INTRODUCTION
We have developed a cell transplantation method in which bone marrow-derived mesenchymal stem cells (MSCs) are cultured and lifted as a cell sheet structure. MSCs are cultured in medium containing dexamethasone (Dex) and ascorbic acid phosphate (vitamin C) to create a cell sheet that could be scraped off as a single sheet. In the present study, we transplanted the dead bone wrapped with the cell sheet into subcutaneous site to show that such sheets could form bone tissue around the dead bone. Then, we applied the cell sheet transplantation method to the nonunion of the femur to evaluate whether the cell sheet enhanced bone formation in a rat model. Cell sheets were transplanted onto fractured femurs of rats without scaffolds to enhance bone formation.

METHODS
1. Cell culture and cell sheet preparation
Bone marrow cells were obtained from the femoral shafts of 7-weeks-old F344 rats by flushing out with 10ml culture medium. The cells were collected in two flasks (75cm²) containing 15ml regular medium of minimal essential medium with 15% fetal bovine serum and antibiotics in a humidified atmosphere of 95% air with 5% CO₂ at 37°C. After being confluent, the primary culture cells were released from the culture substratum using trypsin/EDTA. To make cell sheet, the released cells were seeded at a cell density of 1 × 10² cells/cm² to 10cm dish for subculture with 10nM Dex and VC (L-ascorbic acid phosphate magnesium salt n-hydrate, 82μg/ml) until confluent. The cells were rinsed with phosphate-buffered saline twice and then the cell sheet was lifted by scraper (Fig.1).

2. Dead bone transplantation with cell sheet
To make dead bone, the femurs from F344 rat were irradiated with a single exposure of 60 Gy. Bone fragments of 5mm length were prepared by cutting the above-mentioned bone using a bone micro-saw and transplanted with the cell sheet into subcutaneous site. Four weeks later, the transplanted bones were harvested and fixed in 10% formaldehyde/PBS solution, decalcified in K-CX solution and embedded in paraffin for histological evaluation. Each group involved three samples.

3. Cell sheet transplant to fractured femur
Rat femur nonunion model was made under anesthesia. After transverse osteotomy of the femur, a 21-gauge needle was inserted into the intramedullary femoral shaft from the distal femoral condyle in a retrograde fashion, resulting in loose fixation. The periosteum was removed as much as possible from the distal to proximal sites of the femur. Then, a cell sheet of cultured MSCs was wrapped around the osteotomy site on the left femur (Fig.2). The right femur was treated the same as the left femur, but without cell sheet wrapping. Therefore, left and right femurs were used as sheet and control groups, respectively. Unprotected weight bearing was allowed immediately after the operation. Each group involved 12 hindlimbs.

4. Evaluation of bone union
X-ray photographs were taken under anesthesia, at 2, 4 and 8 weeks postoperatively to evaluate callus formation and bridging bone formation at the fracture site. Animals from each group were sacrificed and the hind limbs were harvested at 8 weeks postoperatively for histological evaluation.

RESULT
The histology of the harvested dead bone transplanted with cell sheet 4 weeks after the transplantation showed newly bone formation around the dead bone, indicating the cell sheet had osteogenic potential. In contrast, no bone formation was observed around the dead bone without cell sheet transplantation.

Radiographs taken at 2 weeks after the sheet transplantation showed callus formation around the fracture site in the sheet group. Bridging callus formation was observed at 4 weeks and the cortical gap disappeared at the sheet-transplanted site at 8 weeks postoperatively, indicating bone union (Fig.3). By contrast, the control group showed no bridging callus formation at the fracture site at 4 and 8 weeks postoperatively resulted in established nonunion of the femur. Histological section of the sheet transplanted femur at 8 weeks showed that the callus on both distal and proximal sites of the femur were united (Fig.4). On the other hand, although the control group also exhibited chondrocytes and endochondral ossification, there was no bridging bone formation at the fracture site, even at 8 weeks postoperatively. The gaps between cortices were filled with chondrocytes and fibrous tissue, resulting in the nonunion of the femur.

DISCUSSION
The literature describes several options such as low intensity ultrasound (LIPUS) for stimulation of bone formation in nonunion. LIPUS has been shown as a less invasive treatment for nonunion to enhance bone formation. However, the success of LIPUS treatment is not always guaranteed. Therefore, we think that a new approach is required for nonunion treatment. We have reported a technique of scaffold-free MSC cell sheet transplantation, in which adhesion molecules on the cell surface and cell-cell interactions remain intact.

First, the present study showed that the cell sheet could form bone tissue when the sheet was transplanted with dead cortical bone, indicating that the cell sheet could supply the osteogenic cells to the dead bone and form bone tissue even on the smooth surface of the cortex. The result led us to think that the sheet could be transplanted to nonunion site of the femur to enhance bone formation. Our results clearly showed that transplanted cell sheet could form a callus around osteotomy site of the femur, which achieved bony bridging across the osteotomy site. This indicated that scaffold-free sheet transplantation can be applied for the treatment of fracture and nonunion in clinical case.