Bone formation in vivo induced by Cbfa1-carrying adenoviral vectors released from a biodegradable porous β-tricalcium phosphate (β-TCP) material

Toshimasa Uemura and Hiroko Kojima

Nanosystem Research Institute (NRI), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba Central-4, Tsukuba, Ibaraki 305-8562, Japan
E-mail: t.uemura@aist.go.jp

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Abstract
Overexpression of Cbfa1 (a transcription factor indispensable for osteoblastic differentiation) is expected to induce the formation of bone directly and indirectly in vivo by accelerating osteoblastic differentiation. Adenoviral vectors carrying the cDNA of Cbfa1/til-1(Adv-Cbf1) were allowed to be adsorbed onto porous blocks of β-tricalcium phosphate (β-TCP), a biodegradable ceramic, which were then implanted subcutaneously and orthotopically into bone defects. The adenoviral vectors were released sustainingly by biodegradation, providing long-term expression of the genes. Results of the subcutaneous implantation of Adv-Cbfa1-adsorbed β-TCP/osteoprogenitor cells suggest that a larger amount of bone formed in the pores of the implant than in the control material. Regarding orthotopic implantation into bone defects, the released Adv-Cbfa1 accelerated regeneration in the cortical bone, whereas it induced bone resorption in the marrow cavity. A safer gene transfer using a smaller amount of the vector was achieved using biodegradable porous β-TCP as a carrier.

Keywords: Cbfa1, β-TCP, adeno virus, DDS

1. Introduction
Genetic engineering for gene therapy has attracted much attention among biologists and clinical doctors because cell sources for tissue engineering are limited and cannot be controlled after implantation. Viral vectors such as adenoviruses and retro viruses are effective transducers of genes to cells. From this viewpoint, studies on the transfection of viral vectors encoding the genes for BMPs, TGF-β and Cbfa1 [1–7] have yielded good results, although clinical applications using mesenchymal stem cell (MSC)-derived cells might be limited by government restrictions in individual countries. A simple method to regenerate bone is the injection of gene transducers such as adenoviruses or retroviruses; however, the injected material is easily dispersed in vivo.

Drug delivery systems (DDSs) using viral vectors as carriers are useful for solving these problems. The carriers should be biodegradable, allowing long-term and effective release. Moreover, the carrier material should be mechanically strong so it can be implanted into bone orthotopically. In this work, we have examined a DDS using β-tricalcium phosphate (β-TCP) as a carrier of adenoviral vectors encoding cbfa1 (Adv-Cbfa1).

Cbfa1 is a key osteogenic transcription factor; it is indispensable for osteoblastic differentiation using Cbfa1-knockout mice, which is completely lacking osteoblasts [8,9]. We have proven that the overexpression of Cbfa1 in rat bone marrow-derived osteoprogenitor cells markedly promoted their rapid differentiation into osteoblasts [5]. The release of Adv-Cbfa1 from β-TCP is expected to stimulate surrounding MSCs to differentiate into osteoblasts.
In this study, adenoviral vectors carrying the cDNA of Cbfa1/til-1 (Adv-cbfa1) were allowed to be adsorbed onto porous blocks of β-TCP, a biodegradable ceramic material, which were then implanted subcutaneously and orthotopically into bone defects. A safer gene transfer using a smaller amount of the vector was achieved using the biodegradable β-TCP as a carrier.

2. Experimental details

2.1. Cells and culture

Experiments were performed in accordance with the guidelines established by the Japanese Government for the care and use of laboratory animals. Rat-bone-marrow-derived osteoprogenitor (RBMO) cells were obtained from the femora of male Fischer 344 (6-week-old) rats using the method of Maniotopoulos et al. [10]. The epiphyses of both sides were removed and the bone marrow cavity was flushed with medium A (Minimal essential medium (MEM) from Nakalai Tesque, Kyoto, Japan, containing 15% fetal bovine serum (FBS) from Sigma, St. Louis, MO, and antibiotic-antimycotic from Gibco, Gland Island, NY) using a syringe. The primary cells were cultured at 37°C in a humidified atmosphere of 95% air with 5% CO₂ until nearly confluent. Subsequently, they were treated with 0.1% trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA), and subcultured under appropriate conditions.

2.2. Construction of recombinant adenoviral vector carrying Cbfa1/til-1 gene

From the total RNA isolated from mouse osteoblasts, cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase. The cDNA of Cbfa1/til-1 was obtained by polymerase chain reaction (PCR) amplification using 5'-ATGCTTCATTCGCCTCACAAAC-3' as the sense primer and 5'-TCTGTTTGGCGGCCATATTGA-3' as the antisense primer specific to cDNA of Cbfa1/til-1 (AF010284). Cbfa1 cDNA was prepared using the thymine-adenine (TA) cloning vector (pCRII-TOPO, Invitrogen). Cbfa1 cDNA was cut with SpeI and EcoRV, blunted, and inserted into the SwaI site of the cosmid vector pAxCALNLw using an Adenovirus Cre/loxP kit (Takara Shuzo Co. Ltd, 6151). The recombinant adenosine was prepared in accordance with the manual of the kit. The virus had a titer of about 10^9 pfu ml^{-1}, and a high infection rate. The recombinant adenoviruses (Adv-til-1) were isolated, and the insertion of each cDNA was confirmed by digestion using restriction enzymes.

2.3. Subcutaneous implantation of β-TCP porous blocks with adsorbed Adv-Cbfa1

The preparation method was described in detail in our previous publication [11]. Porous β-TCP blocks (2 × 2 × 2 mm³, Olympus) were kindly donated by Olympus Optical Co., Ltd. Their physical properties are almost the same as those of Osferion commercially available from Olympus Terumo Biomaterials Co. Ltd. Pores are uniformly distributed at 75% porosity and their average diameters are 200–400 µm. Almost all the pores are interconnected via 100–200 µm paths. The compressive strength of the material is about 2 MPa. The blocks were soaked for 3 h in a solution containing a mixture of recombinant adenoviruses carrying Cbfa1 and Cre recombinase genes (1 × 10^9 pfu ml^{-1}). RBMO cells were harvested by trypsin treatment, adjusted to a density of 1 000 000 cells ml^{-1}, and allowed to be adsorbed onto the blocks under a reduced pressure of 100 mmHg. Treated and untreated blocks were subcutaneously transplanted into the backs of rats. The blocks were removed 3 weeks later and observed by hematoxylin/eosin staining. Figure 1(a) schematically shows the experimental procedure of the subcutaneous transplantation.

2.4. Orthotopic implantation of Adv-Cbfa1 adsorbed β-TCP

Figure 2 shows the procedure used for the orthotopic implantation of Adv-Cbfa1-adsorbed β-TCP porous blocks into rat femurs. Adv-Cbfa1-adsorbed blocks with or without rat-bone-marrow-derived osteoprogenitor (RBMO) cells were implanted for comparison.

For the experiment using simple Adv-Cbfa1-adsorbed materials, β-TCP blocks (2 × 2 × 2 mm³, Olympus Optical Co. Ltd) were soaked for 3 h in a mixed solution of recombinant adenoviruses carrying the Cbfa1 and Cre...
recombinase genes \((1 \times 10^9 \text{ pfu ml}^{-1})\). For transplantation experiment using the composite of Adv-Cbfa1-adsorbed materials and RBMO cells, \(\beta\)-TCP blocks were soaked for 3 h in a mixed solution of recombinant adenoviruses carrying the Cbfa1 and Cre recombinase genes \((1 \times 10^9 \text{ pfu ml}^{-1})\). The RBMO cells were harvested by trypsin treatment, adjusted to a density of 1 000 000 cells ml\(^{-1}\), and allowed to adsorb onto the blocks under a reduced pressure of 100 mmHg.

For the orthotopic implantation of the Adv-Cbfa1-adsorbed materials, the lateral side of the distal end of the femur shaft was exposed and incised surgically. Bilateral bone defects were formed by drilling a hole 2 mm in diameter and 3–4 mm deep on the coronal face at the distal end of each femur. The 2 × 2 × 2 mm blocks were implanted into these holes (right side, Adv-Cbfa1- and Adv-Cre-infected group; left side, control group), and the wounds were sutured. For the experiment with the composite of Adv-Cbfa1-adsorbed materials and RBMO cells, \(\beta\)-TCP blocks were soaked for 3 h in a mixed solution of recombinant adenoviruses carrying the Cbfa1 and Cre recombinase genes \((1 \times 10^9 \text{ pfu ml}^{-1})\). The RBMO cells were harvested by trypsin treatment, adjusted to a density of 1 000 000 cells ml\(^{-1}\) and allowed to adsorb onto the blocks under a reduced pressure of 100 mmHg. The 2 × 2 × 2 mm\(^3\) blocks were implanted into these holes (right side, Adv-Cbfa1- and Adv-Cre-infected group; left side, control group), and the wounds were sutured. After 2 and 6 weeks, the blocks were harvested for histological analysis.

2.5. Histological analysis

Harvested blocks were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in phosphate buffered saline (PBS) with exposure to microwaves. They were then decalcified in 10% EDTA and 100 mM Tris (pH 7.4) for about 1 week, dehydrated with ethanol, and embedded in paraffin. Paraffin sections (5 \(\mu\)m) were prepared, deparaffinized, and stained with hematoxylin-eosin.

3. Results and discussion

3.1. Subcutaneous implantation

Figure 1(b) shows the results of hematoxylin-eosin staining obtained 3 weeks after the subcutaneous transplantation of \(\beta\)-TCP blocks carrying Adv-Cbfa1 and Adv-Cre with RBMO cells into the back. Bone regeneration was observed at the center of the virus-containing block (figure 1(b): upper right, lower right), in which osteocytes were clearly observed. In uncalcified regions, there existed osteoblast-like cells forming a bone matrix. Although osteoblast-like cells were observed in the untreated block, ossification did not proceed (figure 1(b): lower left), and immature bone was found in small areas of pores.

The results clearly indicate that Adv-Cbfa1 strongly activated not only the cells adhered to \(\beta\)-TCP, but also the cells away from the surface of the blocks. The adsorbed Adv-Cbfa1 were probably gradually released from \(\beta\)-TCP and activated almost all the cells in the pores. It was confirmed that Adv-Cbfa1 activated the RBMO cells without the help of the bone tissue at subcutaneous sites.

3.2. Orthotopic implantation

Figure 3(a) shows the results of hematoxylin-eosin staining 2 weeks after the transplantation of \(\beta\)-TCP blocks into femur bone defects. The degree of resorption was greater in pores of the Adv-Cbfa1-adsorbed blocks and ossification had progressed around the pores (figure 3(a): lower left, lower right).

The resorption and ossification were more advanced in the blocks onto which osteoprogenitor cells were added.
Figure 3. Hematoxylin-eosin staining of orthotopically implanted \(\beta\)-TCP blocks with or without osteoprogenitor (RBMO) cells in rat femur bone defects. Images were obtained 2 weeks (a) and 6 weeks (b) after implantation. Bone formation is indicated by yellow ellipses and the unregenerated gap on the cortical bone is indicated by yellow arrows. Scale bar is 1 mm.

Bone regeneration was more rapidly induced by adding RBMO cells to blocks with the Cbfa1-gene-carrying virus. Images taken 2 weeks after the implantation showed marked differences between the blocks with and without RBMO cells—dissolution of the blocks and ossification around the pores were observed in the presence of RBMO cells. Images obtained after 6 weeks showed the ossification of almost all of the remaining blocks. From the observed regeneration of the bone and bone marrow, the addition of RBMO cells together with the virus was found to enhance the effects of the Cbfa1 gene. A viral vector with cDNA of Cbfa1 was allowed to be adsorbed onto \(\beta\)-TCP as a biodegradable ceramic material, which was implanted into bone defects. The sustained release of Cbfa1 promoted bone regeneration.

3.3. Discussion

Transcription factors associated with stem cells have attracted much attention among not only cell biologists but also...
clinicians, particularly since Takahashi et al. reported that induced pluripotent stem (iPS) cells were generated from adult human fibroblasts transfected with four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) [12]. The idea of transfecting with transcription factors to promote the proliferation and differentiation of target cells is not new; however, appropriate carrier materials have not been systematically investigated.

The transfection of Cbfa1 into MSCs strongly affects osteoblastic differentiation from MSCs in vitro and in vivo [5,13]. Moreover, the clinical application of MSC-derived cells is limited by laws of individual countries. An easy method of regenerating bone is a simple injection of gene transducers such as adenoviruses or retroviruses. DDSs using viral vectors as carriers are useful for solving these problems [14]. The carriers should be biodegradable, allowing long-term and effective release.

The carrier materials should have both high mechanical strength and the potential to effectively deliver transfection molecules. Porous hydroxyapatite, β-TCP, and α-TCP are typical calcium phosphate materials with a significant mechanical strength. Porous hydroxyapatite has a good strength for bone regeneration, but is poorly biodegradable. Although mechanically strong, it is not expected to release molecules effectively. Porous β-TCP is weaker, but is biodegradable and appropriate for the release of molecules. According to our comparisons between porous hydroxyapatite and β-TCP (data not shown), the latter has a greater potential for the sustained release of Adv-Cbfa1 inducing rapid ossification in pores. It is difficult to estimate the amount of Adv-Cbfa1 on the surface of β-TCP materials. The advantage of ossification on the β-TCP material suggests that the adsorbed Adv-Cbfa1 is easier to release from β-TCP owing to the difference in adsorption and biodegradability between β-TCP and hydroxyapatite.

Regarding orthotopic implantation shown in figures 3(a) and (b), control data reveal a larger amount of bone was regenerated with RBMO cells than without them; however, without RBMO cells, bone regeneration progressed more slowly in the presence of the already existing stem cells. In the case of using Adv-Cbfa1 adsorbed β-TCP with or without RBMO, the TCP with RBMO cells regenerated a much larger amount of bone than without them, indicating that the effect of Adv-Cbfa1 on RBMO cells is dominant, however, it was difficult to distinguish the new bone originating from RBMO cells from that originating from already existing stem cells.

Furthermore, rapid regeneration of the cortical bone and rapid loss of the blocks in the medullary cavity were observed when porous β-TCP blocks were implanted, as shown in figure 3. Less regeneration in cortical bone and a slower loss of material were observed in the case of using porous hydroxyapatite materials. These findings clearly show the advantages of β-TCP as a carrier of Adv-Cbfa1, as reported in our previous work [5]. It is true that Cbfa1 accelerated the bone regeneration; however, in the bone cavity, osteoclasts induced the resorption of TCP. The mechanism of material resorption in the cavity is as yet unclarified and might be related to self-repair mechanisms.

Although porous β-TCP is weaker than hydroxyapatite, its compressive strength gradually recovers with degradation and replacement by newly formed bone [11,15]. Several applications of β-TCP materials as carriers are expected.

4. Conclusions

Adenoviral vectors carrying cDNA of Cbfa1/til-1 were allowed to be adsorbed onto porous blocks of β-TCP, which were then implanted subcutaneously and orthotopically into bone defects. Results of the subcutaneous implantation suggest that a larger amount of bone formed in the pores of the implant than in the control material. Regarding orthotopic implantation, Adv-cbfa1 accelerated bone regeneration in the cortical bone, whereas it induced bone resorption in the bone marrow cavity. A safer gene transfer with a smaller amount of a vector can be achieved using biodegradable β-TCP as a carrier.

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References

[1] Cheng S L, Lou J, Wright N M, Lai C F, Avioli L V and Riew K D 2001 Calcif. Tissue Int. 68 87
[2] Breitbart A S, Grande D A, Mason J M, Barcia M, James T and Grant R T 1999 Ann. Plast. Surg. 42 488
[3] Franceschi R T, Wang D, Krebsbach P H and Rutherford R B 2000 J. Cell Biochem. 78 476
[4] Mehrara B J, Saadeh P B, Steinbrech D S, Dudziak M, Spector J A, Greenwald J A, Gittes G K and Longaker M T 1999 J. Bone Miner. Res. 14 1290
[5] Kojima H and Uemura T 2005 J. Biol. Chem. 280 2944
[6] Han D, Li J and Guan X 2010 Connect. Tissue Res. 51 274
[7] Chang S C, Lin T M, Chung H Y, Chen P K, Lin F H, Lou J and Jeng L B 2009 Neurosurgery 65 75
[8] Komori T et al 1997 Cell 89 755
[9] Otto F et al 1997 Cell 89 765
[10] Maniatisopoulos C, Sodek J and Melcher A H 1988 Cell Tissue Res. 254 317
[11] Dong J, Uemura T, Shirasaki Y and Tateishi T 2002 Biomaterials 23 4493
[12] Takahashi K, Tanabe K, Ohmuki M, Narita M, Ichisaka T, Tomoda K and Yamanaka S 2007 Cell 131 861
[13] Phillips J E and Garcia A J 2008 Methods Mol. Biol. 433 333
[14] Schek R M, Wilke E N, Hollister S J and Krebsbach P H 2006 Biomaterials 27 1160
[15] Uemura T, Dong J, Wang Y, Kojima H, Saito T, Iejima D, Kikuchi M, Tanaka J and Tateishi T 2003 Biomaterials 24 2277