Use of an osteoinductive biomaterial as a bone morphogenetic protein carrier

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A porous calcium phosphate ceramic, which induced bone formation in soft tissues of dogs, was termed as osteoinductive biomaterial and studied as a carrier of bone morphogenetic protein (rhBMP-2). Cylinder implants (∅ 4 × 5 mm) impregnated with 0, 1, 10 and 40 μg rhBMP-2 were implanted in dorsal muscles of rabbits for five weeks. Histological observation and histomorphometric analysis were performed on thin un-decalcified sections. No bone formation was detected in the implants without rhBMP-2, while mature lamellar bone was found inside the implants with 1 μg rhBMP-2, both on the outer surface and inside the implants with 10 μg and 40 μg rhBMP-2. Little difference in formed bone was found between 1 μg and 10 μg rhBMP-2, but no difference was found between 10 μg and 40 μg rhBMP-2. A significant difference in bone marrow formation was found among 1, 10 and 40 μg rhBMP-2. The more rhBMP-2, the more bone marrow formed. The present results indicate that osteoinductive biomaterial is a good carrier of BMP and high dose of BMP is not necessary for bone formation in clinic.

1. Introduction

Through the investigation of bone induction by decalcified bone matrix in soft tissues, proteins related to bone induction were suggested [1]. Later on, a protein that was termed as bone morphogenetic protein (BMP) was discovered [2] and purified [3]. Now different types of human BMP have been obtained by recombination [4, 5].

A lot of knowledge about bone induction (osteoinduction), bone biology and bone physiology have been obtained from the studies on BMPs. BMPs belong to the transforming growth factor-β superfamily. They have more than 13 subtypes [5, 6]. Among them rhBMP-2, rhBMP-3, rhBMP-4 and rhBMP-7 (OP-1) have evident osteoinductivity and can induce cartilage and *de novo* bone formation in soft tissues [7]. Native BMPs, which are combinations of several subtypes of BMPs, are more active than single recombinant BMP in bone induction [8]. Only small amount of BMPs exist in postnatal life and play a role in bone regeneration of bone injury such as bone fractures. BMPs have other functions than bone induction and even play their roles in the nerve system [9].

Because of its osteoinductivity, BMP has been suggested to be a useful tool for bone repair in large bone defects, non-union and spine fusion [5, 10]. The highly purified native BMPs and the recombinant human BMPs provided the possibility for clinical use of BMPs [5, 11]. However, a carrier is necessary for the clinical application of BMPs, since the highly purified BMPs and recombinant BMPs are water-soluble. Without carriers, they diffuse away quickly *in vivo*, and thus preventing a relative high local BMP concentration as required for bone induction [5, 7].

Different biomaterials have been tested as the carrier of BMPs with regard to their clinically required shapes, their biocompatibility, the required dosage of BMPs, the retention of BMPs in the carriers, the retained release of BMPs and the safety of BMPs [6, 12, 13]. At the moment, no clear preference can be given to polymers [6, 13–15], calcium phosphate ceramics [16–22], bioglass ceramics [23, 24], metals [25, 26], demineralized bone matrix [11] or collagen [10].

In recent years, specific calcium phosphate biomaterials, which induced bone formation in soft tissues of special animal models, have been reported [27–43]. The mechanism of bone induction by calcium phosphate biomaterials was not clear yet and the clinical significance of these osteoinductive calcium phosphate biomaterials was not explored. However, it was supposed that calcium phosphate biomaterials concentrated native BMP *in vivo* for their bone induction [8, 34, 39, 43]. This explanation suggests osteoinductive calcium phosphate biomaterials to be an effective carrier of BMPs.

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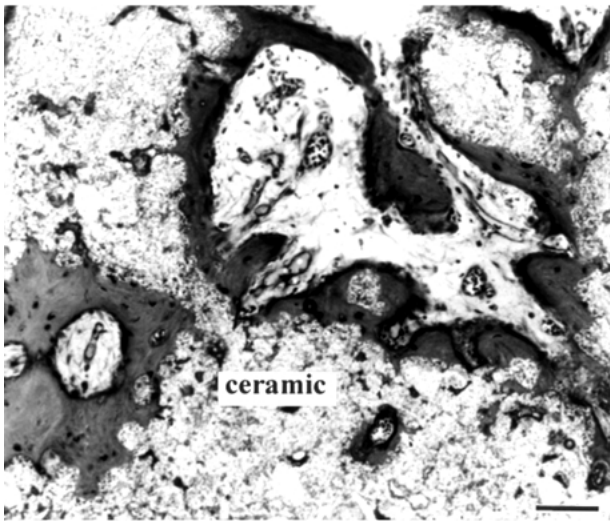

Figure 1 Bone induction by calcium phosphate ceramic in muscle of dogs (60 days). (Un-decalcified section, methylene blue and basic fuchsin staining, bar = 200 μm).

In this study, calcium phosphate ceramic, which induced bone formation in the muscles of dogs [43], was termed as osteoinductive biomaterial and investigated as a carrier of recombinant human bone morphogenetic protein-2 (rhBMP-2) in rabbits in which bone induction by calcium phosphate biomaterials was seldom observed even for a long time (more than three months) [35, 37].

2. Materials and methods

Porous calcium phosphate ceramic with microporous structures, which consisted of hydroxyapatite (HA) and a trace of α-tricalcium phosphate (α-TCP), was obtained by sintering green body (formed with 5% H_2O_2 from wet-synthetic calcium phosphate apatite powder) at 1150 °C for 3 h. The osteoinductivity of this calcium phosphate ceramic was tested in dogs, and bone formation was found in two months (Fig. 1) (details were reported elsewhere [43]).

Ceramic cylinders (∅ 4 × 5 mm) were made, cleaned and dried. RhBMP-2 solutions with the concentration of 4 mg/ml, 1 mg/ml and 0.1 mg/ml were prepared by dissolving rhBMP-2 (Genetics Institute, Cambridge, MA) in sterile distilled water. Four ceramic cylinders were coated each with 10 μl 4 mg/ml rhBMP-2 solution, four ceramic cylinders each with 10 μl 1 mg/ml rhBMP-2 solution and four ceramic cylinders each with 10 μl 0.1 mg/ml rhBMP-2 solution. Ceramic cylinders coated with rhBMP-2 solution were dried at room temperature. Ceramic cylinders without rhBMP-2 were used as controls. The implants, both rhBMP-2 treated and controls, were sterilized with ethylene oxide gas before implantation.

Surgical operation was made on four adult male rabbits (2.5–3.0 kg) under general anaesthesia and sterile condition. In each rabbit, four ceramic cylinders (one ceramic cylinder as control, one with 1 μg rhBMP-2, one with 10 μg rhBMP-2 and one with 40 μg rhBMP-2) were implanted in dorsal muscles. In each side of the dorsal muscles, two implants were implanted at a distance of more than 2 cm.

Five weeks after implantation, the animals were sacrificed. Implants were harvested with surrounding tissues and fixed in 4% buffered formaldehyde (pH = 7.4). The fixed implants were washed with phosphate buffer solution (PBS), dehydrated with series ethanol solution and embedded in methylmethacrylate (MMA). Thin un-decalcified sections (10–20 μm) were made and stained with methylene blue and basic fuchsin for histological observation. Backscattered scanning electron microscopy observation (BSE) was made on some sections coated with carbon (Philips SEM 525).

The formed bone and bone marrow were measured with histomorphometric analysis (KS400 image system, Zeiss). In each section, three images on a crossline were measured and measurement was performed on four sections of each implant. The ratio of bone to macropore area and the ratio of bone marrow to macropore area were calculated from each section which was taken as a sample. With regard to the effect of rhBMP-2 concentration on bone formation and bone marrow formation, paired student *t*-test was made.

3. Results

Mature lamellar bone was found in all implants treated by rhBMP-2 (4 in 4 with 1 μg, 4 in 4 with 10 μg and 4 in 4 with 40 μg), but no bone formation in controls (0/4) at day 35 (Fig. 2). Gross observation showed the difference of bone formation among the treated implants with different concentration of rhBMP-2. In the implants with 1 μg rhBMP-2, bone formation occurred only inside the implants, but bone mass was big. Besides bone, a little amount of bone marrow and a large amount of fibrous tissues were observed in the pores of the implants (Fig. 2B). In the implants with 10 μg (Fig. 2C) and 40 μg rhBMP-2 (Fig. 2D), besides the bone formation inside the implants, a layer of bone was found on the outer surface. Large amount of bone marrow was found. Fibrous tissues decreased in the pores of implants with 10 μg rhBMP-2 (Fig. 2C) and no fibrous tissue was found inside the pores of implants with 40 μg rhBMP-2 (Fig. 2D), compared to that in the implants with 1 μg rhBMP-2 (Fig. 2B).

No cartilage was found. Bone formation was dependent on the pore surface and started directly as bone (Fig. 2). The formed bone contacted directly the surface of calcium phosphate ceramic implants as shown in both the histological observation (Fig. 2) and BSE observation (Fig. 3).

As shown in Fig. 4, 14.5% pore area was covered with bone in implants with 1 μg rhBMP-2, 24.0% in implants with 10 μg rhBMP-2 and 20.0% in implants with 40 μg rhBMP-2. Student *t*-test was made with regard to the difference among different rhBMP-2 concentration. Little difference of bone was found between 1 μg and 10 μg ($p < 0.005$), but no significant difference was found between 10 μg and 40 μg ($p > 0.010$. A significant difference in bone marrow was found between 1 μg and 10 μg rhBMP-2 ($p < 0.0005$), between 10 μg and 40 μg rhBMP-2 ($p < 0.005$). The more rhBMP-2 used, the more bone marrow formed.

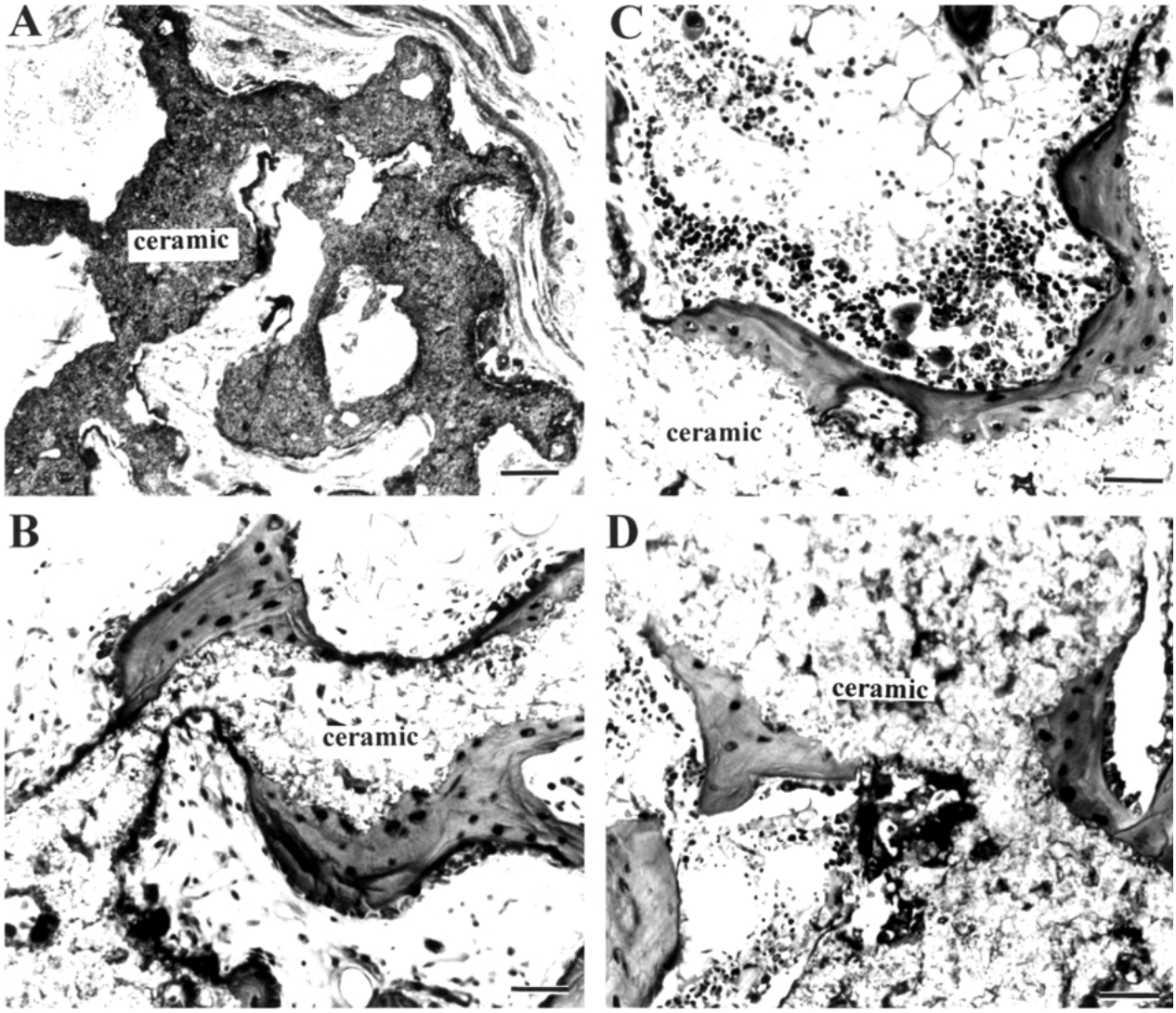

Figure 2 Bone formation in calcium phosphate ceramic with rhBMP-2 in muscles of rabbits (35 days). A, Control without rhBMP-2, bar = 200 μm; B, 1 μg rhBMP-2, bar = 100 μm; C, 10 μg rhBMP-2, bar = 100 μm; D, 40 μg rhBMP-2, bar = 100 μm. (Un-decalcified section, methylene blue and basic fuchsin staining).

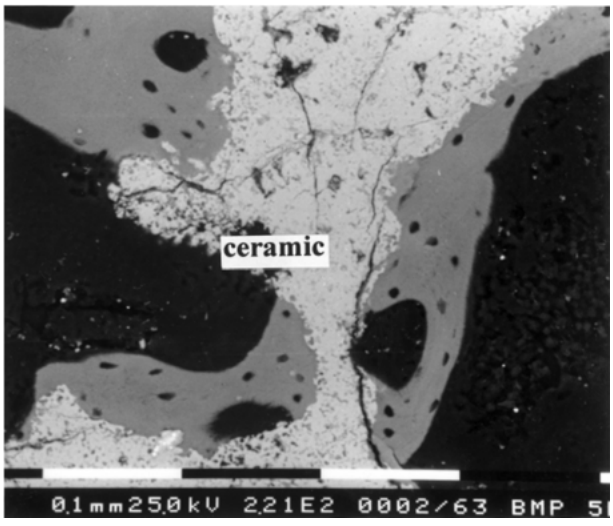

Figure 3 BSE image of bone in calcium phosphate ceramic in muscle of rabbits with 10 μg rhBMP-2 (35 days).

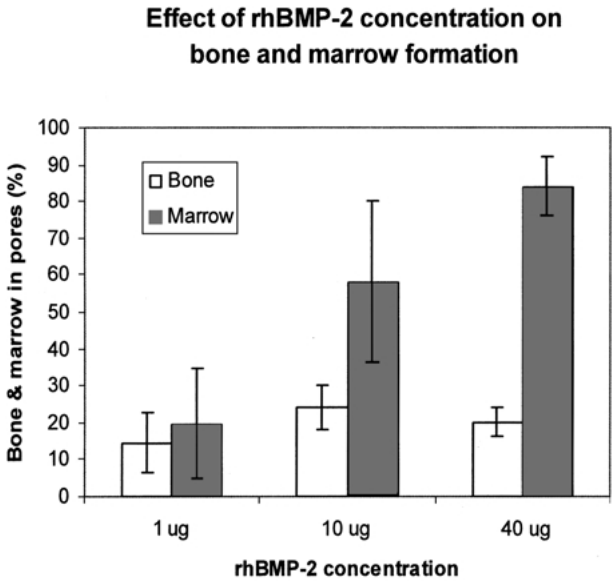

Figure 4 Effects of rhBMP-2 concentration on bone formation and bone marrow formation.

4. Discussion

In agreement with our previous results [35], bone induction by calcium phosphate ceramic is animal-dependent. Although porous calcium phosphate ceramic induced bone formation in the muscles of dogs in 60 days, no bone formation was found when calcium phosphate ceramic cylinders were implanted in the muscles of rabbits at day 35. However, 1 μg rhBMP-2 induced a large amount of bone formation in rabbits in this study. The results indicate that osteoinductive calcium phosphate ceramic is an effective carrier of BMPs.

Not only for the cost of BMPs, but also for the safety of BMPs in clinical use, the dosage of BMP for bone formation, especially the lowest concentration of BMP in biomaterials, is a matter of concern, because BMPs may play roles even in central nervous system [9]. The lowest concentration of BMPs varied in different studies. Sixteen μg rhBMP-2 ml^{-3} in fibrous glass membrane induced bone formation under subcutis of rats [23]; 50 μg rhBMP-2 ml^{-3} in collagen induced random bone formation in the muscles of rats and monkeys [44]; 510 μg rhBMP-2 ml^{-3} in polymer sponge induced bone formation in rats [14]; 2870 μg rhBMP-2 ml^{-3} in biphasic calcium phosphate ceramic (BCP) resulted in spinal fusion of monkeys [16]. In one article 0.5 μg rhBMP-2 was used to induce bone formation when demineralized bone matrix (DBM) was used as carrier in rats and mice, but the size of the implants and thus the concentration of rhBMP-2 was not clear [11]. In this study, 16 μg rhBMP-2 ml^{-3} in osteoinductive calcium phosphate ceramic induced bone that covered 14.5% pore area at day 35. For histologically detectable bone formation, less rhBMP-2 can be used. Based on the results of the present study and the fact that the dose of BMPs for bone formation is much higher in rabbits than that in rats [45], a conclusion can be made that, for bone formation, osteoinductive calcium phosphate ceramic requires the lowest dose of BMPs.

The roles of BMP carriers were emphasized as the retention of BMPs, the sustained release of BMPs, the substrates for the ingrowth of osteogenic precursors in soft tissues and the template for bone formation [13]. While the promotion of bone formation by special BMP carriers themselves was ignored, although the promotion was so evident in some studies [19].

As reported by Urist in 1984, the bone formation induced by a composite of β-tricalcium phosphate ceramic (β-TCP) and 1 mg bBMP was 12 times more than that induced by 1 mg bBMP in gelatin capsule when tested in muscles of mice [19]. The reason for the enhancement of BMP-induced bone formation by β-TCP ceramic was not clear at that time, but our research on osteoinduction of calcium phosphate biomaterials suggested that β-TCP, being osteoinductive in dogs [38, 42], may be, at least partly responsible for the large bone formation in the composite of β-TCP and bBMP in Urist's study. The results shown in present study indicate that osteoinductive biomaterial is a good carrier of BMPs, and the combination of calcium phosphate ceramic and BMPs was very effective for bone formation in soft tissues.

Bone incidence induced by calcium phosphate biomaterials was higher in dogs, pigs, monkeys and baboons than that in rodents [34, 35]. In rabbits, the incidence of bone induction by calcium phosphate biomaterials was less, but bone induction by calcium phosphate biomaterials was also found at longer implantation time [37]. In this study, bone formation did not occur in calcium phosphate ceramic at day 35 in rabbits, but, as shown in dogs, the porous calcium phosphate ceramic had basically the capability to induce bone formation and thus a little rhBMP-2 triggered the bone induction in rabbits. It is likely that the bone formation found in this study in rabbits resulted from the osteoinductivity of both rhBMP-2 and calcium phosphate ceramic.

BMP induced bone formation is usually dose-dependent when other materials without osteoinductivity was used as the carriers. The more BMP, the more bone formed [46]. However, the bone formation in this study was not dependent on the amount of rhBMP-2 used when osteoinductive calcium phosphate ceramic was used as the carrier. Little difference in bone formation was found between 1 μg and 10 μg ($p < 0.005$), and no significant difference was found between 10 μg and 40 μg ($p > 0.01$). The reason for this phenomenon was not clear, but some explanations can be found in the chemical adsorption of BMPs in calcium phosphate biomaterials. When applied onto calcium phosphate ceramic, BMPs were stored in calcium phosphate ceramic by physical adsorption and chemical binding. *In vivo*, a burst release of the physically adsorbed BMPs occurred at first (within minutes and hours) and the sustained release of chemical bound BMPs occurred later due to the replace of the BMP binding site by other proteins [6, 13]. Certainly, the binding sites for BMPs are fixed in a biomaterial. When all binding sites were occupied by BMPs, the surplus of BMPs was stored physically and released at early implantation time. The early released BMPs give no bone formation, thus the formed bone is dependent on the functional BMPs that were chemically absorbed in calcium phosphate ceramic, not on the total amount of BMPs applied. There might be no difference in the chemically absorbed rhBMP-2 in the implants with 1 μg, 10 μg and 40 μg rhBMP-2, while the dose-dependent bone marrow formation indicated that it was not true. Another explanation may be possible. Bone formation induced by BMPs and by calcium phosphate biomaterials went different processes. BMP-induced bone formation started normally as cartilage and the amount of formed bone was determined by the amount of BMPs. Calcium phosphate-induced bone formation started as bone directly and had its own homeostasis [43]. The induced bone by calcium phosphate biomaterials finally remained stable in the implants. It is likely that in this study, calcium phosphate ceramics determined the intramembrous ossification and the amount of the formed bone, rhBMP-2 enhanced calcium phosphate ceramic induced bone formation in rabbits.

Different biomaterials have been tested as the carriers of BMPs. Different phenomenon was found when an osteoinductive calcium phosphate ceramic was used as the carrier of rhBMP-2 in this study due to the osteoinductivity of the biomaterial itself. It should be

noted that osteoinduction of calcium phosphate biomaterials is material-dependent. Although many calcium phosphate biomaterials have been used as BMP carriers [16–22], comparison cannot be made because not all calcium phosphate biomaterials were osteoinductive [38, 40]. In some cases, composites of hydroxyapatite ceramic and bone morphogenetic proteins did not give bone formation and additional collagen must be needed [17].

The dosage of BMPs for clinical use is very important, but it is not easy to find a suitable dosage of BMPs for clinical use because of the dose-dependent bone formation by BMPs as reported before and also because as much as 100 times BMPs were needed for therapeutic benefit in large, long-lived animals than in rodents [47]. When osteoinductive calcium phosphate ceramic was used as a carrier, bone formation was not totally dependent on the amount of BMP and the higher dose was not necessary for bone formation. As shown in this study, 16–160 μg rhBMP-2 ml^{-3} can be selected as the experimental dose in rabbit model. For a 1 cm^3 implant, only 16–160 μg rhBMP-2 is needed. For large animal model like dog, monkey and baboon, the same dosage or even less rhBMP-2 can be selected, because from rodent to dog to monkey to baboon, incidence of bone induction by calcium phosphate biomaterials becomes higher. The dose is not so high, and it assures the safety of BMPs in clinic.

Osteoinduction of calcium phosphates was not generally accepted, and it was ruled out as a general thought [48, 49], but increasing evidence suggested that osteoinductive biomaterials do exist [27–43] and calcium phosphate ceramic even induced bone formation in humans [50]. It is difficult to estimate the clinical significance of osteoinductive biomaterials alone, because the bone induction is animal-dependent and bone formation needs a longer time (>30 days). However, as shown in this study, when combined with rhBMP-2, bone formation occurred easily even in soft tissues. The combination of osteoinductive calcium phosphate biomaterials and BMPs may make bone repair more effective in clinic.

5. Conclusion

The results presented here suggest that osteoinductive calcium phosphate ceramic, which showed bone induction in muscles of dogs, is an effective carrier of BMPs. Low dose of rhBMP-2 as 1 μg (equal to 16 μg rhBMP-2 ml^{-3} implant) induced a large amount of bone formation. Moreover, contrary to that in other BMP carriers, the formed bone was not totally dependent on the dose of BMPs. The present results indicate that a high dose of rhBMP-2 is not necessary for bone formation and may provide the possibility to use BMPs clinically at lower dosage, more effectively and safely.

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