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Osteogenic activity of human periosteal sheets cultured on salmon collagen-coated ePTFE meshes

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Abstract Our animal implantation studies have demonstrated that, after osteogenic processing, cultured human periosteal sheets form osteoid tissue ectopically without the aid of conventional scaffolding materials. To improve the osteogenic activity of these periosteal sheets, we have tested the effects of including a scaffold made of salmon collagen-coated ePTFE mesh. Periosteal sheets were produced with minimal manipulation without enzymatic digestion. Outgrown cells penetrated into the coated mesh fiber networks to form complex multicellular layers and increased expression of alkaline phosphatase activity in response to the osteoinduction. In vitro mineralization was notably enhanced in the original tissue segment regions, but numerous micro-mineral deposits were also formed on the coated-fiber networks. When implanted subcutaneously into nude mice, periosteal sheets efficiently form osteoid around the mineral deposits. These findings suggest that the intricate three-dimensional mesh composed of collagen-coated fibers substantially augmented the osteogenic activity of human periosteal sheets both in vitro and in vivo.

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1 Introduction

The idea of a periodontal regenerative therapy using autologous cultured periosteal sheets was first demonstrated in the canine model where mechanical alveolar bone defects made in the 4th mandibular premolar were repaired by newly formed bone [1]. Following this positive result, we investigated the clinical applicability of this approach in more than 40 patients over 3 years and concluded that this therapeutic methodology is potentially important for stimulating alveolar bone formation at specific sites [2, 3]. Besides clinical effectiveness, this methodology has additional merits. First, periosteal tissue is more accessible for the dentist than any other source of stem cells (e.g., bone marrow cells). Second, neither specialized cell-processing techniques (e.g., enzymatic digestion, cell sorting) nor devices (e.g., expensive bioreactors) are required to prepare the periosteal sheets. Third, periosteal sheets alone (i.e., without any conventional scaffolds) do function as an effective osteogenic "biomaterial" graft. Most non-malignant, adherent cells require appropriate scaffolds to function after implantation, but periosteal sheets do not—probably because they are composed of a complex multicellular layered tissue architecture that possesses appreciable mechanical strength.

In a parallel basic study [4], we elucidated the cellular and molecular mechanism behind how human periosteal sheet-based therapy works at in vivo sites of implantation. The primary mechanism is that mineralized regions formed by osteogenic induction in cultured periosteal sheets

function as seeds of ectopic bone formation at the site where they are implanted. The second, probably complementary, mechanism is that periosteal sheets also provide several important cytokines involved in bone anabolism that influence the recipient's cells near implantation sites. In this way they function as living drug-delivery systems (DDSs).

These basic findings raised the possibility that including an appropriate functional scaffold would further improve the osteogenic potential of cultured periosteal sheet as a graft biomaterial. In this study, we have examined the function of salmon collagen-coated ePTFE (expanded polytetrafluoroethylene) mesh (Vecell 3D-insert®) as a scaffold for periosteal sheets in vitro and in vivo. We have found that this material, which was originally developed as a scaffold for three-dimensional (3D) cell cultures, facilitated formation of multicellular layers and mineral deposits in our periosteal sheet cultures, and that it also facilitated formation of osteoid after subcutaneous implantation of developed sheets.

2 Materials and methods

2.1 Isolation and culture of periosteal tissue segment

Eight healthy and non-smoking volunteers aged 25–36 years participated in this study. As described previously [4], periosteum tissue was aseptically dissected, washed three times in PBS(−), cut into small pieces (2–3 × 2–3 mm), and plated on Vecell 3D-insert® (ϕ30 mm; Vecell Inc., Kitakyushu, Japan) inserted in 6-well plates or 35-mm dishes. After incubating for 15–20 min under dry conditions in a CO_2 incubator, the fresh growth medium [Medium 199 supplemented with 10% FBS, 25 μg/ml L-ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen, Carlsbad, CA)] was added.

For osteogenic induction, cultured periosteal sheets were changed into the induction medium. Osteoinduction medium was prepared by adding 3% (v/v) of a commercially available osteoblast-inducing agent, KE-200 (DS Pharma, Osaka, Japan) [4], which was described by the manufacturer as containing dexamethasone, β-glycerolphosphate, and L-ascorbic acid, at a minimum, to the growth medium.

The study design and consent form were approved in all details by the ethical committee for human subject use at Niigata University Medical and Dental Hospital in accordance with the Helsinki Declaration of 1975 and as revised in 2000. Data variation was found between individuals (as shown in figure statistics), but general characteristics were almost identical from preparation to preparation.

2.2 Vecell 3D-insert® and SEM observation

In a Vecell 3D-insert®, the main component is an ePTFE mesh that is coated with polyamino-acid urethane copolymer (PAU) [5] and thereafter cross-linked with salmon type I atelocollagen. On one end of a plastic tube, this mesh is tensed like drumhead to assemble a cell-insert type culture cup (Fig. 1a). This system was originally developed as a device for three-dimensional cell culture, but is substantially better for multilayered cells, such as periosteal sheet, than conventional culture dishes. This is because under this arrangement, medium nutrients are expected to be supplied from both the upper and lower sides of the forming cell layer. Similarly, cellular metabolites should similarly diffuse away from both sides.

The micro-appearance of the surface of the mesh was observed by a scanning microscopy (Miniscope TM-1000, Hitachi High-Technologies, Tokyo, Japan). Figure 1b shows a SEM observation of the surface indicating that the mesh was composed of thick fibers (X-axis) and thin fibers (Y-axis). The mesh opening was evaluated to be approximately 30 × 2 μm, but thick and thin fibers were randomly overlaid. Therefore, the actual opening size might be a little smaller for cells than the measurement data indicate.

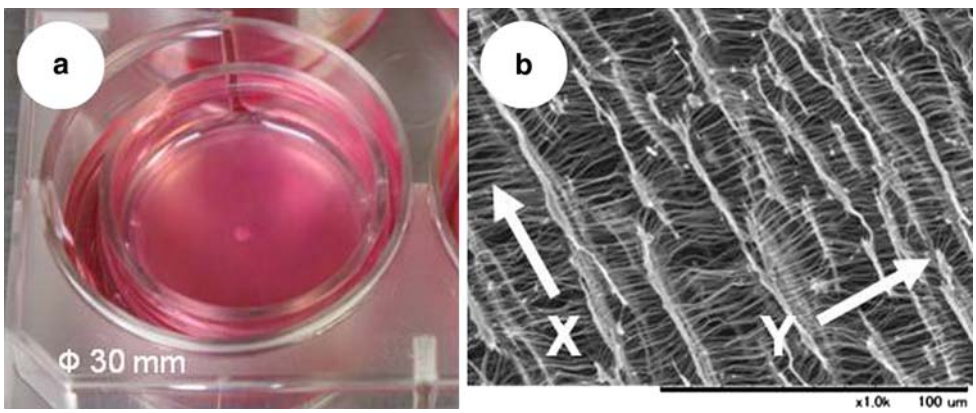

Fig. 1 The macroscopic and microscopic structure of a Vecell 3D-insert®. **a** A macro appearance of Vecell 3D-insert® (ϕ30 mm). **b** SEM observation of Vecell's mesh. X-axis and Y-axis represent the direction of the thick and the thin fibers, respectively

2.3 Examination of ALP expression and in vitro mineralization

For histochemical examination, human periosteal sheets cultured in the mesh were fixed in 10% formalin in 0.1 M phosphate buffer (pH 7.4) and stained with an ALP-staining kit (Muto Chemicals, Tokyo, Japan). For quantitation, image analysis was performed using WinROOF software (Mitani Corp., Fukui, Japan). The cross-sectional areas of ALP-positive cells were measured.

Otherwise, periosteal sheets cultured in the mesh were directly fixed, dehydrated, embedded in paraffin, and sectioned for staining. Periosteal sheets cultured on dishes were scrapped and fixed for paraffin embedding. As described below (Sect. 2.5), sections were made and stained with HE and silver nitrate (for the visualization of calcium deposits).

2.4 Animal implantation study

Balb/c-nu/nu mice (male, age: 5-week old, weight: 15–20 g) were obtained from Japan Charles River Laboratories (Yokohama, Japan) and housed at the Brain Research Institute Center for Bioresource-based Researches, Niigata University, at least 1 week prior to the experiment.

Human periosteal sheets cultured on the mesh were rinsed three times with PBS(−), and the center of mesh containing the original periosteal segment were cut into the size of 5 × 5 mm for implantation. Human periosteal sheets cultured on plastic dishes were rinsed as described above and harvested with a cell scraper. Surgical procedures on nude mice were performed under standard conditions. After disinfecting the dorsal area, a midline skin incision was made, and periosteal sheets alone or periosteal sheets cultured on the mesh were directly implanted. The care and use of animals followed the Guiding Principles for the Care and Use of Animals, as approved by Niigata University.

2.5 Histological processing and histomorphometry

At intervals of 2–8 weeks (every 2 weeks) after implantation, animals were euthanized by an overdose of pentobarbital (Nembutal®; DS Pharma). As described previously [4], implanted periosteal samples were retrieved along with some surrounding connective tissue, fixed in 10% neutralized formalin, dehydrated, embedded in paraffin, and sectioned sagittally at a thickness of 10 μm. Nondecalcified sections were stained with HE or silver nitrate (von Kossa staining) [4]. For image analysis, serial sections were prepared, and a section containing the largest mineralization area was selected. The cross-sectional areas of dark-brown deposits, which represent mineralized tissue, per each view (but expect for the deposits on the mesh) were quantified using WinROOF (Mitani Corp.), as described in the Sect. 2.3.

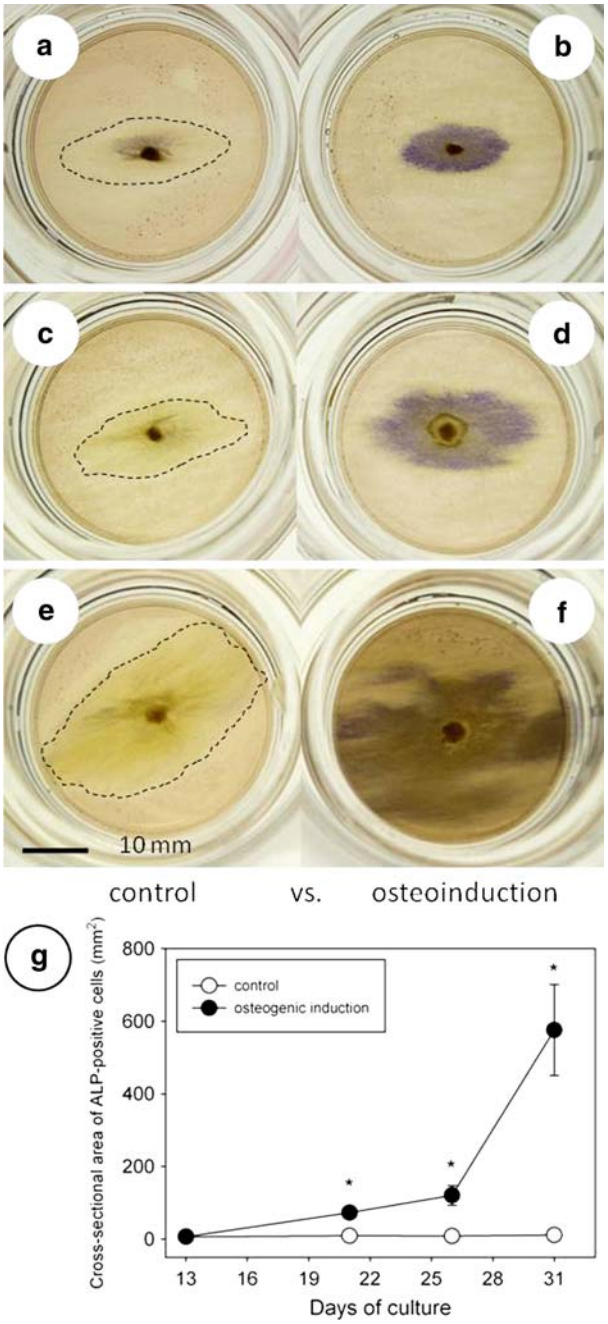

Fig. 2 Time-course of the growth and ALP expression of human cultured periosteal sheets. Periosteal sheets were cultured on Vecell 3D-insert® (ϕ30 mm) in the growth medium continuously (*left* panels) or in the growth medium for the initial 13 days and subsequently in the osteoinduction medium for days indicated below (*right* panels). ALP expression is demonstrated by the *violet* staining. **a** 22 days vs. **b** 13 + 9 days; **c** 26 days vs. **d** 13 + 13 days; **e** 31 days vs. **f** 13 + 18 days. **g** Time-course changes in ALP-positive cross-sectional area in human periosteal sheet cultures. * $P < 0.001$ vs. each corresponding control culture. (Color figure online)

Some sections were assayed for cellular expression of tartrate-resistant acid phosphatase (TRAP) with an ACP staining kit (Muto Chemicals) in the presence of 50 mM

tartrate (Wako Pure Chemicals). The sections were faintly stained with hematoxylin to provide a background stain.

2.6 Statistical analysis

Unless otherwise stated, each experiment was repeated twice, and the results are expressed as mean ± SD of three samples from the representative experiment. Total number of mice used in the representative experiment shown was nine ($n = 9$). Statistical significance of differences between groups was analyzed by Student's *t*-test. *P* values < 0.05 were considered significant.

3 Results

3.1 In vitro observation of the growth and mineralization of periosteal sheets

Figure 2 shows the time-course effects of osteogenic induction on ALP expression by human periosteal sheets cultured in the mesh (ϕ30 mm). Cells migrated from the original periosteum segments grew along the thick fibers, and therefore periosteal sheets became oval ellipsoids, rather than the circular disks obtained in standard tissue culture dishes (data not shown). In cultures receiving osteogenic induction (for the last 9, 13, or 18 days: total 22, 26, or 31 days), most cells within the periosteal sheets were ALP-positive. The time-course changes in ALP-positive cross-sectional areas in both cultures are shown in Fig. 2g. The ALP-positive areas in the osteoinduced periosteal sheet cultures were substantially larger than those of the control (noninduced) cultures at all time points tested. In contrast, the periosteal sheets in control cultures (for 22, 26, or 31 days) proliferated over time somewhat faster than did the osteoinduced periosteal sheets. Thus, the sizes of the control sheets at each time point were slightly larger than those of the osteoinduced periosteal sheets.

Human periosteal sheets cultured in larger plastic dishes (ϕ60 mm) reproducibly grew significantly faster than periosteal sheets cultured in the mesh. In contrast, osteogenic induction (for the last 6 days vs. the last 20 days) seemed less strongly to up-regulate their ALP expression

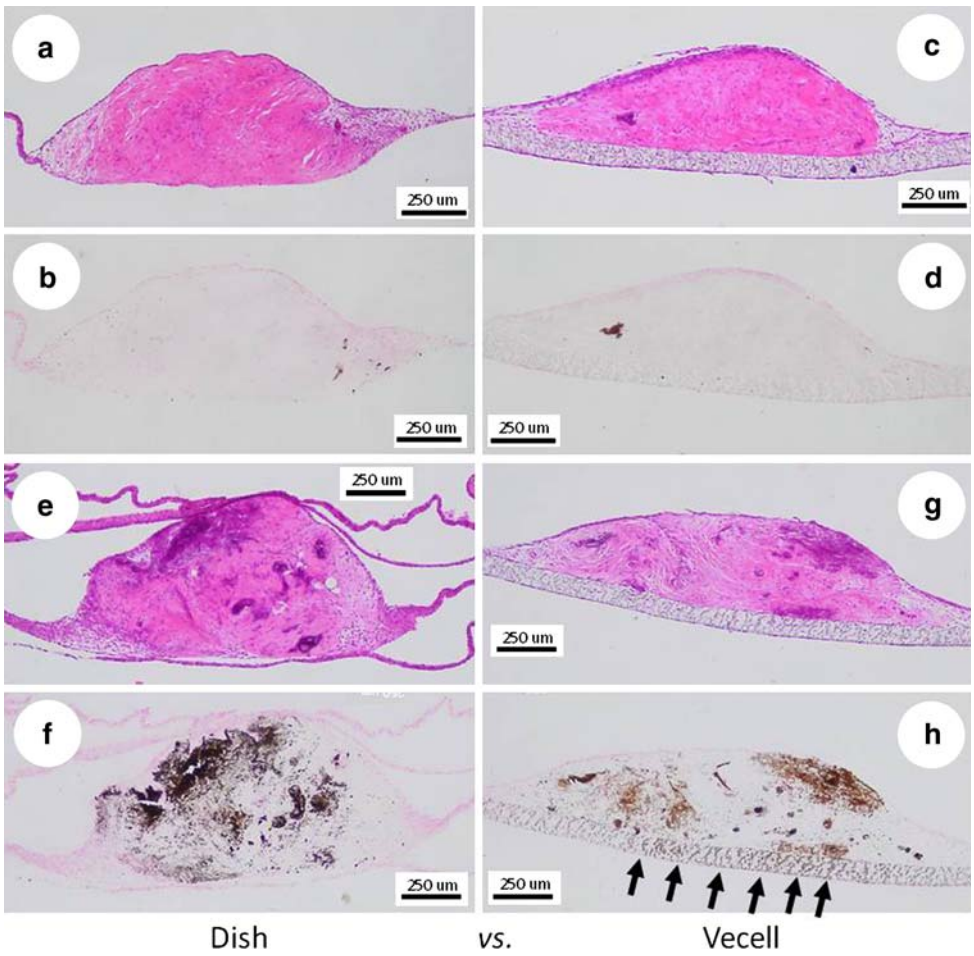

Fig. 3 Photomicrographs of paraffin-sectioned human cultured periosteal sheets. Periosteal sheets were cultured on plastic dishes (**a**, **b**, **e**, **f**) or in Vecell 3D-insert® (**c**, **d**, **g**, **h**) in the growth medium for 13 days, and subsequently in the osteoinduction medium for 10 (**a**, **b**, **c**, **d**) or 16 days (**e**, **f**, **g**, **h**). **a**, **c** HE-staining. **b**, **d** von Kossa staining. *Dark-brown* particles or areas represent von Kossa-positive mineral deposits. *Arrows* indicate fine mineral deposits formed on the mesh fiber networks. Bar = 250 μm. (Color figure online)

in plastic dishes than it did in cultures grown in the mesh (data not shown).

Figure 3a–d shows sagittal sections of human periosteal sheets cultured for 13 days in the growth medium and for additional 10 days in the osteoinduction medium in the mesh and on the dishes (as control). Periosteal cells migrated from the original segments expanded not only horizontally but also vertically: Cells penetrated into the meshes (thickness $\cong$ 100 μm) and reached the other edge so that cellular multilayers in mesh cultures were always thicker than those of the dish cultures. In both cultures, the original segments are enriched in extracellular matrix (ECM), which was eosinophilic, while peripheral regions that consisted of outgrown cells were less eosinophilic. Appreciable mineralization was found only in one or two spots within the original regions of both types of culture.

Formation and growth of in vitro mineralization was observed that depended directly on the time period of the osteogenic induction in both cultures. Figure 3e–h shows that in vitro mineralization was substantially increased when the osteoinduction was expanded to 16 days. The cross-sectional areas of formed osteoid tissue and of in vitro mineralization in the periosteal sheets cultured both on dishes and in the meshes were enlarged (versus Fig. 3a–d). At this time point, diffuse and widely scattered mineral deposits had formed in the original segment of both culture types (Fig. 3e–h). Interestingly, in the mesh cultures, some mineral deposits formed inside the mesh fiber networks where the original segments faced (indicated by arrows: Fig. 3h).

3.2 In vivo histological observation of osteoid formation in implanted periosteal sheets

To evaluate the osteogenic activity of human cultured periosteal sheets, periosteal sheets cultured on dishes with osteoinduction medium and those cultured in the mesh with osteoinduction medium were implanted subcutaneously in

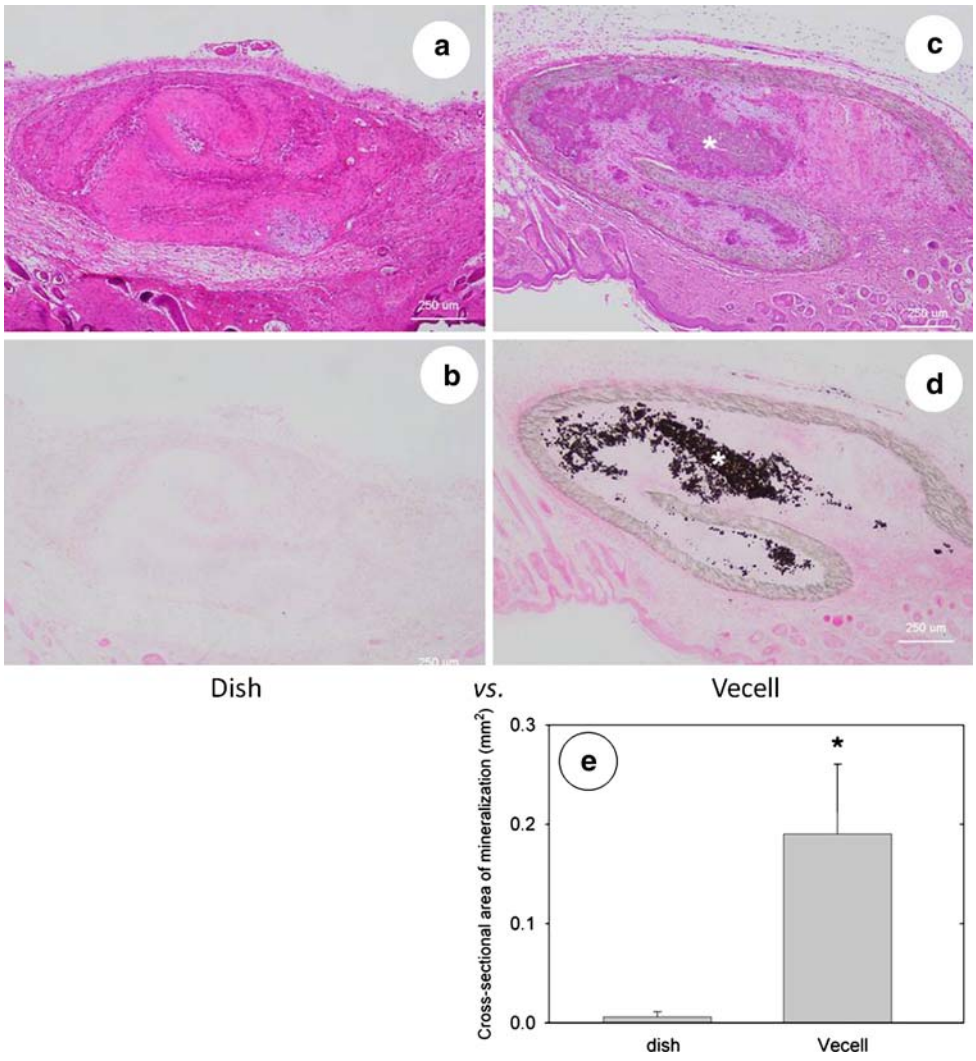

Fig. 4 Osteoid formation by human periosteal sheets at 2 weeks post-implantation. Human periosteal sheets were cultured on plastic dishes (dish; **a**, **b**) or in Vecell 3D-insert® (Vecell; **c**, **d**) with the growth medium for 13 days, and subsequently with the osteoinduction medium for 24 days. Induced periosteal sheets on the dishes (**a**, **b**) were rinsed, harvested, and implanted subcutaneously to nude mice, while induced periosteal sheets in then mesh (**c**, **d**) were rinsed, cut, and directly implanted in combination. **a**, **c** HE-staining. **b**, **d** von Kossa staining. The eosinophilic regions shown in Panels **a** and **c** represent osteoid tissues, and the *dark-brown* regions shown in Panels **b** and **d** represent mineralized tissues. In Panel **d**, substantial mineral deposits were formed on some parts of the mesh fibers. *Bar* = 250 μm. **e** The cross-sectional areas of mineralization formed in the original segments. * $P < 0.01$ versus the dish culture. (Color figure online)

the back of nude mice (Fig. 4). Major osteoid areas with mineral deposits were observed in the original periosteum segments in the mesh-cultured periosteal sheet grafts at 2 weeks post-implantation (Fig. 4c, d). However, mineral deposits were minimal in dish-cultured grafts (Fig. 4a, b). Image analysis revealed that the cross-sectional areas of mineralization per view in the mesh-cultured grafts were significantly larger than those of the dish-cultured grafts (Fig. 4e).

At 4 weeks post-implantation, new mineral-deposit and osteoid formation were also observed in osteoinduced dish-cultured periosteal sheet grafts (Fig. 5a, b). By comparison, the mineral deposits and osteoid had grown substantially in size by this time in the osteoinduced mesh-cultured grafts (Fig. 5c, d). The difference in the mineralized cross-sectional areas per view between the dish-cultured and the mesh-cultured grafts became relatively smaller over time, but was still statistically significant at the end of our experiments (Fig. 5e). Inclusion of a collagen-coated mesh scaffold does accelerate osteogenic activity over at least the first 4 weeks after implantation. In addition, these results suggest again that implanted osteoinduced periosteal sheets, with or without the aid of mesh scaffolding, will continue to mature and express osteogenic activity at the site of implantation.

Of interest is the observation that some parts of the mesh covered with mineral deposits formed in vitro could function as a nucleus of in vivo mineralization. Periosteal sheets were cultured for 37 days in the growth medium (indicated as "control") or for 13 days in the growth medium and for an additional 24 days in the osteoinduction medium prior to implantation. As shown in Fig. 6, osteoid with significant mineralization was formed inside the mesh fiber networks in the osteoinduced periosteal sheets by 2 weeks post-implantation (Fig. 6d, e) TRAP-positive multinucleated cells were also formed along the mesh fibers (Fig. 6f). In the control (no induction) periosteal sheets, neither osteoid nor mineralization was usually found inside the mesh (Fig. 6a, b). In addition, TRAP-positive cells were also absent from all sections of the control cultures (Fig. 6c).

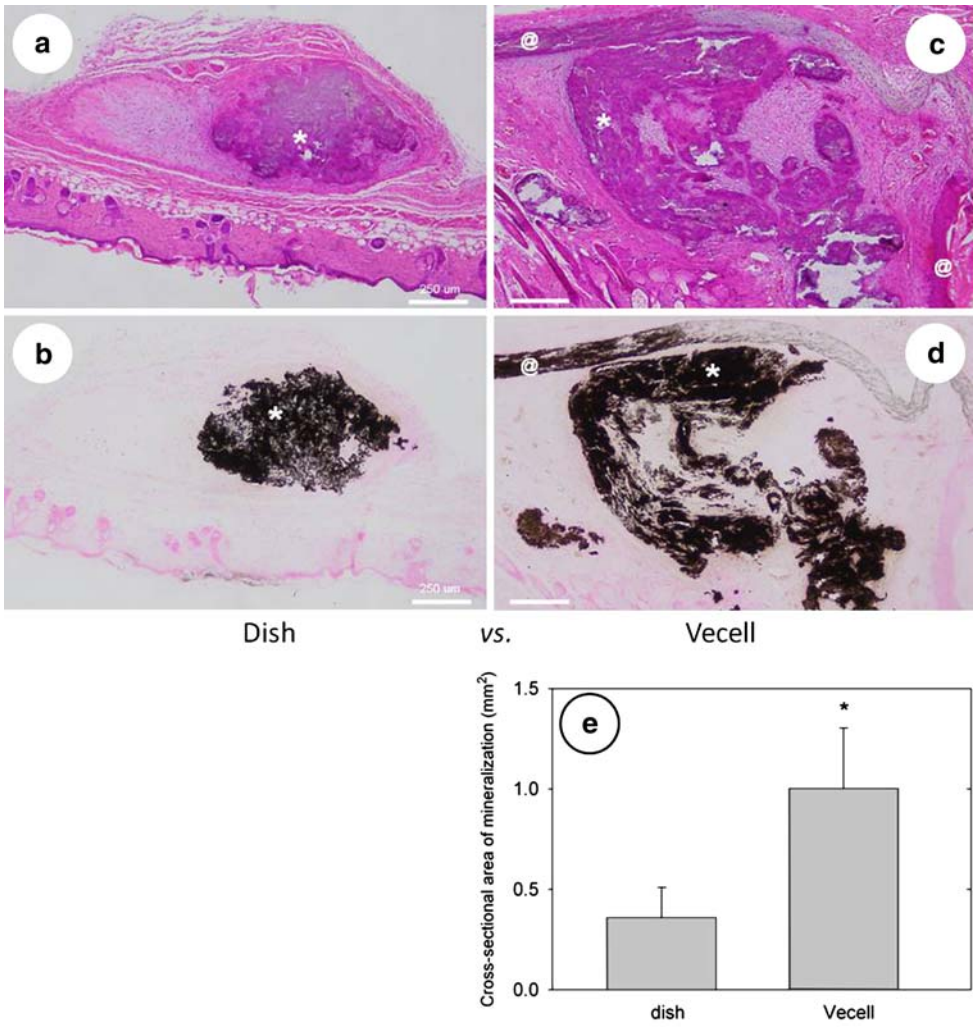

Fig. 5 Osteoid formation by human periosteal sheets at 4 weeks post-implantation. Human periosteal sheets were prepared, osteoinduced, and implanted, as described in the legend of Fig. 4. **a**, **c** HE-staining. **b**, **d** von Kossa staining. The eosinophilic regions shown in Panels **a** and **c** represent osteoid tissues, and the *dark-brown* regions shown in Panels **b** and **d** represent mineralized tissues. In Panel **d**, substantial mineral deposits were formed on some parts of the mesh fibers. *Bar* = 250 μm. **e** The cross-sectional areas of mineralization formed in the original segments. * $P < 0.03$ versus the dish culture. (Color figure online)

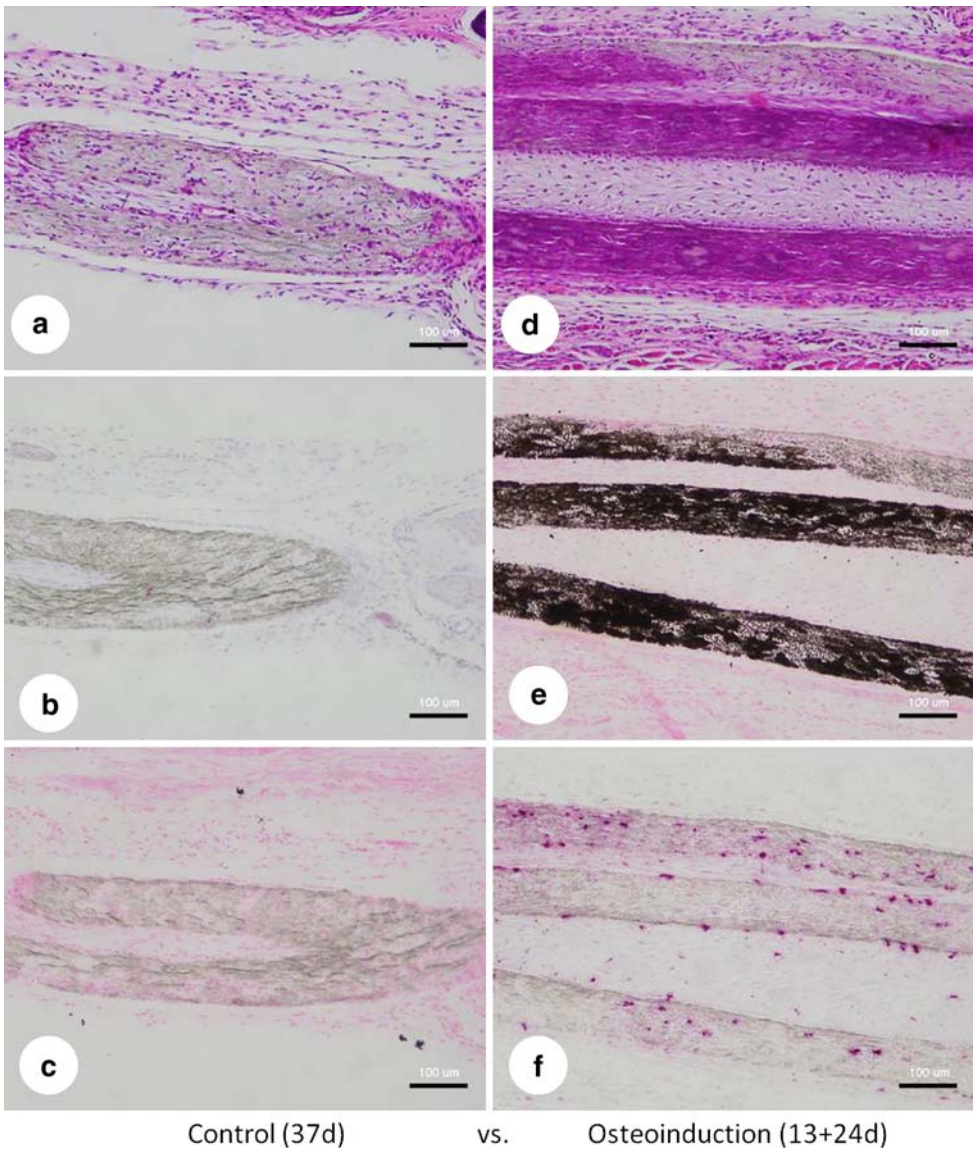

Fig. 6 Matured osteoid formation inside the mesh fiber networks by implanted periosteal sheets at 2 weeks post-implantation. The control non-induced periosteal sheets were cultured in the growth medium for 37 days (control; **a**–**c**), while the induced periosteal sheets were cultured in the growth medium for 13 days and additionally in the osteoinduction medium for a subsequent 24 days (osteoinduction; **d**–**f**). At the end of culture time, periosteal sheets were harvested with the mesh and implanted subcutaneously to nude mice for 2 weeks. **a**, **d** HE-staining, **b**, **e** von Kossa staining, **c**, **f** TRAP-staining. TRAP-positive multinucleated cells were stained *red* in Panel **f**. *Bar* = 100 μm. (Color figure online)

Similar findings were obtained in the samples retrieved at 8 weeks post-implantation. As shown in Fig. 7, osteoid with mineralization was observed in the osteoinduced periosteal sheets, but not in control sheets (Fig. 7d, e vs. a, b). However, in these 8 week samples a few TRAP-positive cells were recruited into control periosteal sheets, but these were notably less than the numbers observed within the osteoinduced periosteal sheets (Fig. 7c vs. f).

4 Discussion

In our previous study [4], we observed that the original periosteum segments are ALP-positive, highly eosinophilic (i.e., have ECM-rich) regions, and that these characteristics could be well maintained throughout periods of culture without appreciable apoptosis or necrosis. In addition, treatments with established osteogenic-inducing agents stimulated in vitro mineralization primarily in these regions and up-regulated ALP activity in more than 80% of the outgrown periosteal cells. We believe that this functional feature is predominantly dependent on the original architecture of the seed segment tissue that is maintained during culture expansion. Cultured human periosteal sheets are still composed of a complex multicellular layered tissue arrangement that contains several heterogeneous cell types. Therefore, from a morphological point of view, our periosteal sheet is clearly distinguishable from simpler "cell-sheets" that are composed of a single-layer of dispersed homogeneous cells.

In this study, we have confirmed that a osteogenic induction is obtained in periosteal sheet cultures grown

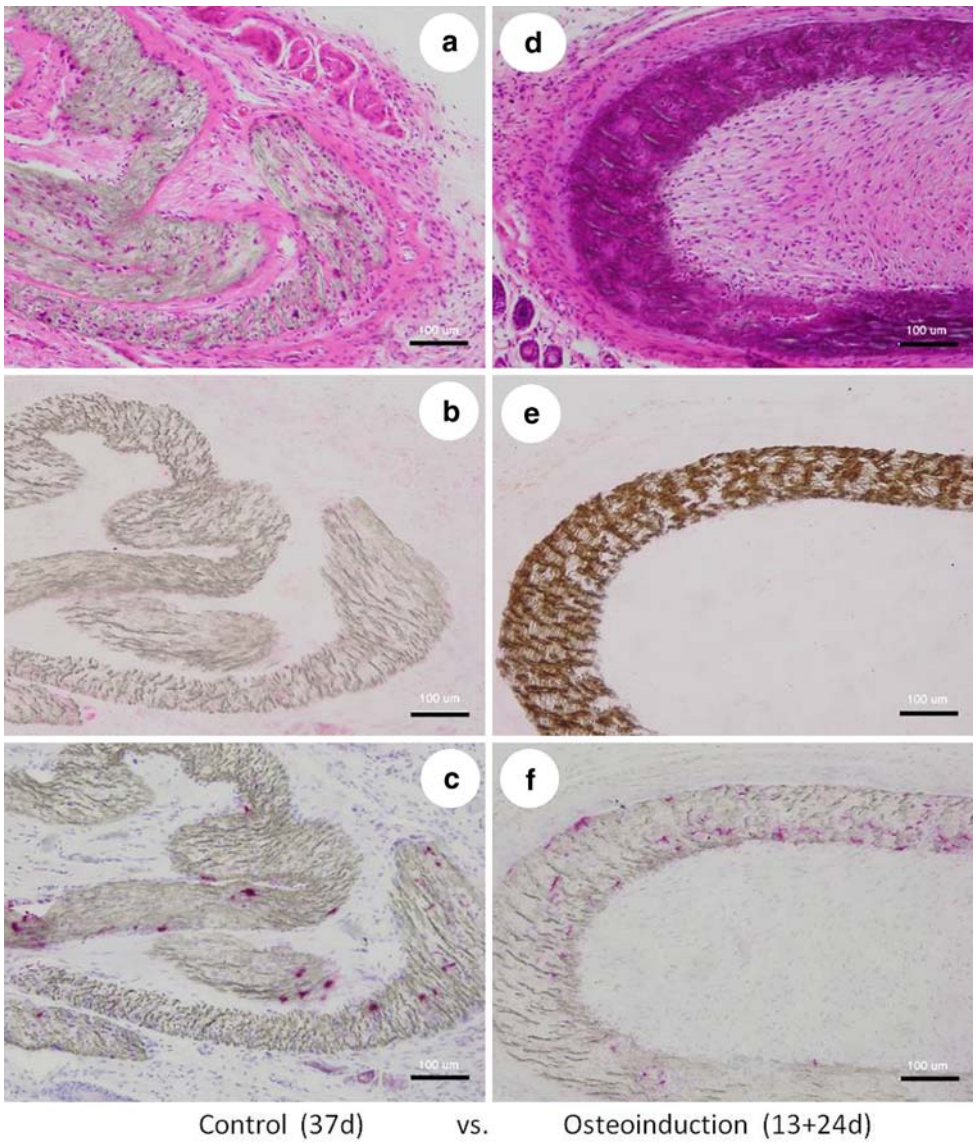

Fig. 7 Osteoid formation by implanted periosteal sheets after 8 weeks. Other than increased implantation time, the experimental protocol is as described in the legend of Fig. 6. HE-staining (**a**, **d**), von Kossa staining (**b**, **e**), TRAP-staining (**c**, **f**). The control data and the data of osteoinduction periosteal sheets are shown in Panels **a**–**c** and **d**–**f**, respectively. *Bar* = 100 μm

in salmon collagen-coated ePTFE mesh that is analogous to what we have observed before. However, the mesh seems to aid in this induction, and both in vitro mineralization and up-regulation of ALP activity were notably enhanced. Consequently, in vivo osteoid formation was also augmented by the mesh at the site of implantation. Therefore, these findings suggest that addition of the mesh augments the osteogenic induction of cultured periosteal sheets resulting in enhancement of the osteogenic activity expressed by grafted periosteal sheets.

We postulate that this successful improvement is probably due to two major merits of the mesh. One is salmon collagen used for coating of mesh fibers. There is no doubt that collagen plays a very important role in biological mineralization [6]. Because of these biological properties and its availability, type I collagen has been applied to preparation of some biomaterials, and this formulation was predicted to facilitate biomineralization in our periosteal sheet cultures. In fact, we have observed that the initial in vitro mineralization corresponded to increased ALP activity and occurred right along the mesh fibers.

The second reason, which would be more important for our purpose, is that the intricate 3D-construction of the fine fiber networks within this mesh provides better space for outgrown cells to form multicellular layers. In a periosteal sheet cultured in a dish, the number of cell-layers was more limited: Outside ϕ10 mm, the number of cell-layers was usually less than 5 (<50 μm). In contrast, the mesh provided additional cell-layers (of at least 100 μm = mesh thickness), in which outgrown cells are expected to produce ECM-rich regions upon osteoinduction. In our following animal implantation experiments, osteoid formation

was found not only in the original regions but also inside the mesh fiber networks of the peripheral regions that were previously mineralized in vitro. In support of this possibility, several articles have repeatedly demonstrated that an appropriate 3D-construction of scaffolding is important for osteoblastic differentiation of precursor cells and subsequent osteogenic activity [7–11].

In a series of our studies using various ectopic osteoid formation systems ([4, 12], Kawase et al., manuscript in preparation), we have found that TRAP-positive multinucleated cells appear after osteoid formation in most cases. Also in this study, these TRAP-positive cells appeared only in the osteoinduced sheet grafts at the initial phases of implantation. However, at 8 weeks post-implantation, significant numbers of TRAP-positive cells infiltrated into the regions surrounding the mesh fibers. Although it is possible that such cells could be attracted to newly formed mineral deposits in preparation for subsequent remodeling cycles, it is much more likely that degraded polyurethane by-products and/or reactive oxygen intermediates [13] have been exposed or generated after the digestion of the collagen fiber-coating by tissue collagenases or proteases. The mesh has now been recognized probably as a foreign body, even in nude mice.

In conclusion, in addition to the overall biological ramifications, this mesh could prove to be important clinically because the current system would enable the preparation of graft material within a short time, e.g., less than a month, and also simplify the handling of biomaterials within the clinic. If this kind of scaffold is approved for clinical use, periodontal regenerative therapy using cultured autologous periosteal sheets will become more widely applied as a promising therapeutic methodology.

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