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From Cell Biology to Tissue Engineering

Effect of short-term betamethasone administration on the regeneration process of tissue-engineered bone

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Summary. Local inflammation at the transplanted site of tissue-engineered bone may cause apoptosis of the transplanted cells, thus negatively affecting bone regeneration. To maximize the efficacy of bone tissue engineering, the local effect of short-term corticosteroid administration at the transplanted site of tissue-engineered bone was studied with respect to the expression of inflammatory cytokines. Compact bone-derived cells from mouse leg bones were isolated, cultured and seeded onto β-tricalcium phosphate granules. The constructs were transplanted to the back of syngeneic mice. Betamethasone sodium phosphate was administered intraperitoneally to an experimental (betamethasone) group, whereas the same amount of saline was administered to a control group. When betamethasone was administered three times (immediately after operation and 12 hours and 24 hours after transplantation), the number of SP7/osterix-positive osteoblasts was larger in the betamethasone group. Three times of betamethasone administration (immediately after operation and 12 hours and 24 hours after transplantation) did not change the number of apoptotic cells and osteoclasts, but showed a slight upregulation of IL-4 and a downregulation of IL-6. However, 7 doses of betamethasone administration (over 7 consecutive days) increased the number of apoptotic cells and osteoclasts, which was correlated with a downregulation of IL-4 and an upregulation of IL-6. TNF-α expression levels showed no significant differences between the two groups. The results showed beneficial effects of 3 betamethasone administrations for bone regeneration therapy but contrary effects when betamethasone was administered 7 times due to the downregulation of anti-inflammatory cytokines (IL-4) and the upregulation of inflammatory cytokines (IL-6). As a conclusion, our results suggested the importance of the cautious usage of corticosteroids to control local inflammation at transplanted sites in bone tissue engineering.

Key words: Cortical bone-derived cells, Mesenchymal stem cells, Corticosteroid, Betamethasone, Bone tissue engineering, Local inflammation

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Introduction

Clinical studies on bone tissue engineering using somatic stem cells have been reported (Kagami et al., 2014; Gjerde et al., 2018). Although the results from those studies are promising, the regeneration efficacy has not always been sufficient (Bajestan et al., 2017). In particular, low survival rates of transplanted cells have limited the efficacy of clinical tissue engineering (Liu et al., 2011; Kanazawa et al., 2013). Past studies have shown deficient tissue regeneration in immuno-competent animals due to inflammatory cytokines and their effects on the survival of transplanted cells (Liu et al., 2011; Kanazawa et al., 2013; Zhang et al., 2015). To maximize the effects of bone regeneration treatment with

cells, strategies against local inflammatory reactions against tissue-engineered bone should be addressed.

Corticosteroids are anti-inflammatory and immunosuppressive (Ayroldi et al., 2012; Cain and Cidlowski 2015). Corticosteroids bind to glucocorticoid receptor α (GRα), translocate into the nucleus and downregulate inflammatory cytokines, chemokines and adhesion molecules *via* competitive inhibition against activator protein-1 (AP-1) and NFκB, thus exerting anti-inflammatory functions (Ayroldi et al., 2012; Cain and Cidlowski 2015). However, long-term steroid administration causes osteoporosis by complex mechanisms, including the inhibition of osteogenic differentiation and the induction of osteoblast apoptosis (Briot and Roux 2015). In addition, osteonecrosis of the long bone is one of the most serious complications associated with corticosteroid administration (Weinstein, 2012a,b). While the long-term administration of corticosteroids has been examined, the effects of short-term administration on the bone regeneration process have not been well studied. As far as we were able to find, the studies were limited to bone regeneration with bone morphogenetic protein (Spiro et al., 2010) and bone remodeling process (Chavassieux et al., 1997). If short-term corticosteroid administration can reduce local inflammation without affecting the survival of osteoblasts, the process could be advantageous for bone regeneration.

Among the various corticosteroids, betamethasone is a derivative of prednisolone, has a 16β-methyl group that enhances the anti-inflammatory action of the molecule (Drugs.com, 2019). Although it has similar side effects with other corticosteroid to increase the risk of osteoporosis by decreasing bone formation and increasing bone resorption (Drugs.com, 2019), it has been commonly used in practice after surgical treatment due to its superior anti-inflammatory effect. Accordingly, we have chosen betamethasone as a promising candidate to test their effect on the regeneration process of tissue-engineered bone.

In this study, a short-term administration of an anti-inflammatory betamethasone was conducted using an immunocompetent animal model. The effects on local inflammation, osteoblast survival and the induction of osteoclasts, which affect the bone regeneration process, were examined.

Materials and methods

Ethical approval and animal care

Experiments using animals were performed in accordance with the guidelines of the National Institutes of Health (NIH), USA, regarding the care and use of animals for experimental procedures and with the approval of the "Matsumoto Dental University Committee on Intramural Animal Use" (No. 209-12).

BALB/c AJc1 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed under stable conditions (22±2°C) with a 12-h dark/light cycle, and with *ad libitum* access to water and food in the animal facility of the Department of High-Tech Center, Matusmoto Dental University, Shiojiri, Japan.

Isolation and culture of mouse compact bone-derived cells (CBDCs)

The cultivation of CBDCs was based on a protocol of our previous publication (Zhang et al., 2015) with modifications. Briefly, female mice (5 to 7 weeks old) were euthanized by cervical dislocation. Bone marrow from the femur and tibia were flushed out and cut into chips of approximately 1~2 mm, placed into 50-ml centrifuge tubes containing 20 ml of α-minimum essential medium with glutamine (α-MEM; Wako, Osaka, Japan), 0.25% collagenase (Wako, Osaka, Japan) and 20% fetal bovine serum (FBS; Biowest, French) and incubated for 45 minutes at 37°C in a bioshaker at a speed of 100 rpm. Released cells were aspirated, washed three times with α-MEM and inoculated on culture plates containing α-MEM with 10% FBS, 1% penicillin-streptomycin-amphotericin solution and 10 ng/ml recombinant human basic-fibroblast growth factor (b-FGF; PeproTech, Rocky Hill, NJ, USA). Non-adherent cells were removed after 72-96 hours. When the cells reached 70-80% confluence, they were detached with 0.25% trypsin-EDTA (Gibco: Life Technologies, Carlsbad, CA, USA) and subcultured. The bone chips were seeded with media in other wells of the same plate to collect additional cells. Medium was changed every 3-4 days. Passage 2 cells were used for the in vivo experiments. The phenotype of CBDCs obtained with this method has been characterized in our previous publications, i.e., positive for mesenchymal stem cell markers, including CD29, CD51, CD105 and Sca-1, and negative for CD45 and CD11b (Zhang et al., 2015; Chen et al., 2019).

Preparation of tissue-engineered bone

The preparation of tissue-engineered bone has been previously described (Zhang et al., 2015). Briefly, twenty-five milligrams of porous beta-tricalcium phosphate (β-TCP) granules (G0 0.5-1.5 mm; OSferion, Tokyo, Japan) were used as a scaffold. Passage 2 CBDCs (2.5×10^5 cells) were seeded onto the scaffolds in 14-ml polypropylene tubes (Greiner Bio One International GmbH, Kremsmünster, Austria). Medium was exchanged the next day for osteogenic-induction medium (basic culture medium supplemented with 100 nM dexamethasone (Sigma, USA), 50 μM L-ascorbate acid phosphate (Wako, Japan) and 10 mM glycerol phosphate disodium salt hydrate (Sigma, USA)) and induced for 14 days. Medium was changed every 3 days.

Implantation and harvesting method of samples

Male BALB/c AJc1-nu/nu mice (6-8 weeks old)

were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg; Somnopentyl®, Kyoritsu Seiyaku Corp., Tsukuba, Japan). The tissue-engineered bones were transplanted to the back subcutaneously. At 7 and 28 days after transplantation, the animals were euthanized, and the samples were harvested. The samples were split into two pieces; one piece was immediately frozen in liquid nitrogen for RNA extraction. The other piece was fixed in 10% neutral buffered formalin solution, decalcified and embedded in paraffin. The samples were cut into 4-μm sections for subsequent procedures.

Betamethasone administration

Betamethasone sodium phosphate (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was injected intraperitoneally for a total of 3 times (immediately after operation and 12 hours and 24 hours after transplantation). For longer administrations, betamethasone sodium phosphate was administered for 7 consecutive days after surgery (immediately after operation and 6 more days after transplantation). The dose was determined based on the dosage for human and calculated depending on body weight. The dose used for histological study was 0.1 μg/g body weight (3 animals for the betamethasone and control groups at each time point and minimum of 3 samples for each group), and for qRT-PCR, low (0.07 μg/g) and high (0.27 μg/g) doses were used (3 animals for low and high dose groups and 8 samples for each group in both 3- and 7-times administrations). In a control group, the same volume of saline was injected intraperitoneally at the same time points (3 animals and 4 samples in both 3- and 7-times administrations).

Immunohistochemical staining and apoptosis detection

The sections were deparaffinized with xylene, and rehydrated with graded ethanol. To retrieve antigens, the sections were treated with sodium citrate buffer, pH 6.0 (trisodium citrate dihydrate; Wako Pure Chemical Industries, Ltd.), in a microwave. To reduce endogenous peroxidase activity, the sections were quenched in 0.3% hydrogen peroxide in 0.01 M PBS, pH 7.4, for 30 min at room temperature. The sections were incubated in PBS with 10% goat normal serum for 30 min at room temperature to eliminate nonspecific binding of antibodies before incubation with primary antibodies, followed by incubation with primary antibodies. The sections were incubated with anti-Sp7/Osterix antibody (dilution 1:2000, #ab22552, Abcam, Cambridge, UK) and anti-TNF-α antibody (dilution 1:400, #ab6671, Abcam, Cambridge, UK) overnight at 4°C. For visualization, a goat anti-rabbit IgG Vectastain Elite ABC Kit and a DAB Substrate Kit for Peroxidase (both from Vector Laboratories, Inc., Burlingame, CA, USA) were used according to the manufacturer's instructions. Subsequently, sections were counterstained with hematoxylin. As a negative control, the primary antibodies were replaced with PBS. Cell apoptosis was visualized by TUNEL staining, which was performed using a TdT In Situ Apoptosis Detection Kit (4810-30-K, RSD, USA) according to the manufacturer's instructions.

Tartrate-Resistant Acid Phosphatase (TRAP) staining

Paraffin sections were deparaffinized, hydrated with graded alcohol, washed three times with PBS, and subjected to TRAP staining. Ten milliliters of TRAP staining reagent were prepared: 5 ml distilled water, 5 ml 0.2 M acetate buffer solution (pH 5.0; Nacalai Tesque, Inc., Kyoto, Japan), 50 μl N,N-dimethylformamide (Sigma-Aldrich Co.), 6 mg Fast Red Violet LB Salt (Sigma-Aldrich Co.), 75 mg L-(+)-Tartaric Acid (Nacalai Tesque, Inc.), and 1 mg Naphthol AS-BI Phosphoric Acid Sodium Salt (Wako Pure Chemical Industries, Ltd.). The pH was adjusted to 5.0 with a 5-M sodium hydroxide solution (Kanto Chemical Co., Ltd., Tokyo, Japan). The sections were incubated in the reagent for approximately 1 h at 37°C, washed three times with water, and a coverslip was mounted with glycerol-gelatin.

Histomorphometric analyses

To analyze the area of immune-positive cells, at least three sections from different layers were selected. Immune-positive cell area was calculated as a percentage of positive area in the whole tissue area using ImageJ (Rasband, 1997-2012; Schneider et al., 2012).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The relative mRNA expressions of inflammatory cytokine genes were examined using qRT-PCR. First, total RNA was extracted from samples using TissueLyser® (Qiagen, Venlo, Netherlands) with TRIzol reagent (Ambion®, Life Technologies, Carlsbad, CA, USA). The appropriate quantity and concentration of isolated RNA were detected using a Nano Drop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Next, RNA samples were reverse transcribed into complementary DNA (cDNA) using oligo (dT) 12-18 primers (Life Technologies), dNTPs (Toyobo Co. Ltd., Osaka, Japan) and ReverTra Ace® (Toyobo Co., Ltd.) according to the manufacturer's instructions. qRT-PCR was performed in a thermal cycler (Thermal Cycler Dice Real Time System II TP-900, Takara Bio, Japan) using SYBR Premix Ex TaqII reagents (Takara Bio, Kusatsu, Japan) according to the manufacturer's protocols. Primer sets (Sigma-Aldrich Co.) used for the PCR experiment are listed in Table 1.

Statistical analysis

Results are expressed as the means ± standard deviation (SD). Statistical analyses were conducted using the Mann-Whitney's U-test (qRT-PCR) or Student's t-test (others). $P<0.05$ was interpreted to denote significance.

Results

Effect of betamethasone administration on the survival of osteoblasts

The localization of SP7/osterix-positive cells, which is a marker for osteoblasts, was analyzed using anti-SP7 antibody (Fig. 1). SP7-positive osteoblasts were located adjacent to β-TCP granules. More positive cells were observed at day 7 compared with day 28 in both groups (Fig. 1a-d) as confirmed by morphometric analysis. The number of osteoblasts in the betamethasone group was larger than that of the control group at day 7 ($p<0.0001$), although the number of osteoblasts rapidly decreased in both groups and no measurable differences were observed by day 28 (Fig. 1e).

Effect of betamethasone administration on apoptosis

Because long-term corticosteroid administration is known to cause osteoblast apoptosis, it was in our interest to detect apoptosis after short-term corticosteroid administration. After 3 times betamethasone administration, apoptotic cells were mainly observed around the β-TCP granules, which corresponded to the location of seeded CBDCs (Fig. 2a). The control group also demonstrated apoptotic cell distributions nearly identical to those in the spheroid group (Fig. 2c). When the betamethasone was administered 7 times, the number of apoptotic cells increased (Fig. 2b), whereas the number of apoptotic cells remained unchanged in the control group (Fig. 2d). This tendency was confirmed by morphometric analyses; the area of apoptotic cells in the betamethasone group was significantly larger than that in the control when steroids were administered 7 times ($p<0.01$) (Fig. 2e).

Effect of betamethasone administration on osteoclastgenesis

Our previous study demonstrated that osteoclast induction occurred at a relatively early phase of bone regeneration process proximate to tissue engineered bone (Zhang et al., 2015). Accordingly, the localization of TRAP-positive cells was investigated. After 3 times betamethasone administration, TRAP-positive cells (osteoclasts) were observed adjacent to β-TCP granules in both the betamethasone group (Fig. 3a) and the control group (Fig. 3b). After 7 times betamethasone administration, the number of osteoclasts in the betamethasone group was more evident than in the control group (Fig. 3c and d). This tendency was confirmed by morphometric analysis (Fig. 3e). The number of TRAP-positive cells in the betamethasone group was not different in the two groups after 3 times betamethasone administration but was significantly larger in the betamethasone group than in the control group after 7 times betamethasone administration (Fig. 3f) ($p<0.05$).

Effect of betamethasone administration on the expression of inflammatory cytokines

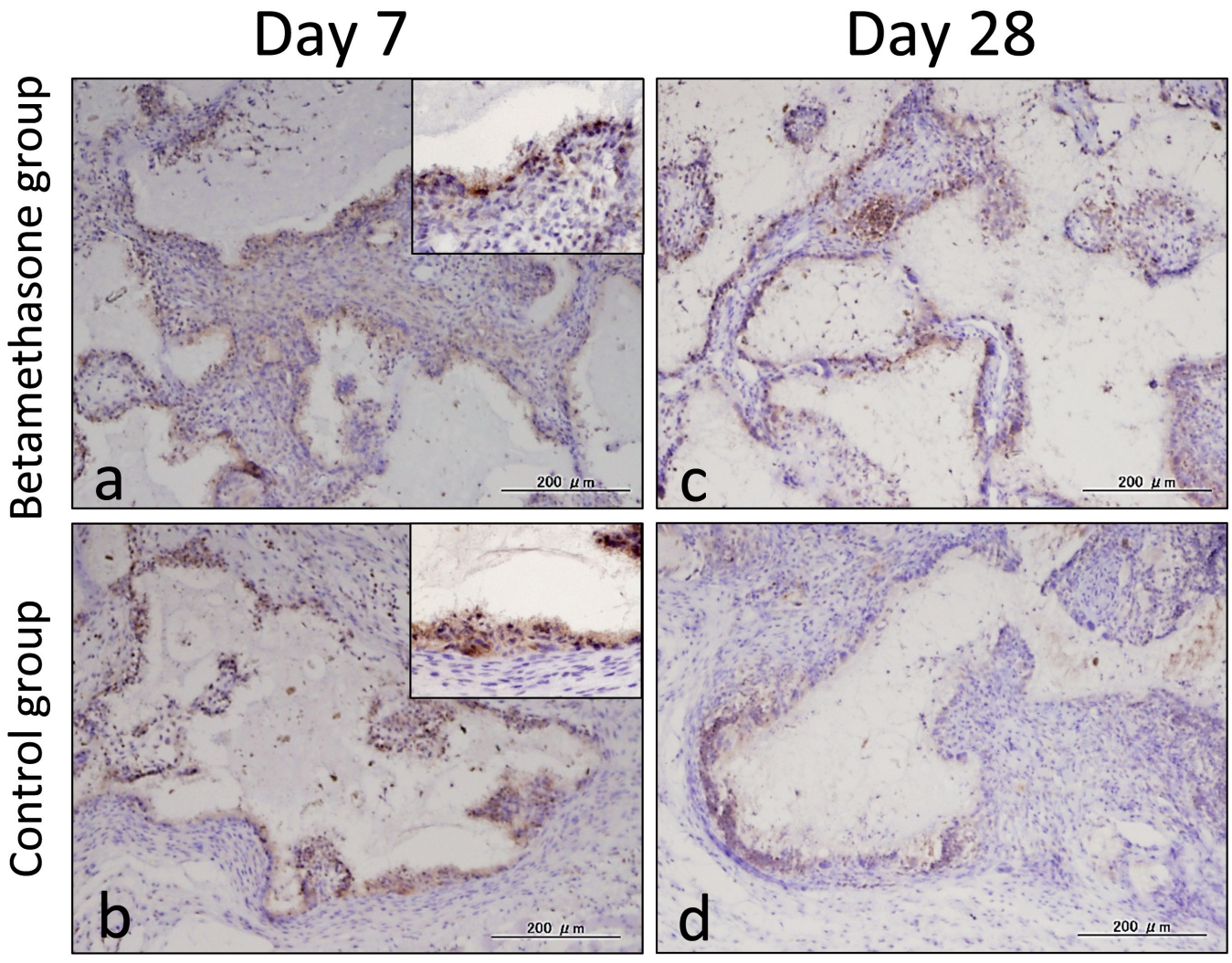
To investigate local immunological reactions, the expressions of IL-2, IL-4, IL-6 and TNF-α in the regenerating tissues were analyzed using qRT-PCR (Fig. 4). IL-2 expression did not show significant differences between the betamethasone and control groups either at 3- or 7-times betamethasone administration (Fig. 4a). IL-4 expression in the high dose betamethasone group was significantly higher than that in the control group when betamethasone was administered 3 times. However, IL-4 expression was significantly lower in the betamethasone group than in the control group when betamethasone was administered 7 times (Fig. 4b). This tendency was more evident at the higher betamethasone dosage. IL-6 expression was lower in the betamethasone group than in the control group when betamethasone was administered 3 times (Fig. 4c). However, IL-6 expression was significantly higher in the betamethasone group when the betamethasone was administered 7 times; this tendency was more evident when the higher betamethasone dosage was used. The level of TNF-α expression was not different between the betamethasone and control groups either after 3- or 7-times betamethasone administration (Fig. 4d).

Effect of betamethasone administration on the expression of TNF-α

Because our previous study showed higher TNF-α expression levels in regenerating tissues in immunocompetent animals than in immunodeficient animals (Zhang et al., 2015), the effects of betamethasone on the distribution of TNF-α-positive cells in regenerating tissues were investigated using an anti-TNF-α antibody. Immunoreactivity for TNF-α was detected at 7 days after transplantation in both betamethasone and control groups (Fig. 5a,b). The intensity of staining decreased after 28 days in both groups (Fig. 5c,d). The positive area was relatively larger in the betamethasone group at day 7, but the difference was not significant (Fig. 5e). The positive area decreased by day 28 in both groups.

Discussion

For animal models of bone tissue engineering, immunodeficient animals, such as nude mice or SCID mice, have been widely used. However, these animals



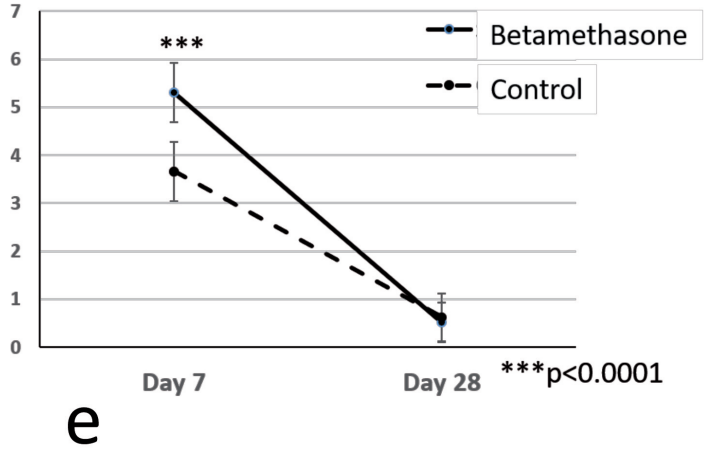


Fig. 1. Localization and SP7-positive cells. The SP7-positive osteoblasts were located adjacent to the β-TCP granules. The positive cell number was relatively larger in betamethasome group at day 7 **(a)** compared with that in control group **(b)**. The number of positive cells decreased by day 28 in both betamethasone group **(c)** and control group **(d)**. This tendency was confirmed by histomorphometric analyses **(e)**.

typically lack physiological immunological reactions, and therefore may not be appropriate to analyze for immunological reactions against transplanted cells or tissues (Zhang et al., 2015). Accordingly, in this study, cells were harvested from immunocompetent mice and transplanted to syngeneic animals. Although

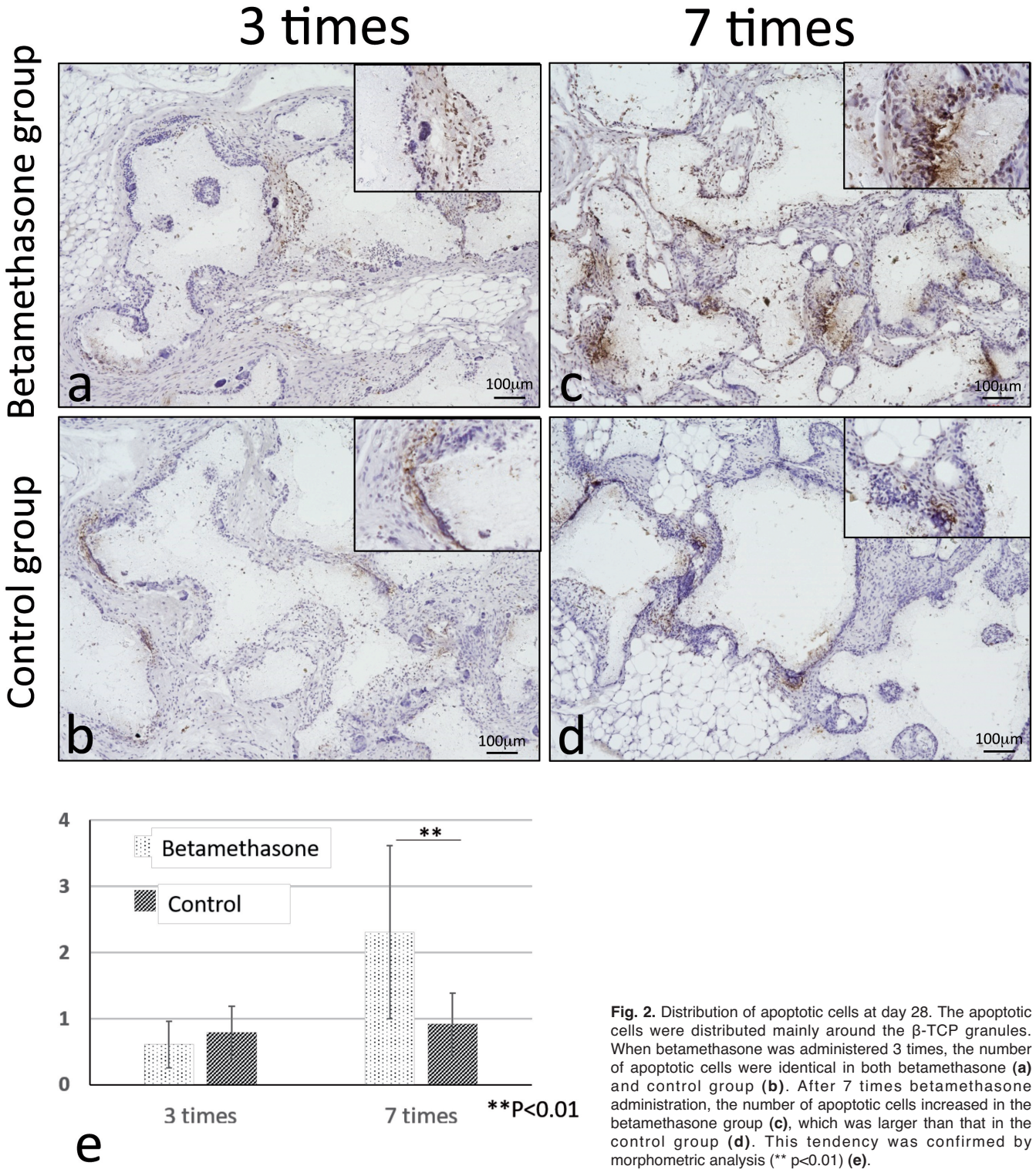


Fig. 2. Distribution of apoptotic cells at day 28. The apoptotic cells were distributed mainly around the β-TCP granules. When betamethasone was administered 3 times, the number of apoptotic cells were identical in both betamethasone **(a)** and control group **(b)**. After 7 times betamethasone administration, the number of apoptotic cells increased in the betamethasone group **(c)**, which was larger than that in the control group **(d)**. This tendency was confirmed by morphometric analysis (** $p<0.01$) **(e)**.

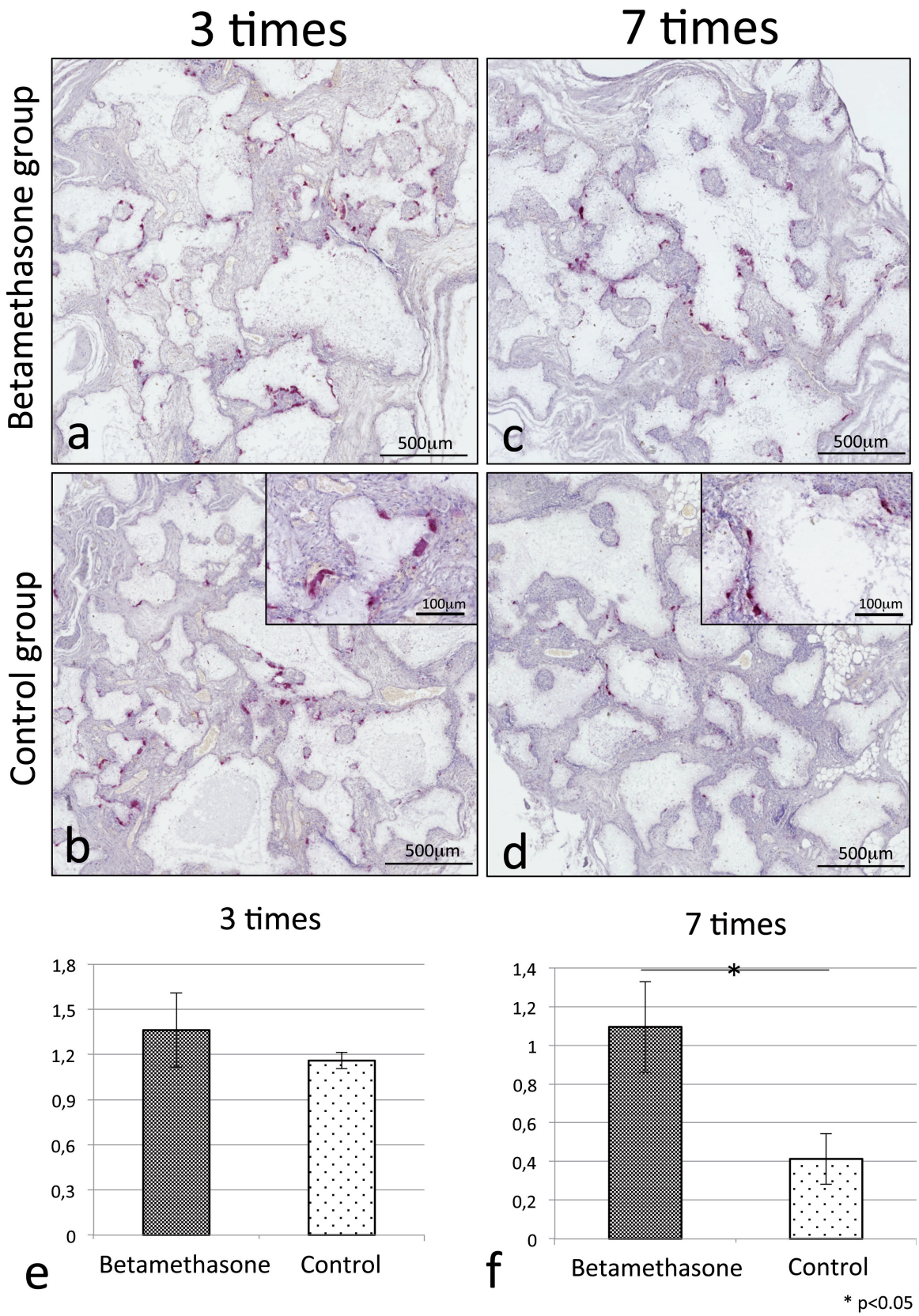


Fig. 3. Localization of TRAP-positive cells at day 28. After 3 times steroid administration, the TRAP-positive osteoclasts were observed adjacent the β-TCP granules in both the betamethasone group **(a)** and the control group **(b)**. After 7 times betamethasone administration, the number of osteoclasts in the betamethasone group was more evident than that in the control group **(c, d)**. This tendency was confirmed by morphometric analyses **(e, f)**. The number of TRAP-positive cells in the betamethasone group was not different between the two groups when betamethasone was administered 3 times **(e)** but was significantly larger in the betamethasone group than in the control after 7 times administration ($p<0.05$) **(f)**.

immunological systems in mice are not completely identical to those in humans, we believe this was a reasonable model to study immunological reactions to transplanted tissue-engineered bone.

Although bone marrow-derived mesenchymal stem (stromal) cells have been widely used for bone tissue engineering, recent studies have shown that CBDCs as an alternative might be a better cell source of mesenchymal stem cells. In particular for mice, CBDCs exert stable proliferation, superior osteogenic differentiation capability and better bone regeneration than those of bone marrow-derived mesenchymal stem cells (Zhu et al., 2010; Cai et al., 2015; Corradetti et al., 2015; Fernandez-Moure et al., 2015; Blashki et al., 2016). Accordingly, CBDCs were used as a cell source for bone tissue engineering animal model (Zhang et al., 2015).

Local inflammatory responses to tissue-engineered bone (transplants) occur due to various causes. Surgical intervention is the primary acute cause of local inflammation. Responses to transplanted scaffolds (as foreign bodies) and reactions to transplanted cells (possibly due to allogenic serum used for expansion) have also been reported to cause immunological reactions (Tonti and Mannello, 2008; Zakrzewski et al., 2014; Wong and Griffiths, 2014). In this study, transplanted cells were derived from syngeneic mice; immunological reactions against transplanted cells were less likely to occur. However, because bovine serum was used for cell culture, the immune responses to cells

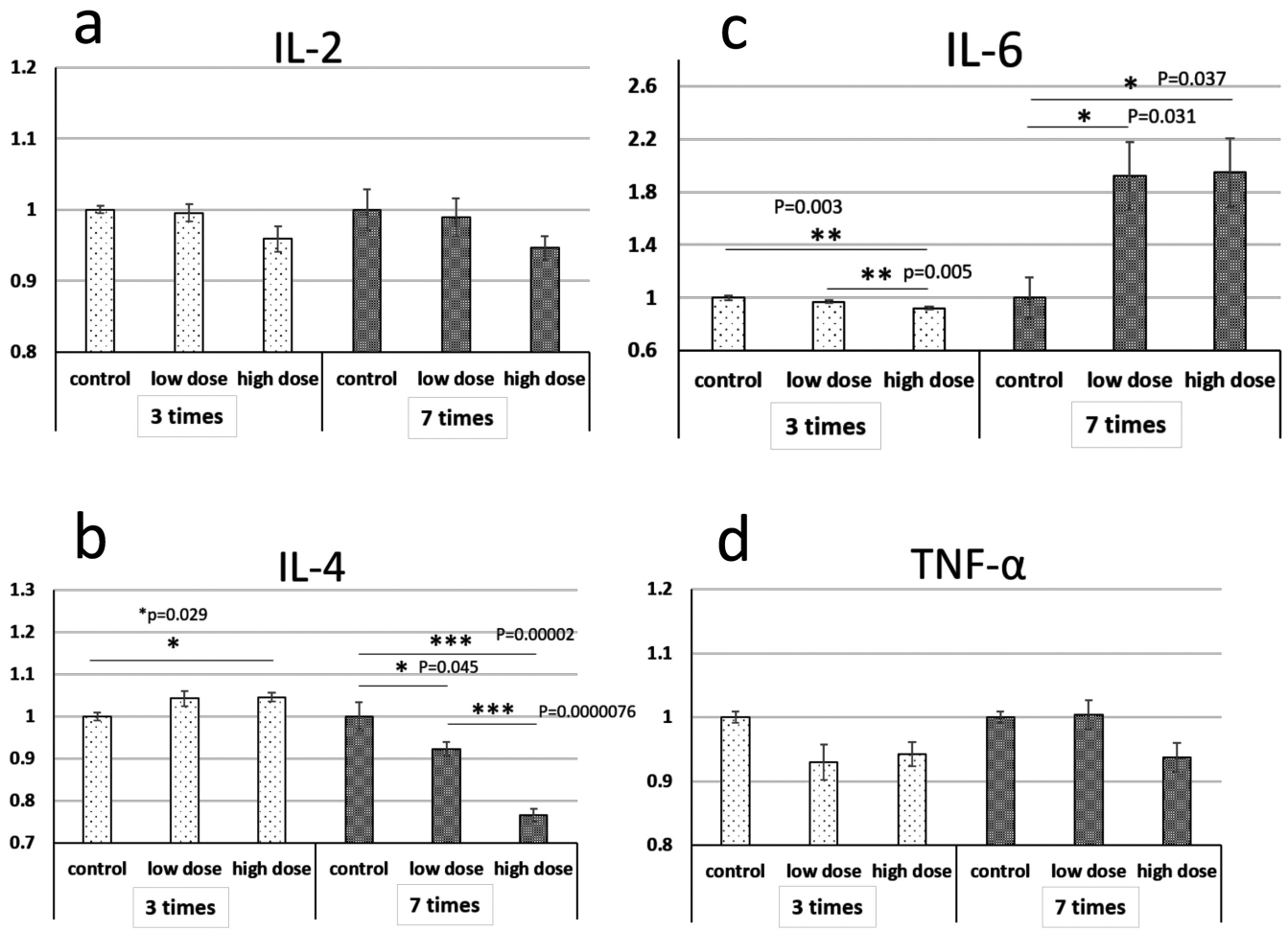


Fig. 4. Expression of IL-2, IL-4, IL-6 and TNF-α in regenerating tissue. IL-2 expression did not show significant differences between the betamethasone and the control groups at either 3- or 7-times betamethasone administration **(a).** IL-4 expression in the betamethasone group was significantly higher than that in the control group when betamethasone was administered only 3 times at high dose, whereas IL-4 expression was lower in the betamethasone group than in the control group when betamethasone was administered 7 times **(b).** This tendency was more evident with higher concentrations of betamethasone. The expression of IL-6 was lower in the betamethasone group than the control group when betamethasone was administered 3 times **(c).** However, IL-6 expression was significantly higher in the betamethasone group when betamethasone was administered 7 times; this tendency was more evident when the higher dose of betamethasone was used. The level of TNF-α expression was not different between the betamethasone and control groups either after 3- or 7-times betamethasone administration **(d)**. *p<0.05, **p<0.01.

cultured in allogenic serum cannot be excluded (Tonti and Mannello, 2008). When carrier scaffolds without cells were transplanted, similar levels of immunological cell infiltration were observed, which suggested the importance of inflammatory reactions against implanted artificial materials (Kanazawa et al., 2013).

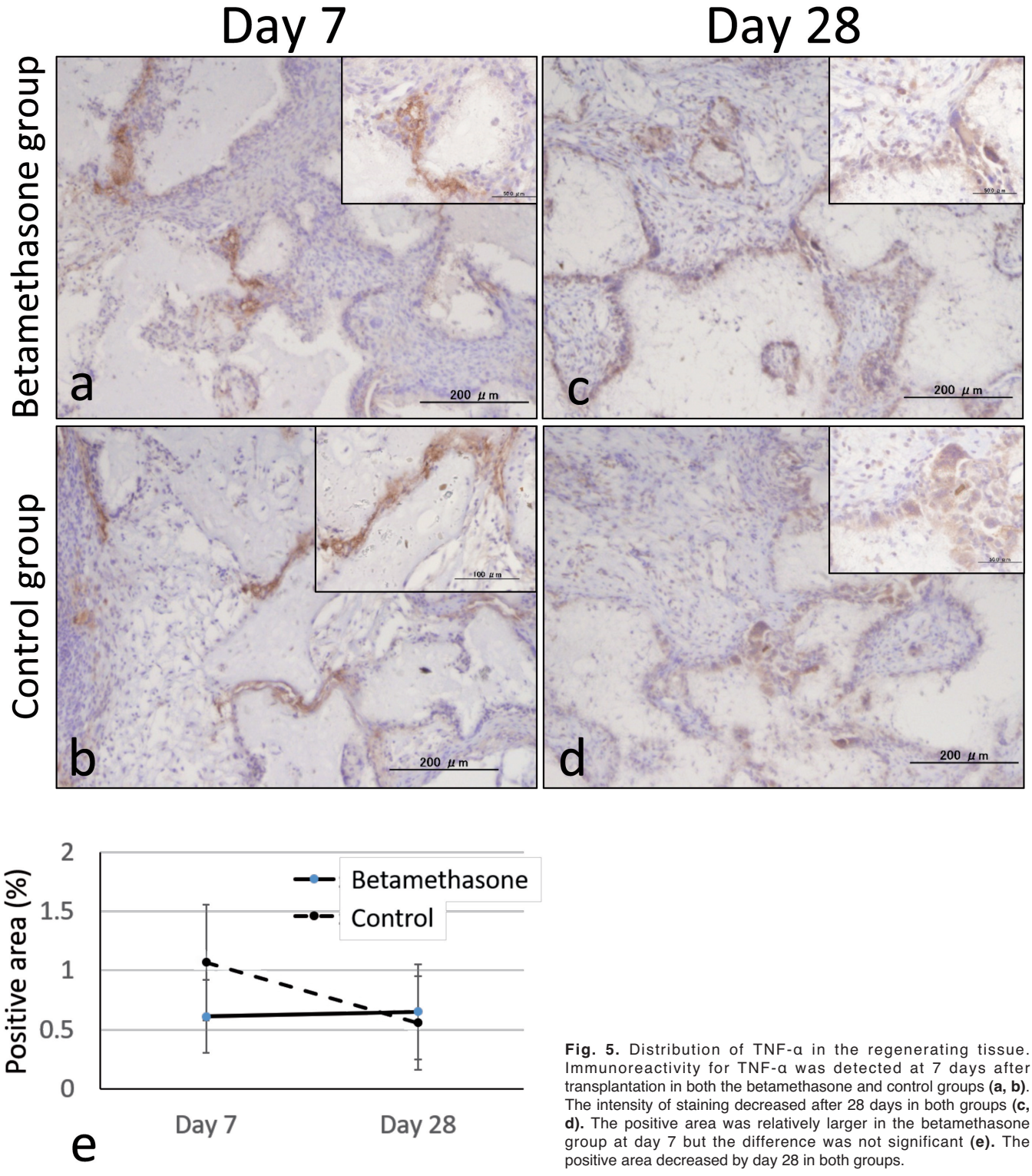


Fig. 5. Distribution of TNF-α in the regenerating tissue. Immunoreactivity for TNF-α was detected at 7 days after transplantation in both the betamethasone and control groups **(a, b)**. The intensity of staining decreased after 28 days in both groups **(c, d).** The positive area was relatively larger in the betamethasone group at day 7 but the difference was not significant **(e).** The positive area decreased by day 28 in both groups.

Corticosteroids have been widely used for anti-inflammatory purposes and for immunosuppression (Ayroldi et al., 2012) and may be beneficial for reducing local inflammation at transplanted sites of tissue-engineered bone. However, corticosteroids are known to induce osteoblast apoptosis, promote osteoclast differentiation, and cause osteoporosis when administered long-term (Weinstein, 2012b). As far as we know, the effects of corticosteroids on bone regeneration have not been reported. We investigated the effects of short-term betamethasone administration, which is commonly prescribed for anti-inflammatory purposes after surgery to treat local inflammation.

The results from this study showed that short-term (3 times within 2 days) betamethasone administration increased the probability of initial osteoblast survival at transplanted sites. However, 7-times betamethasone administration during 7 consecutive days resulted in increased cell apoptosis and osteoclastgenesis acceleration at transplanted sites. Interestingly, even within this short period (7 times administration during 7 days), the typically beneficial, immunosuppressive effects of betamethasone were reversed and harmful (i.e., inducing apoptosis and osteoclastgenesis at the transplanted site). Although the exact time point of this reversal was not shown in this study, the usage of corticosteroids for tissue-engineering approaches may require careful prior evaluation.

To understand the underlying mechanisms of local immunological reactions at the transplantation site after betamethasone administration, the expression of inflammatory cytokines was analyzed. To support the histological and histomorphometric findings, 3-times betamethasone administration increased local IL-4 expression and reduced IL-6 expression. Conversely, 7 doses of betamethasone administration reduced local IL-4 expression and induced higher IL-6 expression. IL-4 was originally defined as a specific Th2 cell product in mice and has been shown to suppress and counterbalance Th1-driven proinflammatory responses in many different disorders (Abbas et al., 1996; van Roon et al., 2001). IL-4 has also been reported to inhibit TNF-α-mediated osteoclast formation in osteoclast precursors in vitro (Fujii et al., 2012). This suggests that IL-4 may have a more important role in osteoclast regulation than TNF-α during early stage bone formation in transplants. IL-6 is known as a mediator of local inflammation and as a factor that induces osteoclast differentiation (Fujii et al., 2012; Mori et al., 2013; Yokota et al., 2014). Accordingly, changes to these cytokine levels could account for immunosuppressive effects after 3 times betamethasone administration and enhanced osteoclastgenesis after 7 times betamethasone administration.

However, explanations for the observed enhanced local inflammatory reaction and induced higher levels of inflammatory cytokine, such as IL-6, after longer betamethasone administration remain unknown and it was one of the limitations of this study. Because osteoblast apoptosis at transplanted sites could be due directly to corticosteroid administration, increased local inflammatory cytokine expression may be the result of cell death and cell death-induced immunological reactions rather than the cause of cell apoptosis. Further studies should elucidate such underlying mechanisms.

In this study, we have chosen betamethasone sodium phosphate. However, there is a different form of betamethasone (betamethasone acetate), which is also used as an injectable suspension (Drugs.com, 2019). Furthermore, it is known that different corticosteroids have different levels of strength, anti-inflammatory effects and half-life, which may also affect the results. It should be kept in mind that the results obtained from this study may not be generalized to other corticosteroids, though most of the known corticosteroids have been reported to show similar tendency to increase a risk of osteoporosis.

The results showed a potential benefit of short-term betamethasone administration to the patients who had the transplantation of tissue-engineered bone. However, a more detailed study should be performed before the results of this study can be applied to patients. First, there is a species difference in immunological reaction and the effect of betamethasone in mice may not be the same as that in human. In particular, the dosage and the time of administration should be adjusted through clinical studies.

In summary, short-term (3 times) betamethasone administration after transplantation of tissue-engineered bone could suppress local inflammation and may increase the survival rate of transplanted cells and reduce osteoclast induction. However, longer (7 times) betamethasone administration showed contrary effects. Overall, our results suggested the importance of the cautious usage of corticosteroids to control local inflammation at transplanted sites in bone tissue engineering.

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Conflicts of interest. The authors state that there are no conflicts of interest regarding this manuscript.

References

Abbas A.K., Murphy K.M. and Sher A. (1996). Functional diversity of helper T lymphocytes. Nature 383, 787-793.

Ayroldi E., Cannarile L., Migliorati G., Nocentini G., Delfino D.V. and Riccardi C. (2012). Mechanisms of the anti-inflammatory effects of

glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways. FASEB J. 26, 4805-4820.

Bajestan M.N., Rajan A., Edwards S.P., Aronovich S., Cevidanes L.H.S., Polymeri A., Travan S. and Kaigler D. (2017). Stem cell therapy for reconstruction of alveolar cleft and trauma defects in adults: A randomized controlled, clinical trial. Clin. Implant Dent. Relat. Res. 19, 793-801.

Blashki D., Murphy M.B., Ferrari M., Simmons P.J. and Tasciotti E. (2016). Mesenchymal stem cells from cortical bone demonstrate increased clonal incidence, potency, and developmental capacity compared to their bone marrow-derived counterparts. J. Tissue Eng. 7, 2041731416661196.

Briot K. and Roux C. (2015). Glucocorticoid-induced osteoporosis. RMD Open. 1, e000014.

Cai Y., Liu T., Fang F., Xiong C. and Shen S. (2015). Comparisons of mouse mesenchymal stem cells in primary adherent culture of compact bone fragments and whole bone marrow. Stem Cells Int. 2015, 708906.

Cain D.W. and Cidlowski J.A. (2015). Specificity and sensitivity of glucocorticoid signaling in health and disease. Best Pract. Res. Clin. Endocrinol. Metab. 29, 545-556.

Chavassieux P., Buffet A., Vergnaud P., Garnero P. and Meunier P.J. (1997). Short-term effects of corticosteroids on trabecular bone remodeling in old ewes. Bone 20, 451-455.

Chen K., Li X., Li N., Dong H., Zhang Y., Yoshizawa M. and Kagami H. (2019). Spontaneously formed spheroids from mouse compact bone-derived cells retain highly potent stem cells with enhanced differentiation capability. Stem Cell International 2019, 8469012.

Corradetti B., Taraballi F., Powell S., Sung D., Minardi S., Ferrari M., Weiner B.K. and Tasciotti E. (2015). Osteoprogenitor cells from bone marrow and cortical bone: understanding how the environment affects their fate. Stem Cells Dev. 24, 1112-1123.

Drugs.com. (2019). Betamethasone Sodium Phosphate and Betamethasone Acetate https://www.drugs.com/pro/betamethasone-sodium-phosphate-and-betamethasone-acetate.html accessed Sept. 20.

Fernandez-Moure J.S., Corradetti B., Chan P., Van Eps J.L., Janecek T., Rameshwar P., Weiner B.K. and Tasciotti E. (2015). Enhanced osteogenic potential of mesenchymal stem cells from cortical bone: a comparative analysis. Stem Cell Res. Ther. 6, 203.

Fujii T., Kitaura H., Kimura K., Hakami Z.W. and Takano-Yamamoto T. (2012). IL-4 inhibits TNF-α-mediated osteoclast formation by inhibition of RANKL expression in TNF-α-activated stromal cells and direct inhibition of TNF-α-activated osteoclast precursors *via* a T-cell-independent mechanism in vivo. Bone 51, 771-780.

Gjerde C., Mustafa K., Hellem S., Rojewski M., Gjengedal H., Yassin M.A., Feng X., Skaale S., Berge T., Rosen A., Shi X.Q., Ahmed A.B., Gjertsen B.T., Schrezenmeier H. and Layrolle P. (2018). Cell therapy induced regeneration of severely atrophied mandibular bone in a clinical trial. Stem Cell Res. Ther. 9, 213.

Kagami H., Agata H., Inoue M., Asahina I. Tojo A., Yamashita N. and Imai K. (2014). The use of bone marrow stromal cells (bone marrow-derived multipotent mesenchymal stromal cells) for alveolar bone tissue engineering: basic science to clinical translation. Tissue Eng. Part. B Rev. 20, 229-232.

Kanazawa S., Fujihara Y., Sakamoto T., Asawa Y., Komura M., Nagata S., Takato T. and Hoshi K. (2013). Tissue responses against tissue-engineered cartilage consisting of chondrocytes encapsulated within non-absorbable hydrogel. J. Tissue Eng. Regen. Med. 7, 1-9.

Liu Y., Wang L., Kikuiri T., Akiyama K., Chen C., Xu X., Yang R., Chen W., Wang S. and Shi S. (2011). Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes *via* IFN-γ and TNF-α. Nat. Med. 17, 1594-1601.

Mori G., D'Amelio P., Faccio R. and Brunetti G. (2013). The interplay between the bone and the immune system. Clin. Dev. Immunol. 2013, 720504.

Riancho J.A., Zarrabeitia M.T. and Gonzalez-Macias J. (1993). Interleukin-4 modulates osteoclast differentiation and inhibits the formation of resorption pits in mouse osteoclast cultures. Biochem. Biophys. Res. Commun. 196, 678-685.

Tonti G.A. and Mannello F. (2008). From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera? Int. J. Dev. Biol. 52, 1023-1032.

Rasband W.S. (1997-2012). ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/.

Schneider C.A., Rasband W.S. and Eliceiri K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671-675.

Spiro A.S., Beil F.T., Schinke T., Schilling A.F., Eulenburg C., Rueger J.M. and Amling M. (2010). Short-term application of dexamethasone enhances bone morphogenetic protein-7-induced ectopic bone formation in vivo. J. Trauma. 69, 1473-1480.

van Roon J.A., Lafeber F.P. and Bijlsma J.W. (2001). Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis Rheum. 44, 3-12.

Weinstein R.S. (2012a). Glucocorticoid-induced osteonecrosis. Endocrine 41, 183-190.

Weinstein R.S. (2012b). Glucocorticoid-induced osteoporosis and osteonecrosis Endocrinol. Metab. Clin. North. Am. 41, 595-611.

Wong M.L. and Griffiths L.G. (2014). Immunogenicity in xenogeneic scaffold generation: antigen removal vs. decellularization. Acta Biomater. 10, 1806-1816.

Yokota K., Sato K., Miyazaki T., Kitaura H., Kayama H., Miyoshi F., Araki Y., Akiyama Y., Takeda K. and Mimura T. (2014). Combination of tumor necrosis factor α and interleukin-6 induces mouse osteoclast-like cells with bone resorption activity both in vitro and in vivo. Arthritis Rheumatol. 66,121-129.

Zakrzewski J.L., van den Brink M.R. and Hubbell J.A. (2014). Overcoming immunological barriers in regenerative medicine. Nat. Biotechnol. 32, 786-794.

Zhang Y., Li X., Chihara T., Mizoguchi T., Hori A., Udagawa N., Nakamura H., Hasegawa H., Taguchi A., Shinohara A. and Kagami H. (2015). Comparing immunocompetent and immunodeficient mice as animal models for bone tissue engineering. Oral Dis. 21, 583-592.

Zhu H., Guo Z.K., Jiang X.X, Li H., Wang X.Y., Yao H. Y., Zhang Y. and Mao N. (2010). A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. Nat. Protoc. 5, 550-560.

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