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Conditioned medium from fresh and demineralized bone enhances osteoclastogenesis in murine bone marrow cultures

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Key words: allograft, autografts, autologous bone, bone regeneration, bone-conditioned medium, demineralized freeze-dried bone, graft consolidation, osteoclast, paracrine, TGF- β

Abstract

Objectives: Osteoclasts rapidly form on the surface of bone chips at augmentation sites. The underlying molecular mechanism, however, is unclear. Soluble factors released from bone chips *in vitro* have a robust impact on mesenchymal cell differentiation. Whether these soluble factors change the differentiation of hematopoietic cells into osteoclasts remains unknown.

Methods: Osteoclastogenesis, the formation of tartrate-resistant acid phosphatase-positive multinucleated cells, was studied with murine bone marrow cultures exposed to RANKL and M-CSF, and conditioned medium from fresh (BCM) and demineralized bone matrix (DCM). Histochemical staining, gene and protein expression, as well as viability assays were performed.

Results: This study shows that BCM had no impact on osteoclastogenesis. However, when BCM was heated to 85°C (BCMh), the number of tartrate-resistant acid phosphatase-positive multinucleated cells that developed in the presence of RANKL and M-CSF approximately doubled. In line with the histochemical observations, there was a trend that BCMh increased expression of osteoclast marker genes, in particular the transcription factor *c-fos*. The expression of *c-fos* was significantly reduced by the TGF- β receptor I antagonist SB431542. DCM significantly stimulated osteoclastogenesis, independent of thermal processing.

Conclusions: These data demonstrate that activated BCM by heat and DBM are able to stimulate osteoclastogenesis *in vitro*. These *in vitro* results support the notion that the resorption of autografts may be supported by as yet less defined paracrine mechanisms.

Bone augmentation is a common procedure in oral, maxillofacial, and orthopedic surgery (Chiapasco et al. 2009; Myeroff & Archdeacon 2011; Buser et al. 2013). Malformation, trauma, and dental implant surgery can make bone grafts necessary for reconstruction surgery (Chiapasco et al. 2009). Autografts have unique characteristics, which make them a favorable material for bone regeneration; they contain living cells that can contribute to bone formation (Bohr et al. 1968), serve as a scaffold (Saulacic et al. 2014), and contain bioactive molecules that are released from the bone matrix upon resorption (Crane & Cao 2014). This trinity of properties is known as osteogenicity, osteoconductivity, and osteoinductivity (Khan et al. 2005; Grabowski & Cornett 2013). It does not rule out that there may be further properties, as yet poorly defined, which add to the existing

knowledge of how autografts modulate bone regeneration.

The paracrine function, or at least the passive release of soluble factors of harvested bone, can be considered the fourth property of autografts and should be distinguished from the endocrine function of orthotropic bone in whole-organism physiology (Karsenty & Ferron 2012). Proteomic analysis detected numerous molecules, including growth factors, in bone-conditioned medium (BCM) (Caballé-Serrano et al. 2014) and has therefore extended existing knowledge of the composition of whole bone (Kupcova Skalninkova 2013; Romanello et al. 2013). Osteocyte-derived molecules are also found in BCM (Brolese et al. 2014), and BCM potently affects the differentiation of mesenchymal cells *in vitro* (Peng et al. 2014). Thus, there is increasing evidence that freshly prepared

Date:
 Accepted 29 January 2015

To cite this article:

Caballé-Serrano J, Schuldt Filho G, Bosshardt DD, Gargallo-Albiol J, Buser D, Gruber R. Conditioned medium from fresh and demineralized bone enhances osteoclastogenesis in murine bone marrow cultures.
Clin. Oral Impl. Res. 27, 2016, 226–232
 doi: 10.1111/clr.12573

bone chips release bioactive molecules, thereby supporting the hypothesis that autografts have a transient paracrine-like function.

Resorption of autografts is a clinical challenge and occurs early during graft consolidation. Multiple osteoclasts rapidly appear when bone chips are placed into bone defects (Saulacic et al. 2014), likely explaining why no residual autografts were detectable 2 years after contour augmentation (Jensen et al. 2014). Resorption occurs with bone harvested from different anatomical regions and placed into different augmentation sites, for example, bone harvested from the iliac crest or ramus and used for sinus floor elevation or ridge augmentation (Spin-Neto et al. 2013; Schmitt et al. 2014). Bone graft resorption is thus not only a clinical principle in regenerative medicine, but also an evolutionary principle removing dead bone after injury or disease. The molecular mechanisms controlling osteoclastogenesis on the surface of bone grafts are unknown. It is possible that molecules released by the bone grafts initiate or at least support the process of osteoclastogenesis.

Osteoclasts originate from hematopoietic stem cells when the key factor – receptor activator of nuclear factor kappa-B ligand (RANKL) – is present (Suda et al. 1999). Osteoclastogenesis also requires the M-CSF receptor (c-fms) (Hofstetter et al. 1992). Osteoclasts are characterized by their multinucleated morphology and the expression of tartrate-resistant acid phosphatase (TRAP), cathepsin k (CatK), and the calcitonin receptor (CTR). Osteoclasts express costimulatory molecules activating the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway (Koga et al. 2004). Osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells 2 (TREM2) are receptors that are associated with the respective adaptor molecules Fc receptor common gamma chain (FcR γ) and DNAX-activating protein 12 kDa (DAP12), respectively. Downstream signaling pathways culminate in the increased expression of transcription factors c-fos and nuclear factor of activated T cells c1 (NFATc1), both master regulators of osteoclastogenesis. Moreover, microphthalmia-associated transcription factor (MITF) and PU.1 can modulate osteoclastogenesis (Sharma et al. 2007). Also relevant are genes that regulate cell fusion, that is, dendritic cell-specific transmembrane protein (DC-STAMP) and the ATPase, H⁺ transporting, lysosomal 38 kDa, and V0 subunit d2 (Atp6v0d2) (Lee et al. 2006; Miyamoto et al.

2012). This study determined the expression levels of the respective genes to understand the role of BCM during osteoclastogenesis *in vitro*.

TGF- β is a heat-stable molecule present in BCM (Caballé-Serrano et al. 2014; Peng et al. 2014) and in conditioned medium from demineralized bone (DCM) (Schuldt Filho et al. 2015) that can considerably increase osteoclastogenesis in bone marrow cultures (Fuller et al. 2000). Thus, current evidence supports the idea that BCM can enhance osteoclastogenesis via a TGF- β -dependent mechanism but not involving c-fos (Fox et al. 2008). Proteomic analysis of BCM (Caballé-Serrano et al. 2014) further revealed other molecules that can stimulate osteoclastogenesis, such as annexin A2 (Menaar et al. 1999) – but maybe more interestingly, also molecules that inhibit osteoclastogenesis, such as apolipoprotein E (Kim et al. 2013), lactotransferrin (Lorget et al. 2002), macrophage migration inhibitory factor (Jacquin et al. 2009), and angiogenin (Morita et al. 2008). Thus, it remains unclear whether the pro-osteoclastogenic or the anti-osteoclastogenic molecules dominate in BCM. The bioassay presented integrates the overall impact of BCM and DCM on the complex process of osteoclastogenesis *in vitro*.

Material and methods

Bone-conditioned medium

Bone-conditioned medium (BCM) was prepared from porcine fresh cortical bone chips as described recently (Caballé-Serrano et al. 2014; Kuchler et al. 2014; Peng et al. 2014). In brief, bone was obtained from adult pigs within 6 h postmortem (Metzger Meinen, Bern, Switzerland). Cortical bone chips were harvested from the mandible and placed into sterile plastic dishes containing DMEM supplemented with antibiotics and antimycotics at 50% (wet weight/volume) (all Life Technologies, Grand Island, NY, USA). BCM was collected after 24 h, filtered sterile, and kept frozen at -20°C . The stocks were thawed immediately before the experiments. BCM was also heated at 85°C for 10 min. If not otherwise indicated, BCM was fourfold. Variations in BCM activity can occur due to various factors, such as techniques used for harvesting, individual characteristics of the animals, and handling of the samples.

Demineralized bone matrix-conditioned medium

Demineralized bone matrix-conditioned medium (DCM) was prepared from porcine cortical bone blocks. Bone blocks were cut from

the buccal side of the porcine mandibles and trimmed to obtain pieces measuring 3 mm in thickness and 12 mm in length. Bone blocks were subjected to defatting and demineralization with 0.5 M HCL at 4°C for 24 h. Defatting was performed twice with acetone and methanol (1 : 1). Residual acid was neutralized by repeatedly washing the bone blocks with demineralized water for 30 min. Following elimination of residual acid, samples were freeze-dried and ground. After 24 h of incubating the demineralized bone matrix with serum-free DMEM containing antibiotics and antimycotics, conditioned medium was filtered sterile and kept frozen at -20°C . Variations on the DCM activity can occur due to various factors, such as processing of the bone.

Osteoclastogenesis in murine bone marrow cultures

Bone marrow cells were prepared by flushing the femur and tibiae of 4- to 6-week-old female mice (strain Balb/c) and seeded at one million bone marrow cells per cm^2 in Eagle's minimum essential medium- α modification (α MEM) supplemented with 10% fetal calf serum (FCS), antibiotics, and macrophage colony-stimulating factor (M-CSF) at 30 ng/ml for 48 h. For osteoclastogenesis, medium was supplemented with M-CSF at 30 ng/ml and soluble receptor activator of nuclear factor kappa-B ligand (RANKL) at 30 ng/ml for 3 days. BCM was used at a fourfold dilution as recently reported (Peng et al. 2014). Recombinant proteins were purchased from Prospecc (Ness-Ziona, Israel). SB431542 was used at 10 μM (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 4 days, histochemical staining for TRAP (Sigma Aldrich, St. Louis, MO, USA) was performed. Cells were considered osteoclasts when positive for TRAP and having three or more nuclei as observed by light microscopy. For the resorption assay, bone marrow cultures were performed on dentin disks, which were cleaned with ultrasound and stained with toluidine blue (1%) to show resorption pits.

Expression of marker genes in murine bone marrow cultures

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Rotkreuz, Switzerland). Reverse transcription (RT) was performed with Transcriptor Universal cDNA Master, and PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) or the FastStart Universal Probe Master Rox on a 7500 Real-Time PCR System (Roche).

Table 1. Primers for RT-PCR

Gene	Forward primer	Reverse primer
m c-fms	gaccatggtgaatggtagg	ggataacgttgatccactg
m c-fos	gcaacttctatgacactgaaacac	tctcttagggctgactgg
m ACTA2	ctctcttcagccatcttctcat	tatagggtggttcgtggatgc
m Atp6Od2	aagcctttgtttgacgtgt	gccagcacattcatctgtacc
m BUB1	tctgtaagtggccagtcatt	tgaattcatgaacactttgattcac
m CTGF	tgacctggaggaaaacattaaga	agccctgtatgtcttcacactg
m DC-Stamp	aagctccttgagaaacgatca	caggactggaaaccagaaatg
m IL11	ctgtggggacatgaactgtg	aggggcaacgactctatctg
m MIK167	gctgtcctaagacaatcatca	ggcgttatccaggagact
m MITF	gacaccagccataaacgtca	ttttcagggtgggtctgc
m MYBL2	gccataaagtcctgggtaac	cacagcattgtcgtctctc
m NFATc-1	ccgtgtctccagaaaataaca	tgtgggatgtgaactcgga
m PLK1	ttgtagttttgagactctgtcg	cagtgcctctctctctgt
m PU.1	ggagaagctgatggcttg	caggcgaatctttcttgc
m RANK	gtgtgtctgttccactg	agatgtcataatgcctctct
m TRAF6	ttgcacattcaggtttttgg	tgcaagtgtcgtgccaag
m β -actin	ctaaggccaaccgtgaaaag	accagaggcatacaggga

Probes for CTR, TRAP, CatK, OSCAR, TREM2, Fc γ , DAP12 and β -actin were obtained from TaqMan Gene Expression Assays service (Applied Biosystems). All other primers were designed with the online Universal Probe Library System (Table 1). The mRNA levels were calculated by normalizing to the housekeeping gene beta-actin using the Δ Ct method.

Western blot analysis

For Western blot analysis, RAW 264.7 cells, kindly provided by Jürg Gertsch (Institute of Biochemistry and Molecular Medicine, University of Bern), were serum-starved overnight and stimulated with fourfold BCM for 30 min. Cells were lysed in SDS buffer containing protease and phosphatase inhibitors (Roche). Cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, binding of the first antibody raised against phospho Smad3, phospho JNK, phospho p38 MAPK (all Cell Signaling Technology, Danvers, MA, USA), phospho ERK, and β -actin (both Santa Cruz Biotechnology, SCBT) was detected with the appropriate secondary antibody (LI-COR Biosciences; Lincoln, NE, USA), directly labeled with near-infrared dyes and visualized with the appropriate imaging system (LI-COR).

BCM incubation with blood fresh-frozen plasma

In indicated experiments, BCM was incubated with human fresh-frozen plasma (Österreichisches Rotes Kreuz, Austria) at 1 : 1. Coagulation was activated by adding 5 U/ml thrombin from bovine plasma (Sigma, St Louis, MO, USA). After 12 h of incubation at 37°C, samples were centrifuged and super-

natant was immediately used in bone marrow cultures at a twofold dilution.

Cell viability and proliferation

For cell viability, bone marrow cells were exposed to BCMh for 24 h and staining was performed (Live-Dead cell staining kit; Enzo Life Sciences AG, Lausen, Switzerland) where green cells are alive and red cells are dead. To further understand the impact of BCMh

on proliferation, expression analysis of relevant genes such as myb-related protein B (MYBL2), serine/threonine protein kinases BUB1 (budding uninhibited by benzimidazoles 1), polo-like kinase 1 (PLK-1), and antigen identified by monoclonal antibody Ki-67 (MKI67) was investigated.

Statistical analysis

Data are reported using dot plots showing all data points and the median. Experiments were repeated at least four times in duplicates. Paired *t*-test was used to analyze RT-PCR data of the controls vs the stimulated groups. *T*-test was calculated with EXEL and *P*-values indicated in the figures. Figure 1 was based on post-ANOVA comparisons with the Tukey's HSD test (SPSS Software, IBM, NY, USA). In Supplement figures, no statistical analysis was used due to a small sample size.

Results

BCMh supports RANKL-induced osteoclastogenesis *in vitro*

To investigate the impact of BCM on osteoclastogenesis, the number of TRAP⁺ MNCs in the presence of RANKL and M-CSF was deter-

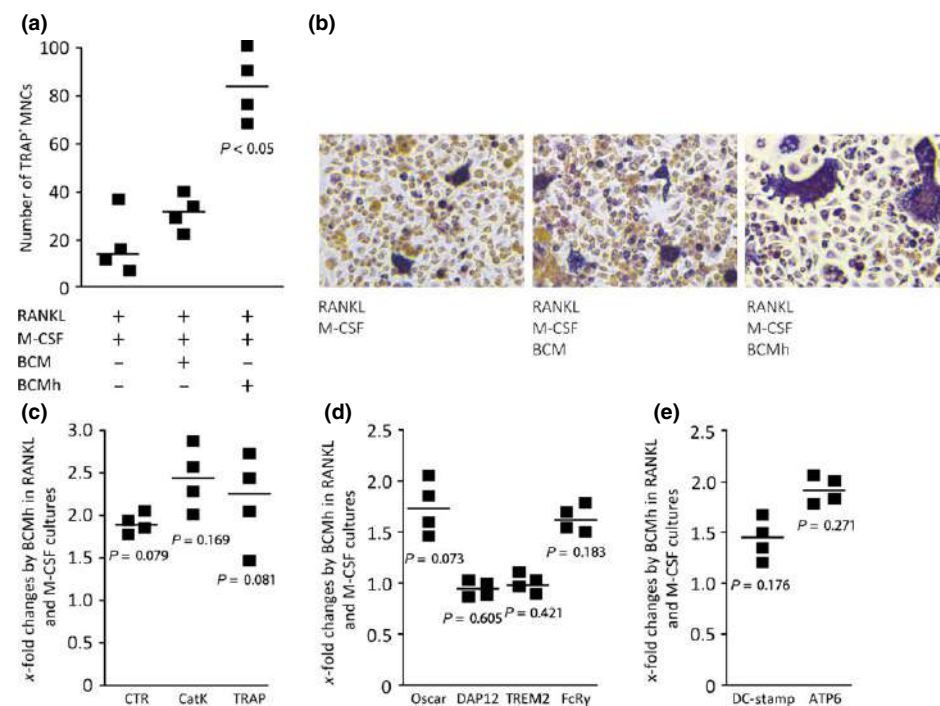


Fig. 1. BCMh supports RANKL-induced osteoclastogenesis *in vitro*. Murine bone marrow cultures were grown in the presence of RANKL and M-CSF supplemented with BCM and BCMh at a fourfold dilution. (a, b) The number and size of TRAP⁺ MNCs increased significantly in the presence of BCMh. Cells counted positive had three or more nuclei. (c) Expression of osteoclast differentiation markers CTR, CatK, and TRAP approximately doubled in the presence of BCMh. (d) BCMh also increased expression of osteoclast costimulatory molecules OSCAR and Fc γ , with the costimulatory molecules DAP12 and TREM2 remaining unchanged. (e) Expression of osteoclast fusion gene DC-Stamp was increased. *P* < 0.05 compared to the two other groups.

mined. As shown in Fig. 1a,b, BCM failed to cause any visible changes in osteoclastogenesis. As TGF- β can enhance osteoclastogenesis (Fuller et al. 2000), TGF- β is found in BCM (Peng et al. 2014), and TGF- β can be activated by heat (Brown et al. 1990), BCM was exposed to 85°C for 10 min. The presence of BCMh increased the number of TRAP⁺ MNCs in the presence of RANKL and M-CSF (Figs 1a,b and S1), but not in the presence of fresh-frozen plasma (Fig. S2). There was also a trend that BCMh increased the expression of osteoclast markers and fusion genes (Fig. 1c,d). A resorption assay performed on dentin disks confirmed the activity of osteoclasts, but no quantification was performed because of the large variations within and between the experiments (Fig. S3). It is noteworthy to mention that most but not all preparations of BCMh supported osteoclastogenesis (data not shown). Together, these findings show that BCMh moderately enhances osteoclastogenesis *in vitro*.

BCMh increases osteoclast signaling molecule expression

To determine whether the effect of BCMh involves increased responsiveness of cells to RANKL or M-CSF, the expression levels of the respective receptors RANK and c-fms were determined. BCMh increased expression of RANK by around twofold but did not increase expression of c-fms (Fig. 2a). There was also a trend that BCMh increased expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, NFATc1, and c-fos (Fig. 2b). Expression of PU.1 and MITF was not changed considerably (Fig. 2b). Taken together, BCMh moderately enhanced the expression of some but not all osteoclast signaling molecules.

BCMh-induced c-fos expression requires TGF- β signaling

In line with a potential role of TGF- β in mediating the effect of BCMh on osteoclastogenesis, blocking of TGF- β R1 kinase with SB431542 decreased c-fos expression (Fig. 3a). TRAP staining showed the suppression of osteoclastogenesis by SB431542 (Fig. 3b). Further support for a potential role of TGF- β signaling in the bone marrow macrophages comes from observations that BCMh increased the respective target genes IL11, ACTA2, and CTGF in an SB431542-dependent manner (Fig. 4a) and that BCMh slightly stimulated phosphorylation of SMAD3 and p38 in a macrophage cell line (Fig. 4b). Together, these data support the assumption that BCMh mediates at least part of its activity via TGF- β signaling.

DCM supports RANKL-induced osteoclastogenesis *in vitro*

To determine whether DCM, previously prepared by acid demineralization, which possibly activates TGF- β (Schuldt Filho et al. 2015), had an effect on osteoclastogenesis, the number of nuclei per TRAP⁺ MNCs in the presence of RANKL and M-CSF was determined (Fig. 5a,b). DCM increased expression of osteoclast differentiation markers CTR, CatK, and TRAP (Fig. 5c). Expression levels of cell fusion markers DC-Stamp and Atp6v0d2 were also significantly enhanced (Fig. 5d). These findings are in line with the basic concept that acid-activated TGF- β supports osteoclastogenesis in the presence of RANKL and M-CSF.

BCMh does not affect cell viability and proliferation

To assess whether BCM could affect cell viability, live-dead staining of cultures supplemented with BCM and genetic expression analysis of cell-cycle-related genes was executed. Live-dead staining, where green indicates viable cells and red indicates dead cells, showed no differences between the groups with and without BCMh (Fig. S4A). To determine whether BCMh affected proliferation of bone marrow cultures, expression analysis of the cell-cycle-related genes MYBL2, BUB1, PLK1, and MIK167 was performed. Expression of cell-cycle-related genes was not affected by BCM (Fig. S4B). Therefore, cells remain viable but are not forced to expand when exposed to BCMh.

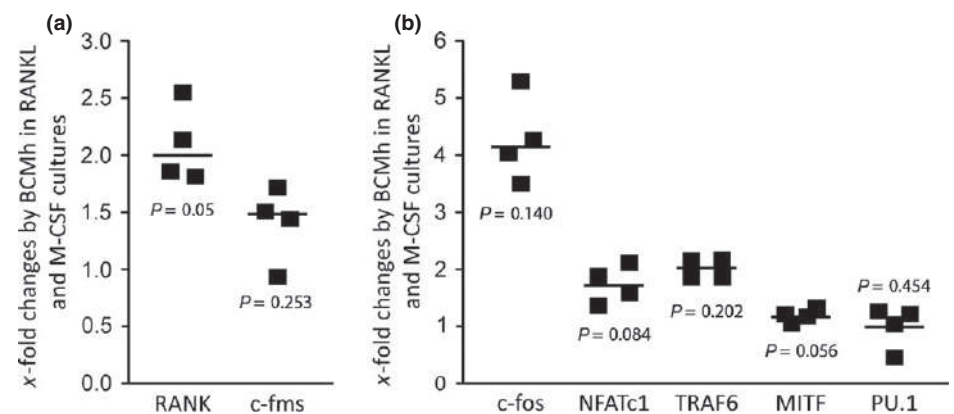


Fig. 2. BCMh increased expression of RANK, signaling molecules, and activity of osteoclasts. To analyze whether the effect of BCMh on bone marrow macrophages was due to increased responsiveness to RANKL or M-CSF, expression of RANK and c-fms receptors was determined. (a) Expression of the RANK receptor was notably increased in the presence of BCMh compared to cultures with RANKL and M-CSF (RM) alone. (b) Expression of TRAF6 and the respective downstream master regulators of osteoclastogenesis, c-fos and NFATc1, was enhanced in the presence of BCMh. Expression of RANK regulators PU.1 and MITF was unchanged.

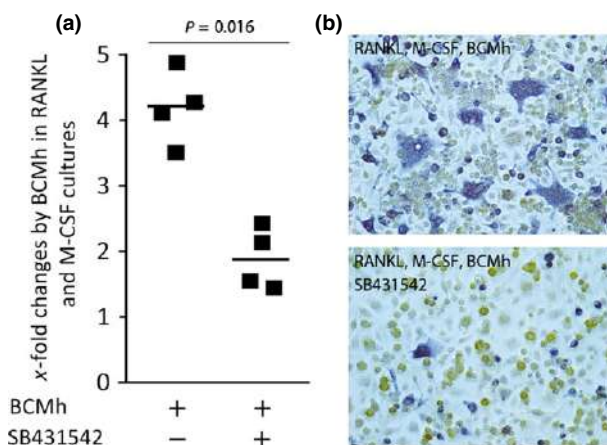


Fig. 3. SB431542 partially blocks the impact of BCMh on c-fos expression. To understand how BCMh exerts its effect on osteoclastogenesis, the TGF- β R1 antagonist (SB431542) was used. (a) SB431542 reduced but did not block the expression of c-fos. (b) Addition of SB431542 to cultures with RM and BCMh abolished the formation of TRAP⁺ MNCs. TRAP staining is representative for two experiments with two replicates.

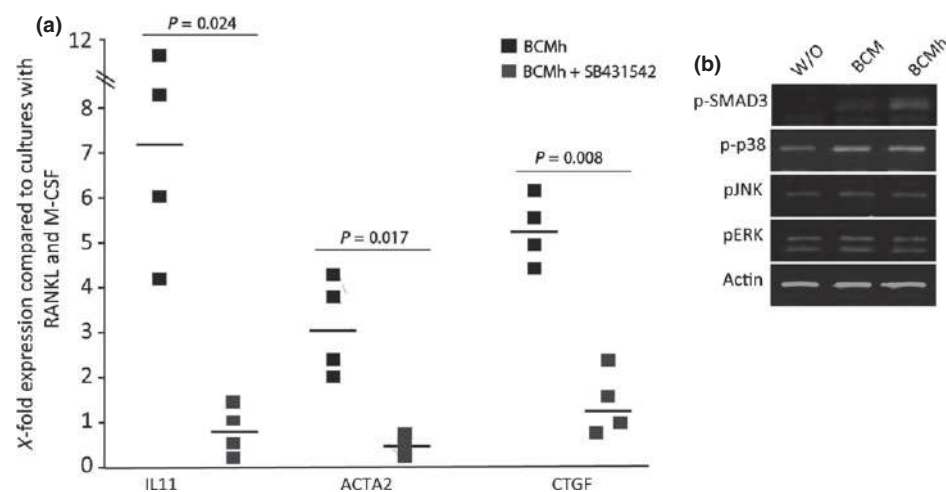


Fig. 4. BCMh does not affect cell viability and proliferation. (a) BCMh increased the expression of respective target genes IL11, ACTA2, and CTGF in bone marrow cultures, which were blocked by the inhibitor for TGF- β 1 kinase, SB431542. (b) BCMh stimulated phosphorylation of SMAD3 and p38 in the macrophage cell line RAW 264.7. Western blot data are representative for three experiments with one replicate.

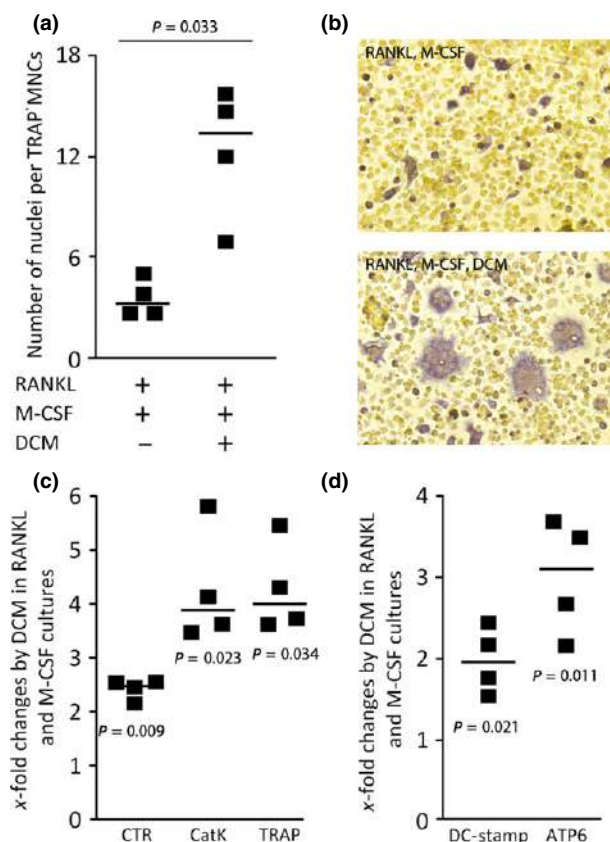


Fig. 5. DCM supports RANKL-induced osteoclastogenesis *in vitro*. Murine bone marrow cultures were grown in the presence of RANKL and M-CSF supplemented with DCM at a fourfold dilution. (a, b) The number of nuclei per TRAP⁺ MNCs was increased significantly in the presence of DCM. (c, d) Expression of osteoclast differentiation markers CTR, CatK, and TRAP was increased up to fivefold in the presence of DCM. DCM also increased expression of fusion genes DC-Stamp and Atp6v0d2.

Discussion

Resorption of autografts and allografts remains a major challenge in implant den-

tistry and other fields dealing with bone reconstruction. Osteoclasts have been detected early, within days, on the surface of transplanted bone (Saulacic et al. 2014). The

underlying molecular mechanism, however, remains unknown. There is reason to suggest that soluble factors released from transplanted bone support osteoclastogenesis, particularly because BCM and DCM contain TGF- β (Peng et al. 2014; Schuldt Filho et al. 2015), and TGF- β supports *in vitro* osteoclastogenesis (Fuller et al. 2000). The results of the present work generally support this notion, but with one premise: BCM had to be exposed to 85°C to enhance osteoclastogenesis. On the basis of these findings, it can be concluded that *in vitro*, soluble factors released from bone chips can enhance osteoclastogenesis.

If we relate our findings to those of other authors, BCM greatly increased IL11 expression in mesenchymal cells, which was inhibited by blocking TGF- β signaling (Peng et al. 2014). Also in the present study, BCMh increased IL11 and other TGF- β target genes in bone marrow cultures, again requiring TGF- β signaling. Increased phosphorylation of the TGF- β signaling molecules Smad3 and p38 was also observed in the present study (Cicek et al. 2011; Lou et al. 2013; Peng et al. 2014). Further support for TGF- β signaling comes from observations that SB431542 reduced BCMh-induced c-fos expression (Wygrecka et al. 2012). Also consistent with the present hypothesis, TGF- β is secreted in a latent form and needs to be activated (Miyazono et al. 1988; Barcellos-Hoff 1996; Koli et al. 2001; Hyytiäinen et al. 2004), for example by heat or low pH (Brown et al. 1990). What was surprising, however, was that TGF- β signaling in mesenchymal cells was achieved without heating BCM (Peng et al. 2014). Also, the effects of recombinant TGF- β are usually stronger than those observed with BCMh (Fuller et al. 2000). Another explanation for the enhanced osteoclastogenesis could be the increased responsiveness to RANKL, as BCMh considerably increased expression of RANK. Thus, the effect of BCM on osteoclastogenesis is likely more complex than simply blaming it on TGF- β .

The clinical relevance of the present findings can be related to the long-recognized need to understand the mechanisms of bone graft resorption. This information can be used to advance current clinical concepts to predict the outcome of bone augmentation. Early resorption is not restricted to bone grafts, however (Saulacic et al. 2014). Early expression of RANKL (Kon et al. 2001) and inflammatory cytokines such as TNF- α (Gerstenfeld et al. 2003), both involved in osteoclastogenesis, is fundamental for fracture healing in mice. Osteoclastogenesis is also a

hallmark of the early phase following tooth extraction (Cardaropoli et al. 2003) and implant insertion (Vasak et al. 2013). Overall, osteoclastogenesis and bone resorption precede osteoclastogenesis and bone formation – which is a general biological principle in bone regeneration. Whether the function of particulated bone, exemplified by the rather weak *in vitro* impact of BCMh and DCM on osteoclastogenesis, explains the rather strong *in vivo* osteoclastogenesis remains to be determined.

Many questions remain to be answered. First, it is unclear whether the stimulation of osteoclastogenesis by BCMh is due to heat-labile inhibitors or heat-activated stimulators of osteoclastogenesis, or a mixture of both. Second, we cannot simulate *in vitro*, if and how BCM will be activated *in vivo*, considering that even if unheated, BCM has potent effects on mesenchymal cells (Peng et al. 2014). Our attempt to use fresh-frozen plasma to simulate the proteases of blood clots, and thereby activate BCM, failed because of the strong inhibition of osteoclastogenesis (Agis et al. 2013). Third, it remains unclear whether BCMh and DCM use the same mechanisms to stimulate osteoclastogenesis. Fourth, it is still uncertain whether the changes in c-fos caused by BCMh, the partial implication of TGF- β signaling, and

the phosphorylation of Smad3 and other kinases have implications for osteoclastogenesis. Fifth, it is surprising that BCM changes the expression of osteoclast genes to almost the same extent that BCMh does, but only the latter preparation visibly supports osteoclastogenesis after histochemical staining of TRAP. Finally, BCM mainly contains proteins released because of mechanical deterioration of the bone matrix and from the remaining viable cells, including osteocytes (Brolese et al. 2014; Caballé-Serrano et al. 2014) – but not selectively those being actively released by osteoclasts during bone resorption (Crane & Cao 2014). Many questions remain unanswered. Nonetheless, the present data provide a scientific basis for more in-depth research in the field of bone regeneration – and specifically, in graft resorption.

Future studies should determine the role of osteocytes as a source of paracrine factors and their role in osteoclastogenesis during bone regeneration in general and in particular the resorption of autologous bone grafts. Osteocytes are important because their apoptosis initiates osteoclastogenesis in mouse models (Tatsumi et al. 2007) and because they also control this process during normal bone remodeling, even being responsible for the adaptation of bone to loading (Nakashima

et al. 2011; Xiong et al. 2011). Nevertheless, we have no direct evidence that osteocytes contribute to the activity of BCMh in osteoclastogenesis; exploring this remains a central aim of future studies. Furthermore, a genome-wide microarray of hematopoietic cells subjected to BCM would possibly provide valuable clues to a deeper understanding of the paracrine function of particulated autologous or demineralized and freeze-dried allogenic bone at regeneration sites.

In conclusion, this pioneer study presents preliminary data on how soluble factors released from bone grafts affect osteoclastogenesis. This information likely serves as one piece of a puzzle allowing us to understand the complex process of graft resorption within the overall sequential process of graft consolidation.

Acknowledgements: We would like to thank Catherine Solioz for skillful technical assistance. Kosaku Sawada helped harvest the bone chips and prepare the BCM. Jordi Caballé-Serrano received a scholarship from the Foundation of Dental Research and Education, Basel, Switzerland. No other external support was received.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Independent preparations of BCMh, with equally enhanced osteoclastogenesis shown by purple TRAP staining, compared to cultures with RANKL and MCSF alone.

Figure S2. BCM incubated with fresh frozen plasma (FFP) failed to reverse negative effects of blood plasma on osteoclastogenesis.

Figure S3. Activity of osteoclasts was assessed by resorption of dentin discs, indicating that also isolated osteoclasts exposed to BCMh were capable of resorbing bone. No attempts were made to quantify dentine resorption.

Figure S4. BCMh does not affect cell viability and proliferation.