Effects of the hierarchical macro/mesoporous structure on the osteoblast-like cell response

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of record](#). Please cite this article as [doi:10.1002/jbm.a.36387](https://doi.org/10.1002/jbm.a.36387).

Abstract

To improve the success of medical devices, implants with strong surface bioactivity are urgently required. Coatings with a macroporous structure produced by micro-arc oxidation (MAO) possess advantages, such as strong adhesion to substrate and excellent resistance to wear and corrosion. Mesoporous structures contain pores with sizes of 2–50 nm, which can endow the biomaterials with the ability to enhance osteogenesis and to be loaded with diverse drugs. Thus, in this study, we aimed to evaluate the effects of both macroporous and mesoporous structures using a hierarchical macro/mesoporous structure to modify the titanium implant surface. The behaviors of SaOS-2 human osteosarcoma cells on the macro/mesoporous structure, including initial adhesion, proliferation, alkaline phosphatase (ALP) activity, and collagen secretion, were investigated. Cells that attached on the macro/mesoporous surface showed the highest cell numbers and greatest spreading area after incubation for 1, 2, and 4 h compared with the polished smooth substrate and macroporous surface in the presence of fetal bovine serum (FBS). However, in the absence of FBS, cell adhesion on the polished substrate, macroporous structure, and macro/mesoporous structure did not differ significantly. Cell proliferation on the macro/mesoporous structure increased compared with that on other surfaces. Furthermore, ALP activity and collagen secretion were enhanced on the macro/mesoporous structure. Our findings provided important insights into the cellular responses to macro/mesoporous structures in the field of implant surface modification.

Keywords: bone regeneration; micro/nanostructure; macro-arc oxidation; mesoporous film; titanium implant

1. Introduction

Cell responses, such as cytoskeletal organization, proliferation, apoptosis, and differentiation, mainly depend on the surface properties and chemical composition of the implant [1]. Micro-arc oxidation (MAO) is employed to modify the titanium implant surface because it can produce an *in situ* ceramic coating on substrate valve metals. This ceramic coating shows strong adhesion to the substrate and excellent resistance to wear and corrosion [2, 3]. MAO is a simple, easily controlled, relatively inexpensive method that is ideally suited to modification of the surface of implants with complex shapes in comparison with other techniques [4]. Additionally, the MAO method can generate micro- or sub-microscale topographies on the titanium implants.

These microstructures significantly modulate cellular behavior *in vitro* and enhance the osteogenic activities of bone-forming cells *in vivo* [5]. Accordingly, the MAO method has many practical advantages as a coating technique and has been successfully used on commercial titanium implant products [6]. Clinical evidence has suggested that micrometer-level surface topography results in greater accrual of bone at the implant surface [7]. However, solo topography at the microscale is unable to fulfill the increasing clinical demand for effective osseointegration. Cells have hierarchical structures in micro-, submicro-, and nanoscale dimensions and contact with micro- and nanoscale topographical features [8]. Nanoscale structures can strongly alter protein interactions with a surface, ultimately affecting cell behaviors.

Irrespective of the surface-adsorbed proteins, cells are remarkable in their ability to sense nanostructures, triggering biological pathways for faster bone healing [9].

As nanoscale structures, mesoporous structures are thought to influence the behaviors of bone cells. Moreover, owing to their high specific surface area, well-ordered pores, and large pore volumes, mesoporous structures have been found to have potential applications in the fields of dental and orthopedic implants [10]. By loading such structures with various agents, such as antibiotics, anti-inflammatory agents, or osseointegration promoters, the mesoporous structures can be endowed with multifunction properties. Evaporation-induced self-assembly (EISA) is a versatile method for development of thin films with a tunable mesoscopic topography on various substrates [11]. By using this method, mesoporous TiO_2 , SiO_2 , and bioglass films with various chemical compositions have been synthesized [12,13]. Mesoporous TiO_2 and SiO_2 films induce the formation of apatite *in vitro* [14,15]. Furthermore, biomechanical stability in a rabbit model was unaffected in the presence of mesoporous TiO_2 film [15]. Bioactive glass in simulated body fluid (SBF) exhibits enhanced bioactivity and antibacterial characteristics due to the increase in pH upon sample dissolution [16]. These results indicate that mesoporous structures established by different methods have excellent bioactivity and biomechanical stability and are promising tools for implant surface modification.

To strengthen the implant-bone interface, multiple methods are available. Hierarchical macro/mesoporous structure topography on the titanium implant surface can benefit from both the advantages of MAO macroporous structures and

mesoporous structures. In our previous study, we reported a highly ordered mesoporous TiO₂ structure on the MAO-modified surface using nonionic block copolymers as structure-directing agents through an EISA process [17]. This synthesis strategy produced a macro/mesoporous structure with uniform hierarchical micro/nanostructured topography on the titanium alloy. However, the osteogenic capacity of bone cells on this macro/mesoporous structure was not investigated. In several reports, hierarchical structures have been shown to exhibit synergistic effects on the enhancement of osteogenic activity when compared with solo topography [18, 19]. Therefore, we assume that modification of the Ti-based alloy surface with macro/mesoporous structures would be beneficial for osseointegration.

Optimization of surface topography is essential in order to improve the integration of prosthesis and host bone. Moreover, combination of MAO and EISA could facilitate the development of macro/mesoporous-structured coatings with various chemical compositions for clinical applications that require rapid osseointegration. Accordingly, in this study, we aimed to evaluate the osteogenic capacity of osteoblast-like cells on the macro/mesoporous structure in comparison with the smooth substrate and macroporous structure. Our findings are expected to contribute to improving our understanding of the influence of mesoporous structures on cell behaviors and the feasibility of applying EISA to tune the adhesion, proliferation, and differentiation of bone cells.

2. Materials and methods

2.1 Preparation of the macro/mesoporous structure

Ti-6Al-4V discs (diameter: 9 mm, thickness: 2 mm) were used in this study. Samples were ground using 1500 # sandpaper and then consecutively treated with acetone, ethanol, and deionized water in an ultrasonic bath for 5 min each (establishing the substrate surface). The MAO process was then conducted on the samples by applying a voltage of 400 V in positive mode and 5 V in negative mode under an alternating current (AC) field for 5 min using the MAO equipment (WHD-20, Haerbin, China) to produce the macroporous structure (establishing the MAT surface). The aqueous electrolyte solution used during the MAO process contained 0.13 M calcium acetate monohydrate ($\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$) and 0.12 M sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$). The frequency and duty of the pulsed AC power were 50 Hz and 50 %, respectively. Subsequently, a mesoporous structure was formed on this macroporous structure without damaging the pores in the micron- and submicron scale (establishing the MA/MET surface) by EISA. Next, 2.1 g titanium (IV) tetraethoxide (TEOT, 95 %; Aldrich, Aladdin, Shanghai, China) as a Ti-precursor, 1.35 mL concentrated hydrochloric acid (HCl) as a suppressant, 0.55 g poly-(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer as a surfactant (Pluronic123, $\text{EO}_{20}\text{PO}_{70}\text{EO}_{20}$; Sigma, St. Louis, MO, USA), and 7.5 mL ethanol (99.5 % EtOH; Aldrich) as a solvent were mixed under vigorous stirring at room temperature for 3 h. Then, the mesoporous layer was deposited on the MAT coatings by dip-coating at a speed of 1.5 mm/s and aged at room temperature overnight. Subsequently, the samples were calcined at 400 °C for 4 h to remove the surfactant. The heating and

cooling rates of the furnace were maintained at ± 1 °C/min. Morphologies of the substrate, MAT, and MA/MET surfaces were observed using field emission scanning electron microscopy (SEM; Merlin Compact, Zeiss, Germany).

2.2 Response of SaOS-2 cells on the macro/mesoporous surface

SaOS-2 cells were seeded on the samples at a density of 5.0×10^4 cells/well. The culture medium was McCoy's 5A basic medium (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco, USA) and 1 % penicillin/streptomycin (Gibco, USA).

The complete medium was refreshed every 2 days.

To confirm the critical role of serum proteins in mediating cellular responses to surface topography, cells on the three surfaces were cultured in the presence or absence of FBS for 4 h. The cells were then fixed with 4 % paraformaldehyde. After washing with Dulbecco's phosphate buffered saline (DPBS; WELGENE, Korea) three times, cells were stained with 5 µg/mL phalloidin-TRITC (Sigma, USA) for detection of the cytoskeleton at 37 °C for 40 min. Subsequently, the cells were stained with 2 µg/mL 4',6-diamidino-2-phenylindole (Sigma, USA) for detection of cell nuclei at room temperature for 15 min. During all steps, cells were protected from light. The cells then were photographed by laser scanning confocal microscopy (Zeiss 710, Germany).

To evaluate the numbers of attached viable SaOS-2 cells on the three surfaces at 1, 2, and 4 h, cells were stained with 2 µM calcein-AM (Dojindo, Japan) for 15 min and then photographed by fluorescence microscopy (Leica, Germany).

The morphologies of attached cells on the three surfaces at 4 h after cell seeding were also observed by SEM. Cells cultured on the three surfaces for 1, 2, and 4 h were fixed with 2.5 % glutaraldehyde solution in DPBS. The dehydration process was conducted in a series of ethanol solutions (30 %, 50 %, 75 %, 80 %, 90 %, 95 %, and 100 % v/v), followed by tertiary butanol (25 %, 50 %, 75 %, and 100 % v/v).

Subsequently, the samples were freeze dried for 1 h before observation by SEM.

Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Japan) on days 1, 3, and 5. CCK-8 reagent was mixed with the complete medium at 10 % (v/v). Two hundred microliters of the mixture was added to each well. The morphologies of cells on the three time points were observed by SEM.

The total intracellular proteins of SaOS-2 cells cultured on the three surfaces for 7 and 10 days were evaluated by BCA protein assays (Beyotime, China) after trypsinization and cell lysis, according to the manufacturer's instructions. The alkaline phosphatase (ALP) activities of the three groups were tested and normalized to the concentration of total protein using an ALP kit (Jiancheng Bio-engineering Research Institute of Nanjing, China).

Collagen secretion of the cells on the three surfaces was stained after culture for 7 days. The cells were fixed with 4 % paraformaldehyde in DPBS solution for 20 min, washed three times, and stained with the Sirius Red staining (Solarbio, China) for 18 h. After washing with DPBS, stains were dissolved in NaOH/methanol solution (0.2 M NaOH:methanol = 1:1, v/v), and optical density values were determined using a microplate reader (EnSpire, PerkinElmer, USA).

3. Results

The morphologies of the three surfaces are shown in Fig.1. The polished substrate showed a smooth surface in both low- and high-magnification images (Fig.1a and b).

In contrast, the MAT group appeared to have many macropores ranging in size from 500 nm to 3 μm (Fig.1c and d), due to the formation of a porous oxide layer resulting from the MAO process. After using the dip coating technique to cover the MAO-treated surface with mesoporous titania fabricated by EISA, the coated layer of mesoporous titania did not affect the macroporous features of the surface (Fig.1e and f). In our previous study, we showed that the macro/mesoporous structure was composed of anatase TiO_2 , similar to the macroporous structure in the current study.

The initial attachment of SaOS-2 cells during the first 4 h was illustrated by live cell staining followed by statistical image analysis (Fig.2). The results indicated that the adherent cell numbers increased with the incubation time. There were significantly more attached cells in the MA/MET group than in the MAT and substrate groups.

After attachment on the surface, cells began to spread well, particularly after 2 days of culture, as shown in SEM images (Fig.3). Notably, most of the cells were still spherical in appearance on all surfaces after 1 h of incubation. However, some cells were fully spread on MA/MET surface. After 2 h of initial adhesion, most of the cells on the smooth substrate surface were rounded in morphology; in contrast, on the MAT and MA/MET surfaces, cell membrane protrusions, which were involved in cell migration and spreading, were observed. At 4 h, polygonal shaped cells were observed

on the MAT and MA/MET surfaces, indicating that the surfaces enhanced cell spreading. Cells attached to the smooth substrate surface showed a polarized shape with elongated pseudopodium.

For investigation of cytoskeleton organization on different surfaces, fixed cells were incubated with TRITC-conjugated phalloidin after culturing for 4 h (Fig.4). On the MA/MET surface, cells were well spread and had the largest areas. The formation of actin fibers on this surface was noticeable for cells on the MAT surface, which showed extension of numerous filopodia, whereas cells on the substrate surface displayed a polar extended shape. In the absence of FBS, cell expansion on all three surfaces was suppressed; indeed, most cells showed rounded shapes, with no evidence of cell membrane protrusions. The numbers of attached cells on the three surfaces were lower than those in samples with FBS added to the culture medium, with no significant difference among all investigated surfaces. Furthermore, the cell area was smaller in the absence of FBS than in the presence of FBS for all surfaces, indicating that the adsorption of serum protein on the surface was critical for cell adhesion and spreading in comparison with variations in surface topography.

SaOS-2 cells on the three surfaces were also examined by SEM at 1, 3, and 5 days after cell seeding (Fig.5a). On day 1, the cells on the substrate were sparsely distributed and less spread. On day 3, the cells on three surfaces were flattened and had elongated lamellipodia. On day 5, the cells on the substrate were elongated along the polishing direction, whereas cells on the MAT and MA/MET surfaces had larger spreading areas. No obvious differences were observed in cell proliferation among the

MAT and MA/MET surfaces. Throughout the entire culture period, an increasing trend in cell growth was observed (Fig.5b). No obvious differences among the three surfaces were observed on day 1. Cell proliferation on MAT and MA/MET surfaces was more substantial than that on the substrate surface on days 3 and 5. The results suggested that cell proliferation was enhanced by the MAT and MA/MET surfaces compared with the substrate surface. However, there were no significant differences between the MAT and MA/MET groups.

Next, we measured ALP activity of SaOS-2 cells cultured for 7 or 10 days (Fig.6a). The cell numbers increased on all surfaces as a function of time. Moreover, ALP activity on day 7 or 10 on the MA/MET surface was notably enhanced compared with the MAT and substrate surfaces. The differences in expressed amounts of ALP activity among different surfaces indicated that the activity of this enzyme was sensitive to the surface topography. Collagen secretion on the three surfaces was evaluated by Sirius Red staining on day 7. More collagen secretion was observed on the MA/MET surface than on the substrate and MAT surfaces (Fig.6b).

4. Discussion

Cellular responses, particularly cell attachment, can be regulated by specific proteins adsorbed on the biomaterial surfaces. Proteins typically adsorb within seconds after they arrive at the surfaces of biomaterials. As a linker between cells and biomaterials, the interactions of surface topographies and serum proteins are crucial for the cytocompatibility of biomaterials [20]. In the present study, we showed that the

macro/mesoporous structure induced a faster and stronger response in SaOS-2 cells than smooth substrate and macroporous structures in the presence of FBS; in contrast, in the absence of FBS, cells on all investigated surfaces display decreased numbers of attached cells, reduced spreading areas, and fewer protrusions of the cell membrane, indicating the crucial role of serum proteins in the promotion of initial cell adhesion on the macro/mesoporous structure. The difference in specific surface area is thought to contribute to the protein affinity of different surfaces. Therefore, the enhancement of cell adhesion could be attributed to the mesoporous structure due to its larger specific surface area than the other two groups [21]. The conformation of adsorbed proteins is also dependent on the surface properties [22]. The mesoporous structure may lead to changes in the conformation of osteoblast-binding extracellular matrix proteins, allowing these proteins to unfold and permitting access to their cell-binding motifs, such as the arginine-glycine-aspartic acid sequence [23]. Receptors on the cell membrane can bind to these cell-binding motifs and transfer information to the nucleus, leading to reorganization of the cell skeleton and creation of new focal contacts [24]. Furthermore, higher degrees of cell spreading are thought to promote increased cytoskeletal tension [25]. Since nanoscale topography is a direct mechanical stimulus altering the biological function of the cells [24], nanoscale mesopores may directly influence cell function.

The primary cell behaviors related to the extracellular matrix can be divided into the following phases: adhesion, detachment for proliferation or migration, re-adhesion, and synthesis of extracellular matrix. Therefore, the quality of adhesion is crucial for

cell proliferation and subsequent processes. Sufficient cell adhesion has positive effects on cell proliferation and differentiation [26], consistent with our findings that the MA/MET surface enhanced cell proliferation compared with that in the MAT and substrate groups. The highest levels of cell differentiation markers (ALP activity and collagen secretion) in the present study were also found on the macro/mesoporous surface. Mesoporous structures possess excellent biocompatibility; mesoporous titania films provide suitable surfaces for MG63 cells to attach and proliferate [27], and silica coatings with highly ordered sub-10 nm porosity accelerate early osteoblast adhesive response [28]. Studies on the nonporous titanium, titanium with mesoporous TiO₂ coating, and titanium with Mg-loaded mesoporous coating have demonstrated that the organized pore distribution, presence of nanoscale roughness, and developed titanium dioxide layer of the 3D mesoporous coating are associated with enhancement of adhesion and osteogenic differentiation *in vitro* [29]. Although the mechanism is not fully understood, topographical cues are known to modulate cell behavior via mechanotransduction [30]. In response to surface topography, changes in cytoskeletal tension will affect mechanotransductive pathways and change nucleus organization, thereby having a direct influence on cellular gene expression profiles [31]. In our previous study, we investigated initial cell adhesion on the MA/MET surface at only 24 h after cell seeding. Notably, the RhoA/ROCK pathway plays a vital role in the early stages of cell adhesion and cytoskeleton organization [17]. Serum response factor (SRF)-dependent skeletal gene expression is reported to be regulated by the RhoA/ROCK and treatment with Y-27632 would lead to reduction in the binding of

SRF [32]. In our work, we studied the effects of FBS on cell adhesion and the results showed the importance of FBS (SRF proteins) to cell adhesion and cytoskeleton reorganization. Considering that cell adhesion and formation of FAK (focal adhesion kinase) are pivotal in cell survival, growth, differentiation and homeostasis [33], the RhoA/ROCK pathway is hypothesized to regulate the further behavior of cells. Studies have indicated that mechanical cues mediated by cell shape regulated bone cell growth and differentiation via the RhoA/ROCK pathway [34-36]. RhoA/ROCK pathway is also found to be essential for recognition of the topography and the transduction of the topography information in cellular responses [37-39]. Based on these previous studies, we postulated that the RhoA/ROCK signalling pathway was involved in the osteoblast-like cell activity induced by the macro/mesoporous structure. Additionally, RhoA kinases mediate mechanotransduction [40] and participate in activating mitogen-activated protein kinase (MAPK) signaling pathways [41]. MAPKs are key mediators of cellular responses to a variety of extracellular stimuli, and three major subgroups of MAPKs have been identified in mammalian cells, namely extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, and p38 [42]. Thus, our findings suggested that macro/mesoporous structures may affect cell proliferation and differentiation through the RhoA/ROCK pathway and the related pathways.

5. Conclusions

Here, we evaluated the topography-induced responses of osteoblast-like SaOS-2

cells grown on the macro/mesoporous structure, with an emphasis on their initial adhesion, proliferation, and differentiation. The results of our study provided important insights into the dependence of topography-induced initial adhesion on the adsorption of proteins in FBS by the macro/mesoporous structure. High sensitivity to micro- and nanoscale topography was observed for proliferation and osteogenic differentiation of SaOS-2 cells. The macro/mesoporous-structured layer showed the ability to enhance the proliferation and osteogenic differentiation of SaOS-2 cells. Our findings provide important insights into the osteogenic capacity of the macro/mesoporous structure, and the results demonstrated that this hierarchical structure may be a promising surface topography for application as orthopedic and dental implants.

Acknowledgement

The authors are grateful for the financial support from National Natural Science Foundation of China (U1605243, 51472139), and International S&T Cooperation of the Ministry of Science and Technology of China (2015DFG52490).

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Figure caption

Fig.1 SEM images of the three surfaces. Substrate (a) and (b), MAT (c) and (d), and MA/MET (e) and (f).

Fig.2 Cell staining and statistical analysis of attached SaOS-2 cells on the substrate, MAT, and MA/MET surfaces at 1, 2, and 4 h. Values are means $\pm$ standard deviations ($n = 3$). One-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method is performed to evaluate differences between these groups. * depicts statistical differences, $*p < 0.05$, $**p < 0.01$.

Fig.3 SEM images of the attached SaOS-2 cells on the substrate, MAT, and MA/MET surfaces at 1, 2, and 4 h.

Fig.4 Staining of the cytoskeleton and nuclei at 4 h after cell seeding in the presence and absence of FBS, representing the dependence of initial cell adhesion on FBS.

Fig.5 Cell morphologies and proliferation on the substrate, MAT, and MA/MET surfaces. Values are means $\pm$ standard deviations ($n = 3$). One-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method is performed to evaluate differences between these groups. * depicts statistical differences, $*p < 0.05$, $**p < 0.01$.

Fig.6 Differentiation of SaOS-2 cells on the substrate, MAT, and MA/MET surfaces. ALP activity (a) and collagen secretion (b). Values are means $\pm$ standard deviations ($n = 3$). One-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method is performed to evaluate differences between these groups. * depicts statistical differences, $*p < 0.05$, $**p < 0.01$.

Fig.1 SEM images of the three surfaces. Substrate (a) and (b), MAT (c) and (d), and MA/MET (e) and (f).

135x153mm (300 x 300 DPI)

Aut

Fig.2 Cell staining and statistical analysis of attached SaOS-2 cells on the substrate, MAT, and MA/MET surfaces at 1, 2, and 4 h. Values are means $\pm$ standard deviations ($n = 3$). One-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method is performed to evaluate differences between these groups. * depicts statistical differences, * $p < 0.05$, ** $p < 0.01$.

700x241mm (300 x 300 DPI)

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Fig.3 SEM images of the attached SaOS-2 cells on the substrate, MAT, and MA/MET surfaces at 1, 2, and 4 h.

241x160mm (300 x 300 DPI)

Author

Fig.4 Staining of the cytoskeleton and nuclei at 4 h after cell seeding in the presence and absence of FBS, representing the dependence of initial cell adhesion on FBS.

317x166mm (300 x 300 DPI)

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Fig.5 Cell morphologies and proliferation on the substrate, MAT, and MA/MET surfaces. Values are means $\pm$ standard deviations (n = 3). One-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method is performed to evaluate differences between these groups. * depicts statistical differences, *p < 0.05, **p < 0.01.

83x30mm (300 x 300 DPI)

Author Mar

Fig.6 Differentiation of SaOS-2 cells on the substrate, MAT, and MA/MET surfaces. ALP activity (a) and collagen secretion (b). Values are means $\pm$ standard deviations ($n = 3$). One-way analysis of variance (ANOVA) followed by post hoc comparisons with least significant difference (LSD) method is performed to evaluate differences between these groups. * depicts statistical differences, * $p < 0.05$, ** $p < 0.01$.

88x33mm (300 x 300 DPI)

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Title:

Effects of the hierarchical macro/mesoporous structure on the osteoblast-like cell response.

Date:

2018-07

Citation:

Zhang, R., Elkhooly, T. A., Huang, Q., Liu, X., Yang, X., Yan, H., Xiong, Z., Ma, J., Feng, Q. & Shen, Z. (2018). Effects of the hierarchical macro/mesoporous structure on the osteoblast-like cell response.. J Biomed Mater Res A, 106 (7), pp.1896-1902. <https://doi.org/10.1002/jbm.a.36387>.

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