Bone-Conditioned Medium Obtained From Calvaria, Mandible, and Tibia Cause an Equivalent TGF-β1 Response In Vitro

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Abstract: Bones with different embryological origin and mode of ossification are supposed to vary in their capacity for supporting graft consolidation. The aim of the current pilot study was to assess the TGF-β1 activity of bone chips obtained from distinct anatomic locations. Conditioned medium was prepared from bone chips harvested from pig calvaria, mandible, and tibia. Human oral fibroblasts were exposed to bone-conditioned medium (BCM) followed by reverse transcriptase polymerase chain reaction of the TGF-β1 target genes. Also an immunoassay for interleukin 11 (IL-11) and TGF-β1 was performed. The impact of BCM on alkaline phosphatase activity was determined with murine MC3T3-E1 osteogenic cells. The authors report here that BCM contains TGF-β1 in the ng/mL range. Bone chips prepared from pig calvaria, mandible, and tibia femur had a similar capacity for increasing the expression of the TGF-β1 target genes IL-11, NOX4, and PRG4. Correspondingly, immunoassays revealed similar production of IL-11 by human oral fibroblasts. Furthermore, conditioned medium obtained from the 3 bones decreased alkaline phosphatase activity in MC3T3-E1 osteogenic cells. These preliminary data demonstrate that particulated bone grafts, regardless of embryological origin, mode of ossification and morphology, release a similar TGF-β1 activity.

Key Words: Allografts, augmentation, bone regeneration, in vitro, TGF-β1

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Bone grafting is a common procedure for bone restoration in various fields of reconstructive surgery including those of oral and maxillofacial surgery as well as implant dentistry. Bone grafts can be harvested from various donor sites, including calvaria,[1–4] mandible,[1–4] and tibia,[5,6] referred to as regional[7] and distant sites,[8] respectively. Cortical bone can be applied as block grafts or as bone chips when milled or harvested with a bone scraper.[9] Block grafts are used for augmentations requiring screw fixation, while bone chips are used to fill containing defects and to reshape the contour. Processing of grafts into bone blocks or bone chips affects the process of graft consolidation; for example, bone blocks have a slower resorption rate compared with bone chips.[9,10] However, there is a debate whether the donor site affects the behavior of bone grafts during the consolidation process.

It is a widely held belief that embryological origin and mode of ossification affect the clinical properties of bone grafts.[4,11,12] At the mandibular base and the alveolar process, Meckel cartilage guides mesenchymal cells originating from the ectodermal neural crest to undergo intramembranous or direct ossification.[13] The flat bones of the cranial vault originate from unsegmented paraxial mesoderm and the neural crest, again by intramembranous ossification.[13] In both developmental processes, epithelial cells have to undergo a transition into mesenchymal cells, which can form bone. The tibia, however, originates from the lateral plate mesoderm where mesenchymal cells form cartilage that undergoes endochondral bone formation.[14] Considering that all bones undergo bone remodeling, the material properties might become similar and independent from developmental aspects.

In our previous studies, we have introduced a bioassay to evaluate the paracrine-like activity of bone chips,[15,16] in addition to proteomic analysis of the conditioned medium.[17] This bioassay reflects the activity of TGF-β released from freshly prepared bone chips or from bone chips undergoing heating,[18] radiation,[19] and exposure to antiseptic solutions.[20] The TGF-β activity is represented by the expression of TGF-β target genes including interleukin 11 (IL-11), proteoglycan 4 (PRG4), and NADPH oxidase 4 (NOX4), all of which play a possible role during bone regeneration and thus graft consolidation. Interleukin 11 is a member of the IL-6 family of cytokines and, together with BMP-2, can accelerate bone regeneration.[21] PRG4 is expressed in the superficial zone of articular cartilage[22] and supports endochondral bone formation.[23] NOX4 generates intracellular superoxide which also modulates osteoblasts BMP-2 activity.[24] Thus, the genes that are regulated by TGF-β activity within bone-conditioned medium (BCM) can target bone cells and theoretically translate into a cellular response that is relevant in graft consolidation.

TGF-β is a central regulator of bone regeneration and remodeling. Reports examined the expression of TGF-β and the respective receptors during fracture healing such as during mandibular distraction osteogenesis.[25] The function of TGF-β in this process, however, is poorly understood. TGF-β2 acts specifically during

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fracture healing to stimulate the migration of osteoblasts as well as the differentiation of osteoblasts and osteoclasts.[26] On the other hand, loss of TGF-β/Smad3 signaling stimulated callus formation by promoting osteogenesis and suppressing chondrogenesis, resulting in faster fracture healing.[27] Locally applied TGF-β1 improves the mineral density of distraction gap and load to failure,[28] and the use of demineralized bone matrix along with TGF-β1 can accelerate bone repair in a tibia fracture open osteotomy model.[29] TGF-β activity within BCM may have an impact on bone regeneration, and it is thus of possible relevance to determine whether the donor site affects the paracrine-like activity of bone grafts.

The goal of the current study is to assess whether there is a difference between embryological origin of various bone grafts on their function by using to TGF-β1 activity.

MATERIALS AND METHODS

Bone-Conditioned Medium

Bone-conditioned medium was prepared as originally described by Peng et al.[15] In brief, bone was obtained from adult pigs within 6 hours postmortem (Fleischerei Leopold Hödl, Vienna, Austria). Bone chips were harvested at room temperature under aseptic conditions from 3 sources: calvaria, mandible, and tibia with a bone scraper (Hu-Friedy, Rotterdam, The Netherlands). Bone chips were placed into sterile plastic dishes containing Dulbecco modified Eagle medium (DMEM) supplemented with antibiotics and antimycotics (all from Invitrogen Corporation, Carlsbad, CA). Five-gram bone chips were incubated with 10 mL serum-free culture medium (50% weight/volume). Bone-conditioned medium was harvested after 24 hours of incubation at 37°C in a humidified atmosphere at 5% carbon dioxide. Bone-conditioned medium was filtered sterile and kept frozen at −80°C. The stocks were thawed immediately before each experiment. The concentration of human TGF-β1 in BCM was measured with an ELISA kit (R&D Systems, Minneapolis, MN).

Primary Gingival Fibroblasts and MC3T3-E1 Cells

Human gingival fibroblasts were prepared from explant cultures of 3 independent donors after approval of the ethical committee of the Medical University of Vienna (EK No 631/2007). Patients consented for obtaining and using gingival fibroblasts from their tissue. Murine osteogenic MC3T3-E1 cells were kindly provided by Oskar Hoffman (Department of Pharmacology and Toxicology, University of Vienna). Cells were cultured in a humidified atmosphere at 37°C in growth medium consisting of DMEM, 10% fetal calf serum, and 1% antibiotics (Invitrogen Corporation). Cells were plated in growth medium at 30,000 cells/cm^2 into culture dishes.

Cell Stimulation

The following day, gingival fibroblasts were exposed to a 20% BCM or recombinant human TGF-β1 (PeproTech; Rocky Hill, NJ) at 5 ng/mL in growth medium for 24 hours, before gene expression analysis was performed. Supernatant was harvested, centrifuged, and stored frozen until subjected for immunoassay. MC3T3-E1 cells were stimulated similarly, but instead for 72 hours, before alkaline phosphatase was measured.

qRT-PCR Analysis and Immunoassay

Total RNA was isolated with the ExtractMe total RNA kit (BLIRT S.A., Gdańsk, Poland). Reverse transcription was performed with SensiFAST cDNA (Bioline, London, UK). Polymerase chain reaction was carried out with the SensiFAST SYBR ROX Kit (Bioline) on a 7500 Real-Time PCR System (both Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). Primer sequences are hIL11_F GGACAGGGAAGGGTTAAAGG, hIL11_R GCTCAGCACGACCAGGAC; hPRG4_F CAGTTGCAGGTGGCATCTC, hPRG4_R TCGTGATTCAGCAAGTTTCATC; hNOX4_F TCTTGGCTTACCTCCGAGGA, hNOX4_R CTCCTGGTTCTCCTGCTTGG; hActin_F CCAACCGCGAGAAGATGA; and hActin_R CCAGAGGCGTACAGGGATAG. The mRNA levels were calculated by normalizing to the housekeeping gene GAPDH using the ΔΔCt method. For the immunoassay, the human IL-11 Quantikine ELISA kit was used (R&D Systems).

Histochemical Staining

For alkaline phosphatase histochemistry, MC3T3-E1 cells were fixed with neutral-buffered formalin and incubated with a substrate solution containing naphthol AS-TR phosphate and fast blue BB salt (Sigma, St. Louis, MO). After rinsing with distilled water, cultures were photographed.

Micro Computed Tomography

Bone chips harvested from calvaria, mandible, and tibia were subjected to micro computed tomography (μCT) with a voxel size of 5 μm using a SCANCO μCT 50 (Scanco Medical AG, Brüttisellen, Switzerland). Scanning was done at 70 kV/100 μAs and an integration time of 500 milliseconds. Bone chips were visualized in Amira 6.0.0 (FEI Company, Hillsboro, OR).

Statistics

Data represent the results of 3 independent preparations of BCM exposed to 3 independent lines of gingiva fibroblasts ending up with n = 6 for each group. The nonparametric Friedman test was used to detect differences in treatment groups with a $P < 0.05$. No statistical test was performed with the alkaline phosphatase histochemistry.

RESULTS

BCM From the 3 Preparations of Bone Chips Increases Expression of TGF-β Target Genes

To determine whether bone chips harvested from calvaria, mandible, and tibia have a similar paracrine-like activity, the corresponding conditioned medium was subjected to bioassays. As expected, BCM provoked a strong increase in the 3 TGF-β target genes IL-11, NOX4, and PRG4. Based on the Friedman analysis, BCM harvested from calvaria, mandible, and tibia equally stimulated gene expression (Fig. 1, Table 1). In support of gene expression, the immunoassay showed no significant differences in

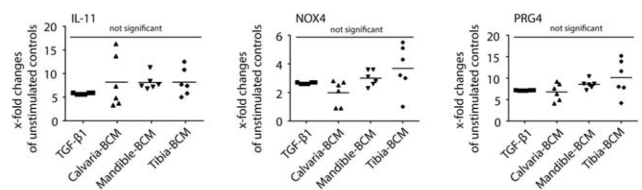

FIGURE 1. Bone-conditioned medium from the 3 preparations of bone chips increases expression of TGF-β target genes. Fibroblasts were exposed to conditioned medium prepared from bone chips harvested from calvaria, mandible, and tibia. Expression of the TGF-β target genes was determined by reverse transcription polymerase chain reaction. Data represent the results of 3 independent preparations of bone-conditioned medium exposed to 3 independent lines of gingiva fibroblasts ending up with n = 6 for each group. The nonparametric Friedman test was used to detect differences in treatment groups with a $P < 0.05$.

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TABLE 1. Relative Gene Expression of Gingival Fibroblasts Exposed to BCM

IL-11	Mean	SD	Median	Min	Max
TGF-β1	5.70	0.10	5.70	5.60	5.90
Calvaria-BCM	8.20	5.50	6.10	3.30	16.30
Mandible-BCM	8.10	1.60	7.60	6.80	11.30
Tibia-BCM	8.20	2.80	7.60	5.00	12.50
NOX4	**Mean**	**SD**	**Median**	**Min**	**Max**
TGF-β1	2.70	0.02	2.70	2.60	2.70
Calvaria-BCM	2.00	0.87	2.30	0.90	2.80
Mandible-BCM	3.00	0.52	3.00	2.30	3.60
Tibia-BCM	3.70	1.63	3.80	1.00	5.50
PRG4	**Mean**	**SD**	**Median**	**Min**	**Max**
TGF-β1	7.10	0.01	7.10	7.10	7.20
Calvaria-BCM	6.80	2.04	6.90	4.10	9.20
Mandible-BCM	8.60	1.05	8.60	7.20	10.40
Tibia-BCM	10.10	4.19	9.80	4.20	15.20

Gingival fibroblasts were exposed to BCM of different origins (calvaria, mandible, and tibia). Recombinant femur and recombinant TGF-β1 (5 ng/mL) served as control. Data represent the fold-change gene expression compared with unstimulated controls of 3 independent experiments with fibroblasts from 2 donors (n = 6).

BCM, bone-conditioned medium; IL-11, interleukin 11; NOX4, NADPH oxidase 4; PRG4, proteoglycan 4; SD, standard deviation.

the release of IL-11 into the supernatant of BCM stimulated human gingival fibroblasts (Fig. 2). Taken together, these results, together with the findings from the immunoassay, demonstrate that bone chips harvested from different anatomic locations exhibit a similar capacity to stimulate TGF-β target genes (Table 2).

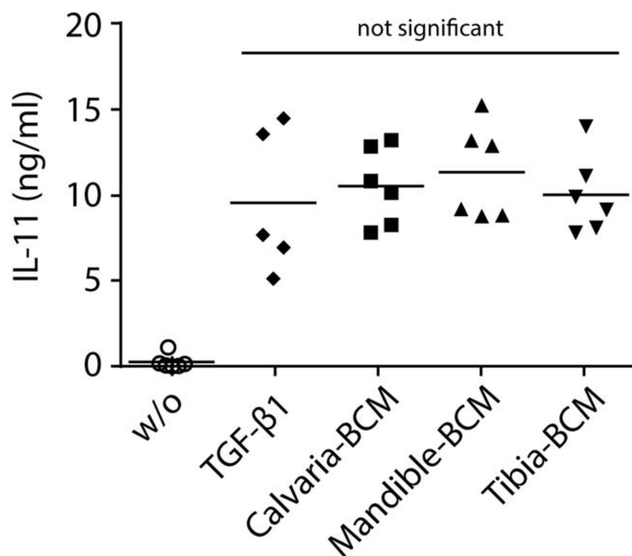

FIGURE 2. Bone-conditioned medium from the 3 preparations of bone chips stimulates IL-11 protein production. Fibroblasts were exposed to conditioned medium prepared from bone chips harvested from calvaria, mandible, tibia, and TGF-β1. IL-11 produced by the fibroblasts and released into the cell culture medium was measured with an immunoassay. Data represent the results of 3 independent preparations of bone-conditioned medium exposed to 2 independent lines of gingiva fibroblasts ending up with n = 6 for each group. The nonparametric Friedman test was used to detect differences in treatments groups with a $P < 0.05$. IL-11, interleukin 11.

TABLE 2. Total Human TGF-β1 of BCM

Preparation	No 1	No 2	No 3
Calvaria-BCM	4.03	3.29	3.52
Mandible-BCM	3.01	3.71	3.82
Tibia-BCM	4.24	4.77	4.85

BCM was prepared from porcine bone from calvaria, mandible, and tibia. Data represent the TGF-β1 amount in ng/mL of 3 independent preparations in total. Static was performed based on a Friedman test with standard weighted-means analysis $P = 0.194$.

BCM, bone-conditioned medium.

BCM From the 3 Preparations of Bone Chips Decreased Alkaline Phosphatase Activity

Next we exposed MC3T3-E1 cells to the BCM and determined alkaline phosphatase activity. Figure 3 demonstrates that BCM from bone chips harvested from calvaria, mandible, and tibia equally suppressed alkaline phosphatase based on histochemical staining. Thus, consistent with the findings of the gene expression changes, histochemical staining of alkaline phosphatase activity further supports the similar activity of BCMs harvested from calvaria, mandible, and tibia.

Bone Chips From Diverse Bone Donor Sites Have Different Morphology

We then sought a morphologic explanation for the similar TGF-β activity of the respective BCMs. We have recently shown that bone chips have a characteristic curled morphology.[9] Thus, it is

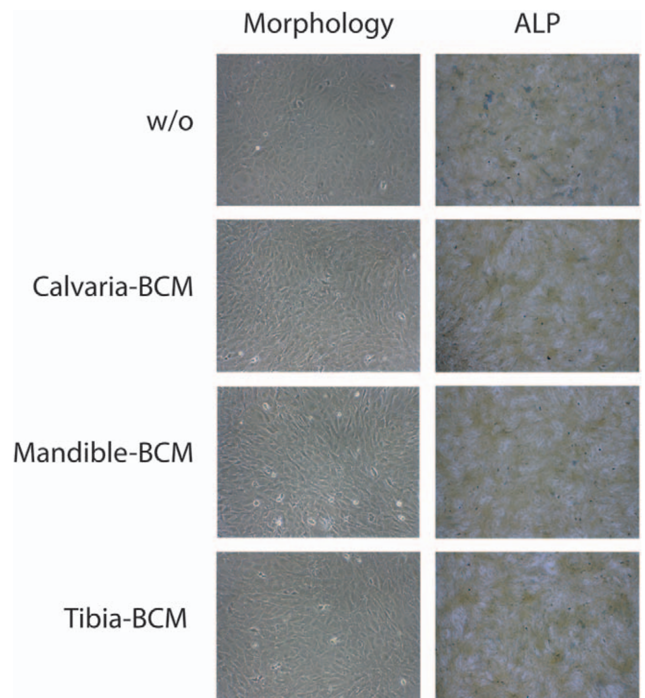

FIGURE 3. Bone-conditioned medium from the 3 preparations of bone chips decreased alkaline phosphatase activity. Mouse MC3T3-E1 osteogenic cells were exposed to conditioned medium prepared from bone chips harvested from calvaria, mandible, and tibia. Alkaline phosphatase activity was determined by histochemical staining. Data represent the results of 1 out of 2 independent experiments.

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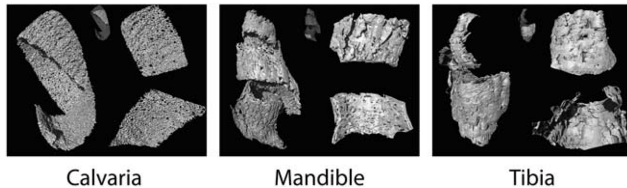

FIGURE 4. Micro computed tomography of 3 preparations of bone chips. Bone chips from calvaria, mandible, and tibia were scanned by micro computed tomography at an isotropic resolution of 5 μm and visualized in Amira software. Data represent the results of 1 out of 2 independent preparations of bone chips.

possible that also bone chips from other sites, including the calvaria and the tibia, have a similar morphology. However, measurement of the bone chips revealed considerable changes in morphology (Fig. 4). The surface of bone chips from mandible and tibia resembled broken sheets consisting of many flat connected bone flakes. These flakes appeared to be larger in the bone chips from the tibia compared with the mandible. Bone chips of the calvaria more closely resembled a slice of trabecular bone without apparent ruptures.

DISCUSSION

There is a widely held belief that the embryological origin and ossification process affects the clinical performance of bone grafts.[4,11,12] This idea has prompted us to investigate the paracrine activity of bone chips obtained from different sites—with the calvaria and calvaria originating form ectodermal neural crest and undergoing intramembranous ossification, while the tibia originates from the lateral plate mesoderm where mesenchymal cells form cartilage that undergoes endochondral bone formation.[13,14] We have now found that the conditioned medium of bone chips harvested from the calvaria, mandible, and tibia caused a comparable increase in the TGF-β target genes IL-11, NOX4, and PRG4 in oral fibroblasts. Likewise, the respective conditioned media of calvaria, mandible, and tibia decreased alkaline phosphatase in the osteogenic cell line MC3T3-E1 to a similar extent. The morphology of the bone chips, however, varies noticeably. Together, these findings establish that the paracrine-like activity of bone chips is independent of embryological origin and the ossification process of the donor site and the graft morphology.

If we relate the findings to those of others, we can refer to our own data on BCM, all representing bone chips from pig mandible[15–20] and mixed samples from human bone.[30] Others have focused on the cellular aspects related to bone chips from different sources including from the mandibles.[31] To study the paracrine-like activity of fresh bone grafts from various sites, no comparative research in vitro is available. Thus, we have to refer to clinical studies describing the properties of grafts from different sites and their impact on the process of graft consolidation and implant survival; for example, atrophic jaws can be grafted with calvarial bone blocks,[1–4] mandibular bone blocks,[3,32,33] and tibial bone blocks.[5,6] Studies on particulated bone grafts or bone chips were performed in self-containing defect or with a titanium mesh, with mandibular bone and tibia.[34] Thus, there is evidence that bones from different sources, all characterized by their cortical nature, show a reliable performance in vivo. Our in vitro data, with the limitation of the in vitro approach, support these clinical observations.

Our findings that the morphology of the bone chips varies considerably depending on the original bone source, were unexpected considering that the surface area of the bone chips would influence the activity of the corresponding conditioned medium. We originally performed the μCT to correlate the surface of the bone chips with the TGF-β response. It would have been reasonable to propose an association of the bone surface and the respective TGF-β activity of the conditioned medium. However, the surface characteristics were rather dissimilar at the nanoscale, so we had to reject this aim. Since this was not the patient, it can be speculated that fibroblasts are already stimulated at a maximum, even though the BCM contains different amounts of TGF-β. Thus, future research should focus on the half-maximal stimulation of TGF-β target genes, rather than exposing cells to 20% BCM. We can thus not rule out that the morphology of the bone chips affects the activity of the conditioned medium. It would be attractive to investigate the impact of the morphology of bone chips, obtained from 1 source, on the activity of the respective conditioned medium in vitro.

The clinical relevance of the present in vitro findings is limited to the paracrine activity of the BCM and the selection of TGF-β target genes, as well as the decrease in alkaline phosphatase activity. Clearly, the model remains preliminary as other bone-derived growth factors besides TGF-β also play a role during graft consolidation, and these growth factors are not considered in our bioassay. TGF-β also has isoforms; however, bone stores around 50-times more TGF-β1 than TGF-β2.[35] It has to be assumed that TGF-β activity is an indicator for possible other factors since it was not the intension of this research to study the myriad of factors in BCM [17] and their respective biological response in a bioassay.[16] The clinical relevance of the TGF-β activity released from bone chips, in particular during the process of graft consolidation, remains a matter of debate as long as we have no preclinical data. Nevertheless, the present in vitro study adds 1 piece to our understanding of bone graft biology, particularly the impact of the anatomic site on graft consolidation.

Future research should investigate the in vivo behavior of bone grafts from different sources such as the calvaria, mandible, and tibia; for example, using bone chips placed in 3-wall defect sites in larger animals for simulations comparison.[36] Other limitations which should be addressed in future research are the process of preparing the bone chips, which was a bone scraper in this study; so possibly a bone mill may change the paracrine-like activity of the bone chips. Moreover, specimens for fibroblasts were obtained from 3 patients only. This is a minor limitation because they were used as a bioassay with 3 independent preparations of bone conditioned medium, together resulting in 9 data points allowing a robust statistical analysis. Also the question as to whether the time between animal euthanasia and the preparation of the bone chips affects the release of TGF-β activity requires further research. Another point of critique may be the cross-species study design. We do not consider this a problem since particular TGF-β is conserved among the species and the bioassays are sensitive and therefore appropriate for our purpose. Ideally, fibroblasts of porcine origin could have been used; however, we have established our system with cells of human origin. This research can be extended to the preparation of conditioned medium from allogenic bone, including demineralized freeze-dried bone, which also holds a TGF-β activity to be released in a similar setting.[37,38] Thus, the present in vitro data should be interpreted with care and the translation of the bioassay into a predictable in vivo response requires additional research. Thus, for the evaluation of bone graft performance a preclinical or even a randomized controlled clinical trial in humans can be proposed.

In conclusion, the findings presented here indicate that bone chips with a different embryological origin release a similar TGF-β1 activity. Therefore, impact of the embryological origin on the clinical performance of bone grafts cannot be explained by a differential release of TGF-β activity.

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