Statistical analysis

GraphPad Prism version 5.03 (GraphPad Software, Inc.) was used to determine whether the differences in cell count, leukocyte count, neutrophil count, monocyte count, fibronectin, collagen type 1 and the amount of birefringent fibrils were statistically significant between groups. First, a Kolmogorov–Smirnov test was used to determine whether the data was normally distributed. The differences between