Serum 1α,25-Dihydroxyvitamin D₃ Accumulates into the Fracture Callus during Rat Femoral Fracture Healing

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ABSTRACT

1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) is thought to be an important systemic factor in the fracture repair process, but the mechanism of action of 1,25(OH)₂D₃ has not been clearly defined. In this study, the role of 1,25(OH)₂D₃ in the fracture repair process was analyzed in a rat closed femoral fracture model. The plasma concentration of 1,25(OH)₂D₃ rapidly decreased on day 3 and continued to decrease to 10 days after fracture. We assessed whether this decrease was based on the accelerated degradation or retardation of the synthesis rate of 1,25(OH)₂D₃ from 25(OH)D₃. After radiolabeled ³H-1,25(OH)₂D₃ or ³H-25(OH)D₃ was injected iv into fractured or control (unfractured) rats, the concentrations of 25(OH)D₃ and 1,25(OH)₂D₃ metabolites were measured by HPLC. The plasma concentrations of these radiolabeled metabolites in fractured group were similar to those in control rats early after operation. However, radioactivity in the femurs of fractured rats was higher than that of the control group. Furthermore, the radioactivity was concentrated in the callus of the fractured group analyzed by autoradiography. 1,25(OH)₂D₃ receptor gene expression was detected early after fracture and, additionally, both in the soft and hard callus on days 7 and 13 after fracture. These results showed that the rapid disappearance of 1,25(OH)₂D₃ in the early stages after fracture was not due to either increased degradation or decreased synthesis of 1,25(OH)₂D₃, but rather to increased consumption. Further, these results suggest the possibility that plasma 1,25(OH)₂D₃ becomes localized in the callus and may regulate cellular events in the process of fracture healing. (Endocrinology 139: 1467–1473, 1998)

REPAIR of long bones after fracture is a unique process that results in the restoration of normal bone anatomy and function after serious injury. The repair process begins with the formation of a large reparative granuloma, called a callus, that surrounds the fracture site. Bone forms in the callus by two distinct processes. First, in intramembranous ossification, osteoblast progenitor cells in the inner layer of periosteum differentiate and synthesize new bone matrix. Bone formed by intramembranous ossification does not restore bone continuity but forms an external buttress for bone tissue that forms subsequently. Second cartilage, which forms at the fracture site immediately adjacent to the intramembranous bone, is invaded by blood vessels and osteoblasts from the new bone and is replaced by a process called endochondral ossification. Bone formation in this portion of the callus is reminiscent of bone formation at the growth plate. Bone formed by endochondral ossification bridges the fracture site, restoring bone continuity. Normal bone anatomy is restored by osteoclast-mediated remodeling of the new bone.

Several systemic factors and hormones are thought to regulate the fracture healing process. However, little is understood about how they regulate the local repair processes and how they control the local cellular events. It is well known that 1α,25-dihydroxyvitamin D₃ (1, 25(OH)₂D₃) influences cell proliferation and differentiation of osteoblasts and chondrocytes via the 1,25(OH)₂D₃ receptor localized in these cells (1–5). Thus, 1,25(OH)₂D₃ is thought to be one of the systemic factors for the fracture repair process. However, the mechanism of action of 1,25(OH)₂D₃ is not clearly defined.

The object of this study was to obtain further information about the role of 1,25(OH)₂D₃ in the process of bone formation and to relate the changes of various metabolites to the process of fracture healing using a rat closed-fracture model.

Materials and Methods

Compounds

1,25-dihydroxy-[26,27-methyl-³H]-vitamin D₃ (³H-1,25(OH)₂D₃, specific activity 172 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK) and 25-hydroxy-[26,27-methyl-³H]-vitamin D₃ (³H-25(OH)D₃, specific activity 155 Ci/mmol from New England Nuclear (Boston, MA). 1,25(OH)₂D₃, 1,24,25(OH)₃D₃, 25(OH)D₃, and 24,25(OH)₂D₃ were synthesized at Teijin Institute for Bio-Medical Research.

Fracture model

Femoral fractures in female Sprague-Dawley rats were produced as described previously (6). Sodium pentobarbital (65 mg/kg body weight) was injected ip, and anesthetized rats were prepared for surgery by shaving and cleaning the lower extremities. With a medial peripatellar incision, the patella was dislocated laterally exposing the femoral condyle. A Kirschner wire (1.1 mm in diameter and 2.7 cm long) was
introduced into the intramedullary canal through the intercondylar notch. The Kirschner wire did not protrude into the knee joint or interfere with motion of the patella. After closing the knee joint, the mid-diaphysis of the pinned femur was fractured by applying a bending force, as described by Bonnarens and Einhorn (7). Radiographs were obtained immediately after surgery, and rats with proximal or distal fractures were excluded from this experiment, so that the only mid-diaphyseal fractures were included in this study. Rats with Kirschner wire in the femur without fracture were served as controls. Rats were permitted full weight bearing and unrestricted activity after awakening from anesthesia. All rats were fed standard rat chow ad libitum (CE-2, Nippon Clea Co., Japan), and maintained on a 12-h light, 12-h dark cycle at 22°C. The experimental protocol was carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University, and Law No. 105 and Notification No. 6 of the government of Japan.

Measurement of plasma Ca, Pi, and 1,25(OH)2D3 concentration

Blood samples were collected, and plasma was obtained for measurement of the levels of calcium (Ca), inorganic phosphorus (Pi), and 1,25(OH)2D3 levels on days 1, 3, 5, 7, 10, 14, and 17 after operation. Plasma Ca and Pi were measured with an autoanalyzer (type 7070, Hitachi Co., Ltd., Japan). Plasma concentration of 1,25(OH)2D3 was measured using the RRA method.

The concentrations of 3H-1,25(OH)2D3 in plasma and various bones after administration of 3H-1,25(OH)2D3

3H-1,25(OH)2D3 (5 mCi/kg) was administered iv to fractured or control rats to examine the profile of 3H-1,25(OH)2D3 and other metabolites in plasma and various bones. Blood samples were collected from the abdominal aorta at 2, 8, and 24 h after administration on day 2, and at 2 and 24 h on 5 and 14 day post surgery. Plasma was separated by centrifugation at 3,000 rpm for 10 min. Plasma and bones were extracted with chloroform/methanol (1/1 vol/vol), and extracts were analyzed by HPLC (HPLC, type LC6A, Shimadzu, Kyoto, Japan). The columns were calibrated with authentic vitamin D3 metabolites. The mobile phase was an isotonic system of methanol/dichloromethane (3/97 vol/vol), and chloroform extract was analyzed by HPLC. The mobile phase was an isotonic system of methanol/dichloromethane (3/97 vol/vol) using a Zorbax Sil column. Each chromatogram was analyzed by UV absorbance at 264 nm and radioactivity measured using a liquid scintillation counter.

Synthesis rate of 3H-1,25(OH)2D3 from 3H-25(OH)D3

To analyze the metabolism of 25(OH)D3, 3H-25(OH)D3 (5 mCi/kg) was administered to rats 2, 5, or 14 days after surgery. Blood samples were collected from the abdominal aorta at 2 h after the administration, and plasma was isolated for measurement of 3H-1,25(OH)2D3 and 3H-24,25(OH)2D3. Plasma was extracted by chloroform/methanol (1/1 vol/vol), and chloroform extract was analyzed by HPLC. The mobile phase was an isotonic system of methanol/dichloromethane (3/97 vol/vol) using a Zorbax Sil column. Each chromatogram was analyzed by UV absorbance at 264 nm and radioactivity measured using a liquid scintillation counter.

Detection of messenger RNA (mRNA) for 1,25(OH)2D3 receptor in the hard and soft calluses

Fracture calluses were harvested 36 h, or 7 or 13 days after fracture. Total cellular RNA was extracted from the whole calluses 36 h after fracture, or from the soft callus and from the hard callus separately on days 7 or 13 as previously described (8). Briefly, tissue remaining on the fracture calluses was carefully removed, and bone marrow was washed from the femur with PBS containing 0.1% diethylpyrocarbonate (DEPC). Specimens were snap-frozen in liquid nitrogen immediately after dissection. Total cellular RNA was extracted from the homogenized samples. The concentration of RNA was determined by spectrophotometric absorption at 260 nm.

Total cellular RNA was used as a template for synthesis of first strand complementary DNA (cDNA) by reverse transcription. A reaction mixture containing oligo(dT), 1 mg of total cellular RNA, dNTPs, and random primers in a total volume of 25 ml was heated at 80°C for 3 min. Reverse transcriptase and RNAse inhibitor were then added and the mixtures incubated at 37°C for 2 h.

The cDNA was amplified as described previously (9). The reaction mixtures were preheated at 94°C for 5 min, then cycled 35 times in a Perkin-Elmer DNA thermal cycler at 94°C for 1 min, 52°C for 2 min, and 72°C for 2 min. The upstream primer corresponded to positions 431–449, whereas the downstream primer corresponded to nucleotides 925–944. As a control, parallel PCR reactions were run for each sample using β-actin primers (11).

Statistical analysis

A statistical analysis was used for data analysis. Values were represented as mean ± sd and the statistical significant of difference between control and fractured groups were determined by Student’s t test for unpaired data or by the Cochran-cox test.

Analysis of excretion after administration of 3H-1,25(OH)2D3

Rats were housed in glass metabolic cages to collect urine and feces at designated times after administration of 3H-1,25(OH)2D3 on day 2 post surgery. Urine was diluted appropriately with water, and appropriately feces were homogenized with water and solubilized with PROTOZOL (New England Nuclear). Radioactivity was measured by a liquid scintillation counter.

Autoradiography of femurs

3H-1,25(OH)2D3 (2.5 mCi/rat) was administered iv to rats to examine its distribution 5 and 14 days after surgery. Femurs were dissected from rats 8 h after administration of 3H-1,25(OH)2D3 and were freed of adherent soft tissue. Whole femurs were embedded in a 3% carboxymethylcellulose solution at −80°C. Thirty- to 40-μm serial sections were cut on PMV Cryo-Microtome 2550 (LKB Co., Stockholm, Sweden). Sections were applied to a plastic tape and exposed to an imaging plate to quantify radioactivity via luminescent energy. Radioactivity in each imaging plate was quantified by the use of Bio-Imaging Analyzer (BAS-2000, Fuji, Tokyo, Japan). The amount of radioactivity was observed as a color image. A dense black color indicated higher radioactivity than a light black color.
Results

The profile of 1,25(OH)₂D₃ concentration in plasma after fracture

Plasma concentrations of 1,25(OH)₂D₃ during the fracture repair are shown in Fig. 1. A dramatic fall in the plasma concentration of 1,25(OH)₂D₃ occurred within 3 days after fracture and persisted until day 10. Subsequently, the concentration of 1,25(OH)₂D₃ in plasma gradually increased, returning to concentrations not significantly different from those in control rats by 17 days after fracture. The dashed area illustrates the plasma levels of 1,25(OH)₂D₃ of intact rats. Plasma Ca concentration increased on day 3 after fracture, and gradually returned to the control level during the next 17 days. However, plasma Pi concentration in both groups showed no difference during the experimental period (data not shown).

Plasma and excretion profiles of ³H-1,25(OH)₂D₃

The plasma and excretion profiles of ³H-1,25(OH)₂D₃ are shown in Figs. 2 and 3. The plasma concentrations of ³H-1,25(OH)₂D₃ in control and fractured rats at 2 and 24 h on days 2, 5, and 14 after fracture gradually decreased time dependently (Fig. 2). As Fig. 2 shows, the plasma concentrations of ³H-1,25(OH)₂D₃ and ³H-1,24,25(OH)₃D₃ in fractured rats were similar to those in control rats. Moreover, on day 2, total radioactivity excreted into urine and feces did not differ between control and fractured rats (Fig. 3). The HPLC pattern of plasma showed no difference between the groups (data not shown). These results suggest that the rapid disappearance of 1,25(OH)₂D₃ from plasma in fractured rats was not associated with a rapid increase in the degradation of 1,25(OH)₂D₃ or a rapid stimulation of excretion into urine and feces due to fracture.
The concentration of $^3$H-1,25(OH)$_2$D$_3$ in femur in control and fractured rats

The concentrations of $^3$H-1,25(OH)$_2$D$_3$ in femur, calvaria, and humerus at 8 h after administration on day 5 post surgery are shown in Fig. 4. The concentration of $^3$H-1,25(OH)$_2$D$_3$ in these bones was measured by HPLC after extraction with chloroform/methanol. The femoral concentration of $^3$H-1,25(OH)$_2$D$_3$ was clearly higher in fracture rats, compared with those in control rats (Fig. 4a). However, the concentration of $^3$H-1,25(OH)$_2$D$_3$ in the calvaria and humerus did not differ between fractured and control rats. Furthermore, Fig. 4b shows that the ratio of femoral to plasma concentration of $^3$H-1,25(OH)$_2$D$_3$ in

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**Fig. 4.** a, The concentration of various bones after administration of $^3$H-1,25(OH)$_2$D$_3$ on day 5 after operation. Each point represents mean ± SD (n = 4 or 5). *P < 0.05 vs. control group. b, The transferred ratio of (femoral concentration of $^3$H-1,25(OH)$_2$D$_3$/plasma concentration of $^3$H-1,25(OH)$_2$D$_3$) on day 5 after operation. Each point represents mean ± SD (n = 4 or 5). *P < 0.05 vs. control group.
fractured rats was 7 times higher than that in control group. These results show that \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) was distributed primarily in femur fracture sites, not other bones in the same animals.

**Localization of \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) at fractured sites by autoradiography**

Figure 5 shows the distribution and the localization of \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) in control and fractured rat femurs, assessed by autoradiography. On days 5 and 14, the radioactivity at the callus in the experimental group was higher and clearly located in calluses, compared with diffuse distribution in control femurs. These data show that \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) is preferentially distributed and localized in the callus in fractured femurs.

**The synthesis rate of \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) from \(^{3}\)H-25(OH)D\(_{3}\)**

The synthesis rate of \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) from \(^{3}\)H-25(OH)D\(_{3}\) in fractured and control rats is shown in Fig. 6. At 2 h after administration on days 2, 5, or 14 post surgery, \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) concentrations in plasma were not significantly different in both groups. Furthermore there was no difference in \(^{3}\)H-1,25(OH)D\(_{3}\) and \(^{3}\)H-24,25(OH)\(_{2}\)D\(_{3}\) between groups. Taken together with data from Figs. 2 and 3, these data suggest that the rapid disappearance of 1,25(OH)\(_{2}\)D\(_{3}\) from plasma early after fracture was not due to the increase of the degradation rate, not due to increase of its excretion, or the decrease of its synthesis.

**Expression of the 1,25(OH)\(_{2}\)D\(_{3}\) receptor mRNA at the fracture site**

1,25(OH)\(_{2}\)D\(_{3}\) receptor mRNA expression was detected in the whole callus 36 h after fracture and in both hard and soft callus on days 7 and 13 (Fig. 7). These results indicate that 1,25(OH)\(_{2}\)D\(_{3}\) receptor gene mRNA expression was initiated just after fracture and that it was subsequently expressed in both cartilage and bone tissues in the fracture callus.

**Discussion**

We demonstrated that the plasma concentration of 1,25(OH)\(_{2}\)D\(_{3}\) rapidly decreased on day 3 and remained low until 10 days after fracture. The rapid disappearance of 1,25(OH)\(_{2}\)D\(_{3}\) in the early stages after fracture was not due to increased degradation or decreased synthesis, but due to an increase in consumption especially at the callus of healing bone. The ratio of femoral to plasma concentration of 1,25(OH)\(_{2}\)D\(_{3}\) in experimental animals was significantly higher than that in control femur, suggesting that transfer of plasma 1,25(OH)\(_{2}\)D\(_{3}\) to femur increased. These data show that 1,25(OH)\(_{2}\)D\(_{3}\) from the plasma is localized in the callus and could regulate the cellular events in the process of fracture healing. This possibility was supported by autoradiography. Our data strongly suggest that 1,25(OH)\(_{2}\)D\(_{3}\) plays a key role in the fracture repair process.

Lidor et al. (12) showed that the levels of \(^{3}\)H-24,25(OH)\(_{2}\)D\(_{3}\) were found to coincide with the formation of cartilaginous tissue in chicks with fractures (12). Their data also showed that during healing process, the plasma levels of \(^{3}\)H-1,25(OH)\(_{2}\)D\(_{3}\) were below normal, but the concentration of
3H-1,25(OH)2D3 increased in the callus, diaphysis, and epiphysis on days 7 to 11 after fracture, compared with the control chicks. These results were similar to our data. 1,25(OH)2D3 is well known to regulate cartilage metabolism because deficiency of this hormone causes calcification disturbance in the growth plate, resulting in so-called rickets. Current studies have shown that this hormone stimulates chondrogenesis by cell proliferation and by promoting matrix protein synthesis (13–15). Additionally, 1,25(OH)2D3 acts directly on osteoblasts, stimulating the synthesis of osteocalcin (16–20) and acts also on osteoclasts to stimulate bone resorption (21–23). Moreover, low doses of 1,25(OH)2D3 or low doses of 1α(OH)D3 have been reported to accelerate healing and promote bone formation and mineralization of experimental fractures in adult rats (24, 25). These studies suggest that 1,25(OH)2D3 is involved in regulation of bone and cartilage formation and remodeling during fracture repair and indicate 1,25(OH)2D3 promotes bone repair.

1,25(OH)2D3 receptor gene expression was detected in the fracture callus just after fracture. This early expression of the receptor in the callus may be one mechanism of decrease of 1,25(OH)2D3 plasma concentrations and increase concentration in the callus.

Autoradiograms of the fracture callus using 3H-1,25(OH)2D3 showed that radioactivity was distributed in the whole callus in both calluses on days 5 and 14. Furthermore, 1,25(OH)2D3 receptor gene expression was detected in both hard and soft calluses on days 7 and 13. This suggests that 1,25(OH)2D3 is involved in the regulation of many cellular events that occur in the callus. On day 5 or 7 in the rat femoral fracture model (8), cartilage formation is initiated at the fracture site, and intramembranous bone formation occurs adjacent to the fracture site. On day 13 or 14, mature cartilage tissue is observed at the fracture site, and mature trabecular (woven) bone tissue is formed adjacent to the fracture site. In the trabecular bone, bone remodeling occurs. In addition, between cartilage and bone tissues, endochondral bone formation is observed on day 14. In this period of fracture process, chondrogenesis in the soft callus and osteogenesis in the hard callus proceed. These data suggest that serum 1,25(OH)2D3 is transferred to the callus to regulate both cartilage and bone formation during fracture repair.

Tauber et al. (26) has reported that blood levels of active vitamin D3 metabolites, 24,25(OH)2D3 and 1,25(OH)2D3, decreases in patients who suffered from prolonged fracture healing or multiple fractures. The decrease of serum 1,25(OH)2D3 concentration in some patients is probably attributed to the consumption of the active metabolites due to accumulation in the forming callus as shown in our experiments.

In conclusion, we demonstrated that serum 1,25(OH)2D3 rapidly accumulated into the fracture callus early after frac-
ture, resulting in a decrease of the plasma concentration. These data show that 1,25(OH)2D3 could regulate the cellular events including bone and cartilage formation in the process of fracture healing.

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