Tissue-type plasminogen activator deficiency delays bone repair: roles of osteoblastic proliferation and vascular endothelial growth factor

Naoyuki Kawaio,1 Yukinori Tamura,1 Katsumi Okumoto,2 Masato Yano,1 Kiyotaka Okada,1 Osamu Matsuo,1 and Hiroshi Kaji1
1Department of Physiology and Regenerative Medicine, Kinki University Faculty of Medicine, Osaka, Japan; and 2Life Science Research Institute, Kinki University, Osaka, Japan

Submitted 13 March 2014; accepted in final form 9 June 2014

Kawaio N, Tamura Y, Okumoto K, Yano M, Okada K, Matsuo O, Kaji H. Tissue-type plasminogen activator deficiency delays bone repair: roles of osteoblastic proliferation and vascular endothelial growth factor. Am J Physiol Endocrinol Metab 307:E278–E288, 2014. First published June 10, 2014; doi:10.1152/ajpendo.00129.2014.—Further development in research of bone regeneration is necessary to meet the clinical demand for bone reconstruction. Recently, we reported that plasminogen is crucial for bone repair through enhancement of vessel formation. However, the details of the role of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) in the bone repair process still remain unknown. Herein, we examined the effects of plasminogen activators on bone repair after a femoral bone defect using tPA-deficient (tPA−/−) and uPA-deficient (uPA−/−) mice. Bone repair of the femur was delayed in tPA−/− mice, unlike that in wild-type (tPA+/+) mice. Conversely, the bone repair was comparable between wild-type (uPA+/+) and uPA−/− mice. The number of proliferative osteoblasts was decreased at the site of bone damage in tPA−/− mice. Moreover, the proliferation of primary calvarial osteoblasts was reduced in tPA−/− mice. Recombinant tPA facilitated the proliferation of mouse osteoblastic MC3T3-E1 cells. The proliferation enhanced by tPA was antagonized by the inhibition of endogenous annexin 2 by siRNA and by the inhibition of extracellular signal-regulated kinase (ERK)1/2 phosphorylation in MC3T3-E1 cells. Vessel formation as well as the levels of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) were decreased at the damaged site in tPA−/− mice. Our results provide novel evidence that tPA is crucial for bone repair through the facilitation of osteoblast proliferation related to annexin 2 and ERK1/2 as well as enhancement of vessel formation related to VEGF and HIF-1α at the site of bone damage.

plasminogen activator; bone repair; osteoblast; cell proliferation; vessel formation

Although autogenous bone graft is the gold standard procedure for the reconstruction of a bone defect, it has several disadvantages, including the limited size of the graft and injury to the donor site (23). Fractures are one of the most frequent injuries of the musculoskeletal system. Optimal treatment of fracture requires knowledge of the complex process of bone repair (10). Therefore, clarification of the mechanism for bone repair and regeneration is necessary to achieve recovery of injured bone.

Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) convert the inactive proenzyme plasminogen into the active serine protease plasmin, which is a pivotal component of the fibrinolytic system (9). The fibrinolytic system has many physiological and pathophysiological functions in mammals beyond its proteolytic effect on thrombi. The fibrinolytic system activates the tissue proteolytic system and modulates the release of growth factors from extracellular matrix, such as vascular endothelial growth factor (VEGF) (22, 31). Previous studies indicate that the tissue fibrinolytic system is involved in the tissue repair process in the skin and liver (2, 5, 27).

tPA and uPA are expressed by osteoblasts and osteoclasts, and these factors are regulated by stimuli with hormones and factors such as parathyroid hormone, prostaglandin E2, and 1,25-dihydroxyvitamin D3 (1, 15, 16, 20, 21, 47). Daci et al. (11) reported that bone mass is increased in tPA and uPA double-deficient mice. The deficiency of PA inhibitor-1 (PAI-1), an endogenous negative regulator of the fibrinolytic system, partially protects against bone loss in estrogen-deficient mice (12). Our recent study also revealed that PAI-1 deficiency protects against diabetic bone loss in female mice (45). These findings suggest that the tissue fibrinolytic system regulates bone metabolism in physiological and pathological conditions. We also demonstrated recently that plasminogen is crucial for bone repair through facilitation of the blood vessel formation related to VEGF, using plasminogen-deficient mice (29). A study by Rundle et al. (42) suggested that the absence of PAI-1 increases the size of the callus as well as the formation of callus cartilage during fracture healing in mice. These findings suggest that the tissue fibrinolytic system is important for the bone repair process. However, details of the role of the tissue fibrinolytic system in the bone repair process still remain unknown.

Therefore, in the present study, we examined the effects of tPA and uPA on bone repair after a femoral bone defect, using mice with tPA or uPA deficiency and their wild-type counterparts to clarify the roles of PAs in the bone repair process.

MATERIALS AND METHODS

Materials. Anti-alkaline phosphatase (ALP) antibody was obtained from Abnova (Taipei, Taiwan). Anti-osterix, anti-CD31, anti-VEGF and, anti-transforming growth factor-β (TGFβ) antibodies were obtained from Abcam (Cambridge, UK). 5-Bromo-2-deoxyuridine (BrdU), collagenase, and dexamethasone were purchased from Sigma (St. Louis, MO). Anti-BrdU antibody was supplied by Chemicon (Billerica, MA). Recombinant human tPA and PD-98059 were supplied by Promega (Madison, WI). Anti-human tPA and PD-98059 were supplied by Promega (Madison, WI). 1,25-dihydroxyvitamin D3 (1, 15, 16, 20, 21, 47). Daci et al. (11) reported that bone mass is increased in tPA and uPA double-deficient mice. The deficiency of PA inhibitor-1 (PAI-1), an endogenous negative regulator of the fibrinolytic system, partially protects against bone loss in estrogen-deficient mice (12). Our recent study also revealed that PAI-1 deficiency protects against diabetic bone loss in female mice (45). These findings suggest that the tissue fibrinolytic system regulates bone metabolism in physiological and pathological conditions. We also demonstrated recently that plasminogen is crucial for bone repair through facilitation of the blood vessel formation related to VEGF, using plasminogen-deficient mice (29). A study by Rundle et al. (42) suggested that the absence of PAI-1 increases the size of the callus as well as the formation of callus cartilage during fracture healing in mice. These findings suggest that the tissue fibrinolytic system is important for the bone repair process. However, details of the role of the tissue fibrinolytic system in the bone repair process still remain unknown.

Therefore, in the present study, we examined the effects of tPA and uPA on bone repair after a femoral bone defect, using mice with tPA or uPA deficiency and their wild-type counterparts to clarify the roles of PAs in the bone repair process.
wick, NJ) and Calbiochem (Darmstadt, Germany), respectively. Etoposide, Alizarin Red S solution, cetylpyridinium chloride, and culture media were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT).

Animals. Male and female mice with tPA (tPA<sup>−/−</sup>) or uPA (uPA<sup>−/−</sup>) gene deficiency and their wild-type counterparts (tPA<sup>+/+</sup> and uPA<sup>+/+</sup>, respectively), provided initially by Prof. D. Collen (University of Leuven, Leuven, Belgium), were bred in the Kinki University Faculty of Medicine animal facility. The genetic background of all mice was mixed C57BL/6J (75%) and 129/SvJ (25%) (7, 8). We obtained male and female mice with heterozygous tPA (tPA<sup>+/−</sup>) or uPA (uPA<sup>+/−</sup>) gene deficiency by cross-breeding every fifth generation of tPA<sup>−/−</sup> and tPA<sup>+/−</sup> mice or uPA<sup>−/−</sup> and uPA<sup>+/−</sup> mice, which were used for breeding to obtain homozygous breeding pairs. tPA<sup>−/−</sup>, uPA<sup>−/−</sup>, and their wild-type counterparts (tPA<sup>+/+</sup> and uPA<sup>+/+</sup>, respectively) obtained from homozygous breeding pairs were used in in vivo and ex vivo cell culture experiments in the present study. All genotypes of mice used for those experiments were determined by PCR analysis of tail DNA. To minimize the effects of mouse strain differences, age-matched and weight-matched male tPA<sup>−/−</sup>, tPA<sup>+/−</sup>, tPA<sup>+/+</sup> and uPA<sup>−/−</sup>, uPA<sup>+/−</sup>, and uPA<sup>+/+</sup> mice, each 8 wk old and weighing 22–25 g, were used for in vivo quantitative computed tomography (qCT) and histological analysis of hematopoietin and eosin-stained sections. Male and female tPA<sup>+/−</sup> and tPA<sup>+/−</sup> mice, each weighing 18–25 g and 7–9 wk old, were used in the other experiments. All experiments were performed according to the guidelines of the National Institutes of Health and the institutional rules for the use and care of laboratory animals at Kinki University. The protocol was approved by the Experimental Animal Welfare Committee of Kinki University (permit no. KAME-23-023).

Bone defect model. A bone defect was induced in the mice according to the previously described method (29). Briefly, under anesthesia induced by 2% isoflurane, the anterior skin over the mid-femur of the right leg was longitudinally incised for 5 mm in length. After the muscle was split, the surface of femoral bone was exposed. Thereafter, a hole was made using a drill with a diameter of 0.9 mm. The hole was irrigated with saline to prevent thermal necrosis of the margins. The incised skin was then sutured in a sterile manner, and the anesthesia was discontinued. The body temperature was maintained at 37°C during surgery using a heating pad.

In vivo qCT analysis. In vivo qCT analysis was performed according to the guidelines of the American Society for Bone and Mineral Research (4). The mice were anesthetized using 2% isoflurane and their femurs were scanned using an X-ray CT system (Latheta LCT-200; Hitachi Aloka Medical, Tokyo, Japan). The parameters used for the CT scans were as follows: tube voltage, 50 kVp; tube current, 500 μA; integration time, 3.6 ms; axial field of view, 48 mm, with an isotropic voxel size of 48 μm. Images were generated by integration of two signal averages for the femur. The total scan time was ~5 min for the femur. Volume-rendered three-dimensional CT images were reconstructed using VGStudio MAX2.2 (Nihon Visual Science, Tokyo, Japan). The area of the bone defect in the femur was quantified for the femur. Volume-rendered three-dimensional CT images were reconstructed using VGStudio MAX2.2 (Nihon Visual Science, Tokyo, Japan). The area of the bone defect in the femur was quantified using VGStudio MAX2.2 (Nihon Visual Science, Tokyo, Japan). The area of the bone defect in the femur was quantified for the femur.

Histological analysis. The mice were anesthetized using pentobarbital sodium (50 mg/kg ip) on days 4 and 7 after surgery. The femur was removed, fixed in 4% paraformaldehyde, demineralized in 22.5% formic acid and 340 mM sodium citrate solution, and embedded in paraffin. Thereafter, 4-μm-thick sections were obtained. The sections were processed for hematopoietin and eosin staining. Immunostaining with ALP, osterix, BrdU, or CD31 was performed as described previously (25, 26, 29). Briefly, the sections were incubated with anti-ALP antibody at a dilution of 1:100, anti-osterix antibody at a dilution of 1:200, anti-BrdU antibody at a dilution of 1:300, or anti-CD31 antibody at a dilution of 1:100, followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase. Positive signals were visualized using a tyramide signal amplification system (Perkin-Elmer, Waltham, MA). These sections were photographed under a fluorescence microscope (E800; Canon, Tokyo, Japan) with a charge-coupled device camera. The numbers of ALP-positive and osterix-positive cells per 0.1 mm<sup>2</sup> of the microscopic fields were quantified in a blinded evaluation. The number and the total luminal area of CD31-positive vessels per 0.1 mm<sup>2</sup> of the microscopic fields were quantified using ImageJ in a blinded manner. The sections were stained with tartrate-resistant acid phosphatase (TRAP), using a TRAP staining kit (Wako Pure Chemical Industries) to determine osteoclasts (29). The number of TRAP-positive multinucleated cells (MNCs) per 1 mm of bone surface was measured at the damaged site of the femur in a blinded evaluation. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to determine apoptotic cells using an in situ cell death detection kit, fluorescein (Roche Diagnostics, Tokyo, Japan). The number of TUNEL-positive cells per 0.1 mm<sup>2</sup> of the microscopic fields was quantified in a blinded evaluation. The sections were processed for Alcian blue and toluidine blue staining to determine cartilage formation (29). The area of cartilage matrices, including proteoglycans and glycosaminoglycans, was quantified by measuring the Alcian blue-positive area and the metachromatic area in the sections stained with toluidine blue using image-processing software (Mac SCEROPE; Mitani, Fukui, Japan) in a blinded evaluation, as described previously (29).

Analysis of cell proliferation in osteoblasts in vivo. The mice received eight intraperitoneal injections of BrdU at 100 mg/kg every 12 h immediately after the production of the femoral bone defect, and they were euthanized on day 4 (25, 28). Double-staining for BrdU and osterix was performed as described above. The number of BrdU and osterix double-positive cells per 0.1 mm<sup>2</sup> of the microscopic fields was quantified in a blinded evaluation.

Quantitative real-time PCR. Total RNA was isolated from the tissues and cells using a RNasey Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions (29). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA). The incorporation of Green into double-stranded DNA, which was performed using an ABI Step One Real-Time PCR System (Applied Biosystems), was assessed by quantitative real-time PCR. The PCR primers are listed in Table 1. The specific mRNA amplification of the target was determined as the CT value, which was followed by normalization with the glyceraldehyde-3-phosphate dehydrogenase level.

Western blot analysis. A 5-mm piece of femur containing the damaged site and an intact femur from the contralateral side were removed and homogenized in a tissue lysis buffer (Cell Signaling Technology) supplemented with protease inhibitors. The cultured cells were also lysed with the same buffer. Total protein levels were determined using a BCA assay reagent (Pierce, Rockford, IL). The same amount of protein aliquots was separated on 10% polyacrylamide-gels, and the proteins were transferred to a nitro-cellulose membrane. The membrane was blocked and incubated with the appropriate primary antibody and the enhanced chemiluminescence select detection system (GE Healthcare, Tokyo, Japan) according to the manufacturer’s instructions.

Preparation of primary osteoblasts. Calvarial osteoblasts were obtained from tPA<sup>−/−</sup> and tPA<sup>+/−</sup> mice according to the previously described method (29). Briefly, calvaria was removed from 3-day-old mice, cleaned to remove soft tissue, and digested four times with 1 mg/ml collagenase and 0.25% trypsin for 20 min at 37°C with gentle agitation. The cells from the second, third, and fourth digestions were plated and grown in Eagle’s minimum essential medium (α-MEM;
Table 1. Primers used for real-time PCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx2</td>
<td>5′-AAATGCTCTCCGCTTTATAG-3′</td>
</tr>
<tr>
<td></td>
<td>5′-TGCTAGTCCGCTGGCAGTTG-3′</td>
</tr>
<tr>
<td>Osterix</td>
<td>5′-AGGCGACATTGAGCAAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-CGGCTGGTGGTCTTCTT-3′</td>
</tr>
<tr>
<td>ALP</td>
<td>5′-ATCTTGGTTCGTCCTCAGT-3′</td>
</tr>
<tr>
<td></td>
<td>5′-TTCGCGGTTAGCGACAO-3′</td>
</tr>
<tr>
<td>Col I</td>
<td>5′-GCTCAAATGTTTGAGCAAG-3′</td>
</tr>
<tr>
<td></td>
<td>5′-TTGGAAGGCAACTTGTTAG-3′</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>5′-CTTGAACGTCTGAAGAAGCCTCA-3′</td>
</tr>
<tr>
<td></td>
<td>5′-GCGGAGTCTCTCTACTTCTT-3′</td>
</tr>
<tr>
<td>Annexin 2</td>
<td>5′-ACCAATTGCTGCTGAGA-3′</td>
</tr>
<tr>
<td></td>
<td>5′-GGCTTCTTGGTCCTTCT-3′</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>5′-CTCGCTACCTTCATGCGACCC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-AGATGCTGTCATGCTGAGACCT-3′</td>
</tr>
<tr>
<td>Col II</td>
<td>5′-CTCGCTACCTTCATGCTGAGA-3′</td>
</tr>
<tr>
<td></td>
<td>5′-TTGAGCCTGGATGAGCA-3′</td>
</tr>
<tr>
<td>Col X</td>
<td>5′-TGGTAGGCTCTGATTAAGAAGC-3′</td>
</tr>
<tr>
<td></td>
<td>5′-CATGAGGCACACTTAGGAATCTGAGA-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AGGGGCTGTTGGAAGATGG-3′</td>
</tr>
</tbody>
</table>

ALP, alkaline phosphatase; Col I, type I collagen; Col II, type II collagen; Col X, type X collagen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

alpha modification) with 10% FBS. The medium was supplemented with 1% penicillin-streptomycin and changed twice/wk.

To culture primary osteoblasts in hypoxic conditions, confluent cells were cultured for 12 h in hypoxic conditions induced using an AnerPack KENIK system (Mitsubishi Gas Chemical, Tokyo, Japan).

The control cells were cultured for 12 h under normoxia.

**Culture of osteoblastic MC3T3-E1 cells.** Mouse osteoblastic MC3T3-E1 cells were provided by Dr. H. Kodama (Ohu Dental College, Koriyama, Japan). The cells were cultured in α-MEM with 10% FBS and 1% penicillin-streptomycin. The medium was changed twice/wk.

**Preparation of primary chondrocytes.** Articular chondrocytes were obtained from mice as described previously (19). Briefly, articular cartilage was removed from 5-day-old tPA+/− and tPA−/− mice, cleaned to remove soft tissue, and digested with 3 mg/ml collagenase in Dulbecco’s modified Eagle’s medium (DMEM) for 45 min at 37°C. The cartilage was further digested overnight with 0.5 mg/ml collagenase in DMEM at 37°C. Cells were seeded in a six-well plate (1.0 × 10^5 cells/well) and grown in DMEM with 10% FBS for 6 days. The medium was supplemented with 1% penicillin-streptomycin and changed twice/wk.

**Statistical analysis.** Data are expressed as means ± SE. Statistical significance was evaluated using Student’s t-test for comparisons of two groups and ANOVA, followed by Tukey’s test for multiple comparisons. The significance level was set at P < 0.05.

**RESULTS**

**Delayed bone repair after a femoral bone defect in tPA−/− mice.** Bone repair was significantly delayed in tPA−/− mice on days 7 and 14 compared with tPA+/− mice (Fig. 1, A and B).

Conversely, the bone repair was comparable between uPA+/− and uPA−/− mice (Fig. 1, C and D). In hematoxylin and eosin-stained sections, bone repair was found to be delayed in tPA−/− mice compared with tPA+/− mice (Fig. 1E). Although the newly generated bone tissues were observed on day 7 in tPA+/−, tPA−/−, uPA+/−, and uPA−/− mice, less tissue was observed in tPA−/− mice than in tPA+/− mice (Fig. 1, E and F). No fibrin clots were observed in the blood vessels on day 24.
14 after a femoral bone defect in tPA+/+, tPA−/−, uPA+/+ and uPA−/− mice (data not shown). These results indicate that tPA, but not uPA, plays a crucial role in bone repair.

Decrease in the number of osteoblasts at the damaged site after a femoral bone defect in tPA−/− mice. We examined the number of osteoblasts and TRAP-positive MNCs at the damaged sites. The numbers of ALP-positive cells and osterix-positive cells in tPA−/− mice were significantly decreased on days 7 and 4, respectively, compared with tPA+/+ mice (Fig. 2, A–D). The number of BrdU and osterix double-positive cells in tPA−/− mice was significantly decreased compared with tPA+/+ mice (Fig. 2, E and F). Conversely, TUNEL-positive cells were scarce at the damaged sites in tPA+/+ and tPA−/− mice on day 4, and there was no significant difference in the number of TUNEL-positive cells between tPA+/+ and tPA−/− mice (tPA+/+ mice: 0.53 ± 0.34 cells/0.1 mm²; tPA−/− mice: 0.36 ± 0.26 cells/0.1 mm²; n = 5, P = 0.393). The number of TRAP-positive MNCs was not significantly different between tPA+/+ and tPA−/− mice on day 7 (Fig. 2, G and H). The levels of osteogenic marker mRNA, including Runx2, osterix, ALP, type I collagen, and osteocalcin, were not significantly different in the damaged femurs of tPA+/+ and tPA−/− mice (Fig. 2I). These results suggest that the proliferation of osteoblasts is impaired by tPA deficiency during bone repair.

Roles of tPA in osteoblast proliferation, apoptosis, and differentiation. We examined the roles of osteoblasts in the impaired bone repair caused by tPA deficiency using primary calvarial osteoblasts from tPA+/+ and tPA−/− mice. Cell proliferation was analyzed by BrdU incorporation, trypan blue staining, and the WST-1 assay in calvarial osteoblasts. BrdU incorporation was significantly reduced in tPA−/− mice compared with tPA+/+ mice (Fig. 3A). Trypan blue staining showed that the number of trypan blue-negative calvarial osteoblasts was significantly less in tPA−/− mice compared...
with that of \(\text{iPA}^{+/+}\) mice (Fig. 3B). The WST-1 assay also showed that the metabolic activity of mitochondria was decreased in \(\text{iPA}^{-/-}\) mice compared with \(\text{iPA}^{+/+}\) mice (Fig. 3C). Next, we analyzed apoptosis and cell viability by TUNEL staining, trypan blue staining, and the WST-1 assay in calvarial osteoblasts. Dexamethasone and etoposide increased the number of nonviable osteoblasts obtained from \(\text{iPA}^{+/+}\) and \(\text{iPA}^{-/-}\) mice (Fig. 3, D–F). No significant differences were observed in apoptosis and cell viability in osteoblasts between \(\text{iPA}^{+/+}\) and \(\text{iPA}^{-/-}\) mice (Fig. 3, D–F). Taken together, these results suggest that cell-autonomous tPA is important for cell proliferation in osteoblasts.

Previous studies indicate that tPA activates intracellular signaling pathways directly through its binding proteins, apart from proteolytic activation of plasminogen (35–38). Therefore, we examined the involvement of annexin 2, a binding protein of tPA (38, 40), in tPA-enhanced osteoblast proliferation. The levels of annexin 2 in the damaged femurs were similarly elevated in \(\text{iPA}^{+/+}\) and \(\text{iPA}^{-/-}\) mice (Fig. 4A). Next, we examined the effects of a reduction in the level of endogenous annexin 2 by siRNA on tPA-enhanced proliferation in mouse osteoblastic MC3T3-E1 cells. Although exogenous tPA treatment enhanced BrdU incorporation in MC3T3-E1 cells, a reduction in endogenous annexin 2 by siRNA antagonized the proliferation enhanced by tPA treatment (Fig. 4B). Since tPA activates ERK1/2 signaling through annexin 2 (40), we next examined the involvement of ERK1/2 signaling in the tPA-enhanced proliferation of MC3T3-E1 cells. tPA increased the phosphorylation of ERK1/2 within 30 min in MC3T3-E1 cells, and a mitogen-activated protein kinase kinase inhibitor, PD-98059, suppressed the phosphorylation of ERK1/2 caused by tPA treatment (Fig. 4C). PD-98059 significantly suppressed the
increased BrdU incorporation induced by tPA treatment (Fig. 4D). Next, we examined the involvement of annexin 2 in the phosphorylation of ERK1/2 caused by tPA treatment in MC3T3-E1 cells. The reduction in endogenous annexin 2 by tPA deficiency on vessel formation. The number and luminal area of vessels at the damaged site in tPA−/− mice were significantly decreased compared with tPA+/+ mice (Fig. 7, A–D). These results indicate that vessel formation was impaired by tPA deficiency during bone repair.

VEGF plays a crucial role in vessel formation, and its expression is regulated by several growth factors, such as TGFβ and BMP-2, and HIF-1α at the damaged site after bone injury (3, 13, 43). Therefore, we examined the levels of these factors during bone repair. Increases in the VEGF protein levels in damaged femurs were suppressed in tPA−/− mice, according to Western blot analysis (Fig. 7E). Moreover, although the level of HIF-1α increased markedly in the damaged femur of tPA+/+ mice, its increase was not observed in tPA−/− mice (Fig. 7E). The increases in the protein and mRNA levels of TGFβ and BMP-2 in the damaged femur were not different between tPA+/+ and tPA−/− mice (Fig. 7E, F–H). Finally, we examined the effects of hypoxia on the levels of VEGF and HIF-1α in calvarial osteoblasts from tPA+/+ and tPA−/− mice. The increases in the HIF-1α and VEGF levels induced by hypoxia were suppressed in calvarial osteoblasts derived from tPA−/− mice compared with those derived from tPA+/+ mice (Fig. 7I).

**DISCUSSION**

An established function of tPA is the activation of plasminogen. Recently, we reported that plasminogen deficiency impairs the formation of bone tissues at the damaged site during bone repair in mice (29). On the other hand, plasminogen deficiency enhanced mineralization and the levels of osteogenic markers in primary osteoblasts, suggesting that plasminogen suppresses the mineralization and differentiation of osteoblasts (29). Therefore, the effect of plasminogen on bone repair was impaired by tPA deficiency during bone repair.

**Effect of tPA deficiency on chondrogenesis.** There were no significant differences in the cartilage matrix between tPA+/+ and tPA−/− mice in sections stained with Alcian and toluidine blue on day 7 after a femoral bone defect (Fig. 6, A and B). The levels of aggrecan and types II and X collagen mRNA were similar in the damaged femurs between tPA+/+ and tPA−/− mice (Fig. 6C). These results indicate that tPA deficiency does not affect chondrogenesis during bone repair. Next, we examined the effects of tPA deficiency on chondrocytes. The levels of aggrecan and types II and X collagen mRNA were not significantly different in primary articular chondrocytes from tPA+/+ and tPA−/− mice (Fig. 6D).

Decrease in vessel formation at the damaged site during bone repair in tPA−/− mice. Since vessel formation is a critical event for bone repair, we examined the effects of tPA deficiency on vessel formation. The number and luminal area of vessels at the damaged site in tPA−/− mice were significantly decreased compared with tPA+/+ mice (Fig. 7, A–D). These results indicate that vessel formation was impaired by tPA deficiency during bone repair.

**DISCUSSION**

An established function of tPA is the activation of plasminogen. Recently, we reported that plasminogen deficiency impairs the formation of bone tissues at the damaged site during bone repair in mice (29). On the other hand, plasminogen deficiency enhanced mineralization and the levels of osteogenic markers in primary osteoblasts, suggesting that plasminogen suppresses the mineralization and differentiation of osteoblasts (29). Therefore, the effect of plasminogen on bone repair was impaired by tPA deficiency during bone repair.
repair might be due to a non-cell-autonomous effect in osteoblasts in vivo but not its direct effects on osteoblasts. tPA is expressed in osteoblasts and enhanced by several stimuli with hormones and factors (1, 15, 16, 20, 21). In the present study, tPA deficiency decreased the number of BrdU and osterix double-positive cells at the damaged sites during bone repair. Moreover, the proliferation of osteoblasts from tPA-deficient mice was less than that from wild-type mice. These findings suggest that the effect of tPA on bone repair is due to a cell-autonomous effect on proliferation in osteoblasts.

Fig. 4. Involvement of annexin 2 and ERK1/2 signaling in the tPA-enhanced proliferation of MC3T3-E1 cells. A: Western blot analysis of annexin 2 in the damaged and contralateral intact femur on day 4 after a bone defect in tPA+/+ and tPA−/− mice. The results represent experiments performed on 6 mice in each group. B and D: proliferation of MC3T3-E1 cells, as assessed by BrdU incorporation assay. MC3T3-E1 cells were transfected with control (Cont) and annexin 2 siRNA (B). MC3T3-E1 cells were treated with tPA (10 μg/ml) and BrdU for 24 h (B, left, and D). Levels of annexin 2 mRNA in control siRNA and annexin 2 siRNA-transfected MC3T3-E1 cells (B, right). Data represent the mean ± SE of 4 experiments. **P < 0.01; *P < 0.05. C and E: Western blots analyses of ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) in MC3T3-E1 cells. MC3T3-E1 cells were treated with PD-98059 (20 μM) and DMSO for 1 h (C). MC3T3-E1 cells were transfected with control siRNA and annexin 2 siRNA (E). These cells were treated with tPA (10 μg/ml) for 30 min. The results represent experiments performed independently 3 times.

Fig. 5. Effects of tPA deficiency on the levels of osteogenic markers and mineralization in primary osteoblasts. A: relative mRNA levels of Runx2, osterix, ALP, Col I, and OCN in calvarial osteoblasts derived from tPA+/+ and tPA−/− mice. B: quantification of ALP activity in calvarial osteoblasts derived from tPA+/+ and tPA−/− mice. C: mineralization of calvarial osteoblasts from tPA+/+ and tPA−/− mice. Images of alizarin red-stained cells (C, left) and quantification of alizarin red staining by measurement of the absorbance of the extracted stain (C, right). Data represent the mean ± SE of 3 (A and C) and 5 experiments (B). **P < 0.01; *P < 0.05.
An increasing number of studies suggests that tPA plays several pathophysiological roles without activation of plasminogen (37, 38). The present study suggests that the effects of tPA on bone repair are cell-autonomous effects on the proliferation of osteoblasts. Moreover, tPA deficiency did not affect chondrogenesis at the damaged site during bone repair in mice. On the other hand, in our previous study, the effects of plasminogen on bone repair were due to non-cell-autonomous effects in osteoblasts, and plasminogen deficiency decreased chondrogenesis at the damaged site during bone repair in mice (29). These findings suggest that the effects of tPA on proliferation in osteoblasts are independent of the fibrinolytic system during bone repair. Previous studies indicate that tPA activates intracellular signaling pathways directly, including ERK1/2 signaling in a noncatalytic manner, apart from its proteolytic activation of plasminogen (35–38, 40). Ortiz-Zapater et al. (38) reported that tPA induces the proliferation of pancreatic cancer cells by a noncatalytic mechanism through annexin 2, a cell surface receptor for extracellular matrix molecules such as tenascin-C, and proteolytic enzymes, including tPA and cathepsin B (33). Annexin 2 is expressed in osteoblasts and mesenchymal stem cells (24, 39), and it is involved in several osteoblastic phenotypes, including 1α,25-dihydroxyvitamin D3-mediated augmentation of the proliferation of osteoblasts after mechanical stress, osteoblastic mineralization, and receptor activator of nuclear factor-κB ligand expression (18, 30, 34). We showed that the increases in the levels of annexin 2 in the damaged femurs were similar in wild-type and tPA-deficient mice in vivo. However, the proliferation enhanced by tPA was antagonized by the reduction in endogenous annexin 2 levels and by an inhibitor of ERK1/2 phosphorylation. Moreover, the phosphorylation of ERK1/2 caused by tPA was suppressed by the reduction in endogenous annexin 2 levels. Taken together, these results indicate that tPA facilitates the proliferation of osteoblasts partly through annexin 2 and ERK1/2 pathway during bone repair.

Our data indicate that the mRNA levels of osteogenic genes at the damaged site after a femoral bone defect were similar between wild-type and tPA-deficient mice in vivo, although tPA deficiency impaired the proliferation of osteoblasts during bone repair after a femoral bone defect in mice. On the other hand, tPA deficiency suppressed the levels of Runx2, osterix, and type I collagen mRNA as well as mineralization in ex vivo primary osteoblast cultures from mice. Therefore, we cannot rule out the possibility that some factors might counteract the suppression of osteoblast differentiation through cell-autonomous effects by tPA deficiency in a non-cell-autonomous manner during the bone repair process.

We showed that tPA deficiency decreased vessel formation at the damaged site, suggesting that a decrease in vessel formation is involved in impaired bone repair caused by tPA deficiency. Moreover, we showed that an increase in the VEGF levels at the damaged site was suppressed in tPA-deficient mice, suggesting that vessel formation by increased VEGF expression occurred in a tPA-dependent manner at the damaged site of the femur during bone repair.

VEGF expression is regulated by several factors, including HIF-1α, TGFβ, and BMP-2 (3, 13, 43). In the present study, tPA deficiency suppressed increases in the HIF-1α level in the damaged femurs, although it did not affect the increases in TGFβ and BMP-2 induced by the bone defect. These findings indicate that tPA induced the expression of VEGF through HIF-1α, but not TGFβ and BMP-2, at the damaged femur after bone defect. The results obtained from tPA-deficient mice in the present study contrast with our previous findings in plasminogen-deficient mice, which showed that plasminogen contributes to vessel formation through an increase in TGFβ, but not HIF-1α, during bone repair (29). Therefore, we speculate that tPA might contribute to vessel formation at the damaged site during bone repair in a manner independent of the tissue plasminogen system. In the present study, we showed that the increases in the HIF-1α and VEGF levels induced by hypoxia were suppressed in calvarial osteoblasts from tPA-deficient mice compared with wild-type mice. These findings also suggest that cell-autonomous tPA is involved in the increases in the HIF-1α and VEGF levels in osteoblasts. Further studies are necessary to clarify the details in mechanisms of tPA-dependent angiogenesis at the damaged site during bone repair.

Plasminogen activators have redundant functions in the activation of plasminogen (32). As for bone, a previous...
study showed that individual tPA-deficient and uPA-deficient mice displayed nonskeletal phenotypes, but tPA and uPA double-deficient mice displayed growth retardation (8). Daci et al. (11) revealed that tPA and uPA double-deficient mice exhibited increased bone formation. In the present study, we revealed that bone repair after a femoral bone defect was impaired by tPA deficiency, but not uPA deficiency in mice with the same background, indicating that tPA, but not uPA, participates in bone repair in mice. Previous studies suggest that uPA rather than tPA is in general relevant to the tissue repair process in the skin and liver by generation of plasmin (2, 28, 32). As for bone healing, Popa et al. (41) reported recently that uPA deficiency impaired the osteoclast-mediated remodeling of fracture cartilage, although no significant differences were observed in any of the parameters of the bony callus during bone repair. Furlan et al. (17) showed that uPA receptor-lacking mice displayed increased bone mineral density, decreased bone size, increased osteogenic potential of osteoblasts, and decreased osteoclast formation and function, although it did not cause developmental disorders in other studies (6, 14). However, unlike the repair of skin and liver, our findings suggest that tPA, but not uPA, plays a primary role in the bone repair process in mice. Moreover, our findings suggest that the effects of tPA deficiency on bone repair result from a lack of tPA but not difference in the genetic background of mice. In conclusion, our results provide novel evidence that tPA, but not uPA, is crucial for bone repair in mice. The present study indicates that tPA contributes to the proliferation of osteoblasts related to annexin 2 and ERK1/2 in a cell-autonomous manner, thereby leading to the enhancement of bone repair.

GRANTS
This study was partially supported by a grant from the Osaka Medical Research Foundation for Intractable Diseases, Kinki University Research, and a Grant-in-Aid for Scientific Research (C: 25460305) to N. Kawao and (C: 24590289) to H. Kaji from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

DISCLOSURES
The authors declare no conflicts of interest, financial or otherwise.
AUTHOR CONTRIBUTIONS
N.K. and H.K. conception and design of research; N.K., Y.T., K. Okumoto, M.Y., and K. Okada performed experiments; N.K. and H.K. analyzed data; N.K., O.M., and H.K. interpreted results of experiments; N.K. and H.K. prepared figures; N.K. and H.K. drafted manuscript; N.K., O.M., and H.K. edited and revised manuscript; N.K. and H.K. approved final version of manuscript.

REFERENCES


