Effects of Callus Formation during Bone Repair in Tibia-Injured Rats on Hematopoietic Stem Cells Around the Callus

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Summary

During bone repair, immature bone, called callus, is formed in the bone marrow. On the other hand, bone plays a role in hematopoiesis involving hematopoietic stem cells (HSCs). Previous studies have shown that an increase in adipose and fibrous tissue in the bone marrow leads to a decrease in HSCs. However, whether this is also affected by callus has not been examined. In this study, as part of a basic study to investigate the relationship between HSC and callus, we histologically examined the effect of callus formed at the time of fracture on the surrounding HSC. Twelve 6-week-old male rats were used as material. When rat is at 7 weeks of age, the rats were classified into three groups: one group in which no bone injury was created in the tibia, and two groups in which the rats were euthanized 1 week and 2 weeks after bone injury After the experiment, tibiae were removed from rats in each group, and various histological specimens were prepared for observation. In normal bone marrow, only a large number of bone marrow cells stained blue by toluidine blue staining were observed, and CD34 immunoreactivity was clearly observed in a wide area of bone marrow. In bone marrow 7 days after bone injury, a large number of callus producing metachromasia were observed, and CD34 immunoreactivity was markedly reduced

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compared to the condition observed in normal bone marrow. In bone marrow 14 days after bone injury, the amount of callus was less than 7 days after bone injury, and CD34 immunoreactivity was increased compared to 7 days after bone injury, similar to normal bone marrow. Callus formed in the bone marrow during the repair process of bone injury were thought to contribute as a negative factor to the increase or decrease of CD34 immunoreactivity, suggesting a transient decrease in hematopoietic stem cells. It was suggested to Callus formed in the bone marrow after fracture decrease hematopoietic stem cells around callus.

Keywords：Fracture

Introduction

A fracture is a partial or complete breakdown of bone continuity, but is characterized by time-dependent regeneration. Normally, the fracture area is restored to a normal state through an inflammatory phase[1], a repair phase[2] and a remodeling phase[3]. On the other hand, such bone repair process transiently results in the formation of less mature bone, called callus, in the bone marrow of the fracture site[4-6].

Bone not only controls the support and movement of the body, but also plays a role in hematopoiesis[7]. Hematopoiesis occurs primarily in the bone marrow inside the bone, where a wide variety of blood and immune cells are continuously produced. All of these cells produced in the bone marrow have in common the differentiation and maturation from hematopoietic stem cells (HSCs).

On the other hand, it has been reported that a decrease in HSCs is induced by an increase in adipose tissue[8] and fibrous tissue[9] in the bone marrow, but whether this also affects callus formation has not been examined. As a basic study to investigate the relationship between HSCs and callus, the purpose of this study was to histologically examine the effect of callus formed as a result of fracture on HSCs around the callus.

Materials and Methods

Animal

Six-week-old (6W) male rats (Wistar strain, 12 rats, Nippon Bio Supp. Center, TYO, JPN) were used in this study. All rats were brought in and pre-reared for 1 week under specific pathogen-free conditions, kept on a 12-hour light/dark cycle. Rats were kept up to 4 rats per gauge and fed ad libitum with water and food (Oriental Yeast Co. LTD, TYO, JPN). At

the end of the pre-rearing period (at 7W for rats), rats were classified into two groups: rats euthanized by CO2 gas inhalation (n=4) and rats subjected to bone injury (n=8) to simulate bone fractures. Bone-damaged rats were euthanized in the same manner as above at 1 week (n=4) and 2 weeks (n=4) after bone injury. The tibiae were promptly removed from both euthanized rats and immersed and fixed in 4% paraformaldehyde solution. Various histological specimens were then prepared and observed by light microscopy (Fig. 1). This experiment was approved by the Animal Experimentation and Research Ethics Committee of the Graduate School of Life Design, Toyo University (TYO, JPN, No. 2021-17).

Creation of Bone injury models

Bone-injured rats were anesthetized (1.0ml/100g) with three anesthetics (medetomidine hydrochloride 0.15 mg/kg + midazolam 2.00 mg/kg + putorphanol tartrate 2.50 mg/kg) injected intraperitoneally before surgery. The skin and muscle of the anteromedial surface of the rat tibia were incised vertically with a scalpel approximately 1.0 cm to expose the cortical bone of the anteromedial surface of the proximal tibial diaphysis. The exposed bone surface was damaged with a dental hand motor fitted with a drill approximately 2.00 mm in diameter (Fig. 2). After bone injury, the incised skin was sutured with suture. Rats were allowed to recover spontaneously from the anesthetized state after administration of 0.3 mg/kg of repetan and kept normally in the cage.

Non-decalcification specimen in toluidine blue

Samples were embedded in Ligolac resin. The specimens were polished and stained with toluidine blue.

Demineralized paraffin sections

Samples were demineralized in 8% EDTA for 3 weeks and then embedded in paraffin. Paraffin-embedded samples were sectioned into sections approximately 4㎛ thick.

Immunohistochemistry

Paraffin sections were paraffinized with Lemosol, dehydrated with ethanol, and blocked with 3% bovine serum albumin (WAKO, TYO, JPN) for 60 minutes. Sections were then reacted with primary antibody of CD34, a marker of HSC (BIW, MN, US; ×100) for 60 minutes at room temperature. After completion of that reaction, fluorescent secondary antibodies (Abcam, Cambridge, UK; ×100) were treated on the sections for 30 minutes.

Finally, nuclear staining was performed using DAPI (Abcam).

Morphometric measurements

Callus area and CD34 positive cell count were measured using Win ROOF.

Results

Normal

First, in normal bone marrow, only a large number of bone marrow cells stained blue by toluidine blue staining were observed. Immunoreactivity of CD34 was clearly observed in a wide area of the bone marrow (Fig. 3a, b, g, h).

7 days after bone injury

Next, observation of the bone marrow 7 days after bone injury revealed a large number of callus in the bone marrow that had developed metachromasia; the CD34 immunoreactivity was markedly reduced from the condition observed in normal bone marrow (Fig. 3c, d, g, h).

14days after bone injury

Furthermore, when bone marrow was observed 14 days after bone injury, smaller amount of callus was observed in the bone marrow than was observed 7 days after bone injury. The CD34 immunoreactivity was increased compared to 7 days after bone injury and resembled normal bone marrow (Fig. 2e, f, g, h).

Discussion

In bone fractures, callus are transiently formed in the bone marrow during repair[1–6]. HSCs are present in the bone marrow as a source of hematopoiesis, and their numbers decrease in the bone marrow due to the increase of adipose[8] and fibrous tissue[9]. Thus, we expected that bone marrow callus formed during fracture repair would result in a decrease in periprosthetic HSCs. We first observed normal bone marrow. In the bone marrow, we observed only a large number of bone marrow cells that stained blue with toluidine blue stain. In contrast, bone marrow at 7 days after bone injury showed many callus with extensive metachromasia, and bone marrow at 14 days after bone injury showed a smaller amount of callus than at 7 days after bone injury. In contrast, extensive and distinct immunoreactivity for CD34, a marker of HSC, was observed in normal bone marrow.

However, after 7 days of bone injury, CD34 immunoreactivity was markedly reduced compared to the normal state. On the other hand, 14 days after bone injury, CD34 immunoreactivity was increased compared to 7 days after bone injury and recovered to a state similar to normal bone marrow. In summary, it was understood that the callus formed in bone marrow increased after 7 days of bone injury and then showed a decreasing trend after 14 days of bone injury. On the other hand, CD34 immunoreactivity showed a tendency to decrease over 7 days after bone injury and then increase 14 days after bone injury. Therefore, callus formed in the bone marrow during the repair process of bone injury were considered to contribute as a negative factor to the increase or decrease of CD34 immunoreactivity. This suggests that callus formed in the bone marrow during fracture repair transiently decrease hematopoietic stem cells. Decreased hematopoietic function leads to systemic consequences such as anemia, immunodeficiency, and bleeding tendency. In other words, it goes without saying that HSC is essential for biological maintenance. Although this study did not go as far as hematological studies, the possibility that the decrease in HSC associated with fractures may affect systemic hematopoiesis cannot be ruled out. In summary, future studies should extensively examine the possibility that fracture may affect hematopoietic function.

Conclusion

Our results suggest that callus formation associated with fracture decreases HSCs around the callus.

Acknowledgments

We would like to express our deepest gratitude to our professors for their guidance and encouragement, and to the graduate students in our laboratory for their cooperation in this research.

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Figure Legend

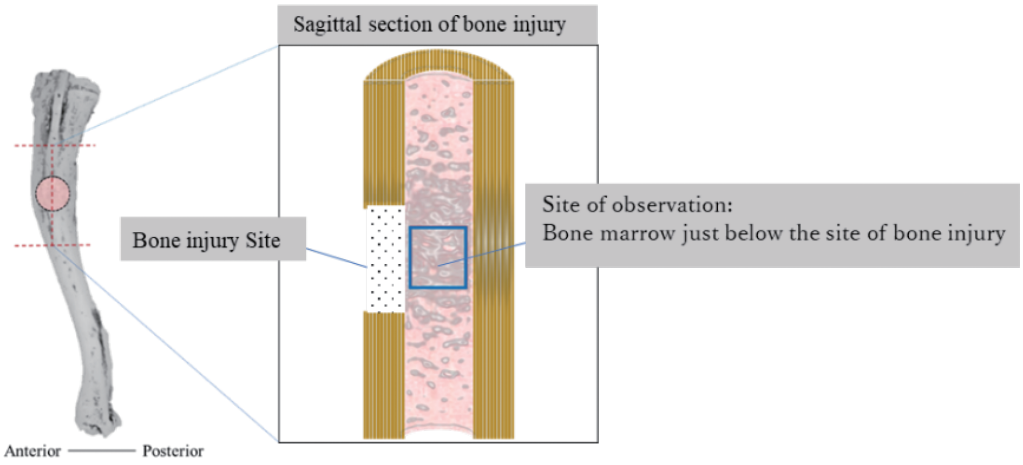

Fig1. Schematic diagram of the observation site

Observation of the bone marrow cavity just below the bony foramen.

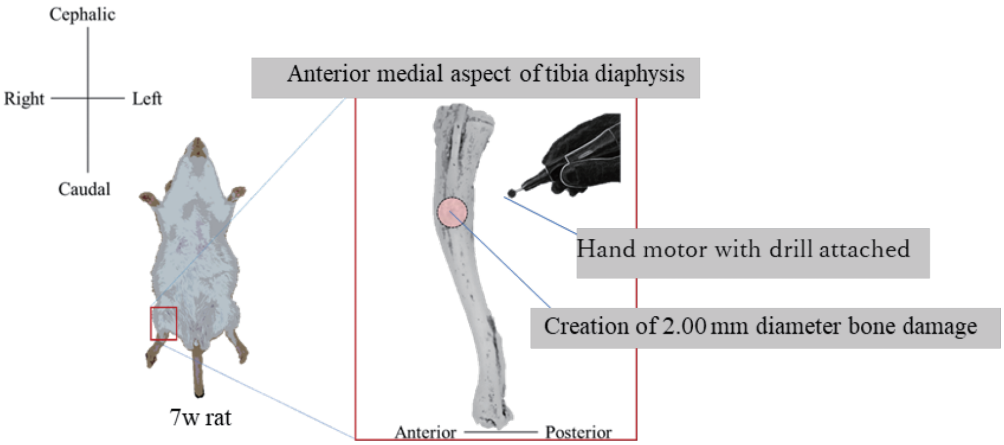

Fig2. Preparation of rat models of bone injury

A bone injury of approximately 2.00 mm diameter was created on the medial aspect of the diaphysis of the rat tibia.

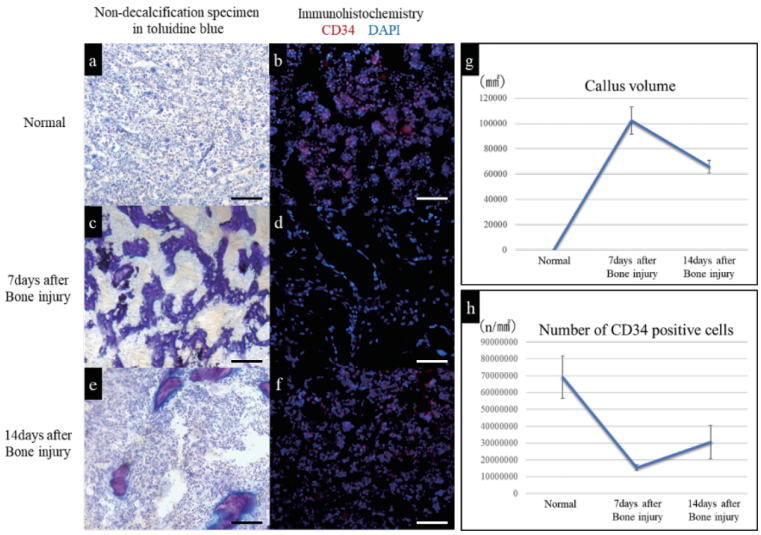

Fig3. Changes over time in callus and CD34 after bone injury (bar=20μm, n=4)

In normal bone marrow, only a large number of bone marrow cells stained blue by toluidine blue staining were observed, whereas after 7 days of bone injury, a large number of callus were observed extensively. Furthermore, the number of callus was reduced in the bone marrow at 14 days after bone injury compared to the bone marrow at 7 days after bone injury. Small amounts of callus were observed. On the other hand, CD34-positive cells were observed extensively and clearly in normal bone marrow, but significantly less than in normal bone marrow on day 7 after bone injury. Furthermore, CD34-positive cells increased on day 14 after bone injury compared to day 7 after bone injury and recovered to a state similar to normal bone marrow.

脛骨損傷ラットにおいて骨修復時に形成される仮骨が仮骨周囲の造血幹細胞に及ぼす組織学的影響

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要旨

【背景】骨修復の際、骨髄では仮骨と呼ばれる未熟な骨が形成される。一方、骨は造血幹細胞（HSC）が関与する造血の役割を担っている。これまでの研究で、骨髄に脂肪組織や線維組織が増加することは、HSCの減少に繋がることが知られている。しかし、これが仮骨形成によっても影響を受けるか否かは、これまで検討されていない。本研究は、HSCと仮骨の関連性について究明する基礎的研究の一環として、骨折時に形成される仮骨が周囲のHSCに与える影響を組織学的に検討した。【材料および方法】材料として、6週齢の雄性ラット12匹を用いた。ラットは7週齢時に、脛骨に骨損傷を作製しない群、骨損傷後1週間および2週間で安楽死させる群の3群に分類した。実験後、各群のラットから脛骨を摘出し、各種組織学的標本を作製して観察した。【結果】正常骨髄では、トルイジンブルー染色で青く染色された骨髄細胞のみが多数観察され、CD34免疫反応は骨髄の広範囲に明瞭に観察された。骨損傷7日後の骨髄では、メタクロマジーを生じる仮骨が多数観察され、CD34免疫反応は正常骨髄で観察された状態に比べて著しく減少していた。骨損傷14日後の骨髄では、骨損傷7日後よりも仮骨の量が少なく、CD34免疫反応は骨損傷7日後よりも増加し、正常の骨髄に類似した。【考察】骨損傷の修復過程で骨髄内に形成される仮骨は、CD34免疫反応の増減に対して負の要素として寄与すると考えられたことから、造血幹細胞を一過性に減少させることが示唆された。【結論】骨折後の骨髄で形成される仮骨は、仮骨周囲のHSCを減少させることが示唆された。

キーワード：骨折