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ORIGINAL ARTICLE

A novel three-dimensional bone chip organ culture

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Abstract
Objectives The objective of this study was to develop a 3D bone chip organ culture model. We aimed to collect in vitro evidence of the ability of vital bone chips to promote new bone formation.
Materials and methods We developed a 3D in vitro hypoxic bone chip organ culture model. Histology of the bone chips was performed before and after culture and immunohistochemistry after 3-week culture. The 3D culture supernatants were tested for the presence of pro-angiogenic growth factors, TGFβ1, GADPH, bone alkaline phosphatase, osteocalcin, osteonectin, osteopontin, bone sialoprotein and collagen type I.
Results Histology after culture revealed bone chips in a matrix of fibrin remnants and a fibrous-appearing matter. Collagen type I- and IV-positive structures were also identified. Cells could be seen on the surface of the bone chips, with spindle-shaped cells bridging the bone chip particles. Pro-angiogenic growth factors and TGFβ1were detected in the 3D cell culture supernatants. The transcripts for osteocalcin, bone sialoprotein and collagen type I were revealed only via PCR.
Conclusions Our results indicate that bone chips in our 3D organ culture remain vital and may stimulate the growth of a bone-forming matrix.
Clinical relevance The use of autogenous bone chips for oral and maxillofacial bone augmentation procedures is widespread in clinical practice. The rationale for this is that if bone chips remain vital in vivo, they could provide an environment promoting new bone formation through growth factors and cells. This 3D culture method is an essential tool for investigating the behaviour of bone chips.

Keywords Bone chips · Angiogenesis · 3D culture · Organ culture

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Introduction

Bone augmentation of the oral and maxillofacial region is a widely used technique and is often necessary for many aesthetic and reconstructive dental procedures when bone loss has occurred [1]. Intra- or extraorally harvested autogenous bone is the most commonly used material for bone augmentation. Despite recent developments in bone replacement materials, autogenous bone is still considered to be the gold standard for regeneration procedures [2, 3].

Autogenous bone chips can be easily collected during the normal preparation of the operative site [4] with the use of bone collectors, bone scrapers or drilling systems [5]. Notably, it has been demonstrated that particulated autogenous bone from the mandible is sufficient for maxillary sinus floor augmentation [6]. Using autogenous bone minimises patients' physical stress and donor site morbidity, and saves time and costs. Moreover, bone chips from a membranous bone source have been shown to undergo less resorption than enchondral bone [7, 8]. This is probably due to the higher amount of cortical bone [9], which counteracts the

problem of a rather pronounced resorption after implantation, as observed in bone grafts from the iliac crest.

Our aim was to collect in vitro evidence that if the autogenous bone chips remain vital, they could provide cells as well as growth factors, such as pro-angiogenic factors, thus providing an environment which could promote new bone formation. To address this issue experimentally, we developed a 3D in vitro bone chip organ culture model. Both the vitality of the bone chips and the presence of pro-angiogenic factors and other factors triggering bone formation were investigated.

Materials and methods

Study design and donors

The present study was performed in adherence to the Declaration of Helsinki and the Good Clinical Practice guidelines, and the study protocol was approved by the Ethics Committee of the hospital (no. 550, 01.01.2006). Patients undergoing implant insertion according to standard protocols between 2006 and 2008 were used as donors for this study. Bone chips were collected and used for augmentation, and if excess bone was available, it was used for the present experiments. One single sample was obtained from each of 42 patients, all of whom had given written informed consent.

Bone chip harvesting: initial method

In ten cases (group A), bone chips were harvested from different sites of the mandible and maxilla using piezosurgery with a piezosurgery instrument or a round bur. An Osseous Coagulum Trap® bone collector was used to collect the bone chips. Sterile water was used for cooling. To reduce bacterial contamination of the samples, the bone chips were rinsed with 0.1 % chlorhexidine.

Bone chip harvesting: revised method

In 32 cases (first 16 cases—group B; last 16 cases—group C), bone chips were harvested from the mandible and maxilla with a twist drill and an Osseous Coagulum Trap® bone collector. Cooling was performed using physiological saline; thereafter, the bone chips were regularly rinsed with physiological saline. In this revised method, the bone chips were not rinsed with 0.1 % chlorhexidine.

Short-term 2D organ culture

The 2D culture of bone chips was established, using the culture protocol of Clausen et al. [10], to assess whether vital bone cells could feasibly be attained. From each of the group A samples collected with the initial method and from the group B samples collected with the revised method, two to four bone chips were placed per well in 24-well culture plates and fixed using Tissuecol DuoS Fibrin glue (Baxter, Unterschleissheim, FRG). To fix the bone chips, they were washed twice in 1× phosphate-buffered saline (PBS) and resuspended in osteoblast medium (Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20 % foetal calf serum (FCS) and containing thrombin (500 I.E./mL, diluted 1:5 in osteoblast medium; Baxter)). In order to form a stable clot, the thrombin-challenged chips were placed on top of 10-μL drops of fibrinogen containing the osteoblast medium (70–110 mg fibrinogen/mL, diluted 1:5 in osteoblast medium; Baxter) that were placed onto the bottom of a Costar 24-well plate (no. 3527; Vitaris, Baar, Switzerland). Once a clot had formed, 1 mL of medium was added and the bone chips were maintained in a humidified incubator at 37 °C, 5 % O_2 and 5 % CO_2.

3D Organ culture and supernatant collection

The group C samples harvested with the revised method were used to develop 3D fibrin bone chip organ cultures. In contrast to the 2D culture, bone chips were completely embedded in a fibrin matrix which has been shown to improve bone chip culture [11]. To imitate the physiological sequence of bone healing, culture conditions for the 3D cultures were adapted for endothelial osteoblast cell culture conditions according to Unger et al. [12]. Bone chips were washed twice in 1× PBS. One small micro-laboratory spoon (approximately 0.1 mL; Z648299 Micro Spoon & Spatula Set, Sigma-Aldrich, Buchs, Switzerland) of bone chips was mixed with 500 μL of complete endothelial medium (no. EBM-2 CC 3156; Lonza, Basel, Switzerland) enriched with 100 μL of thrombin solution in a 50-mL Falcon Bioreactor (87050 TPP, Omnilab, Mettmenstetten, Switzerland) and incubated for 20 min at room temperature. After 100 μL fibrinogen (1:5 diluted in endothelial medium) was added, the sample was mixed and incubated for another 10–15 min at room temperature in order for a stable fibrin clot to form. The remaining fluid was removed, 5 mL complete endothelial medium was added and the samples cultured in a humidified incubator at 37 °C, 5 % O_2 and 5 % CO_2 for 3 weeks. The medium was changed twice a week. In the second week of culture, the endothelial medium was replaced by a mixture of complete endothelial medium and osteoblast medium (ratio, 1:1). In the last week, the cultures were kept in osteoblast medium only.

Measurement of metabolic activity

2D culture

Measurement of the metabolic activity of the bone chips in 2D culture was performed using the alamarBlue™ test daily from day 1 to day 5, inclusive. The samples were also

evaluated microscopically for the outgrowth of cells after 1 and 5 days of culture (see Electronic supplementary material, ESM for further details of the metabolic test).

3D culture

Measurement of the metabolic activity of the group C bone chip constructs from the 3D culture was performed using the alamarBlue™ test at the end of the 3-week incubation period. Three 100-μL aliquots of the supernatant were measured in a fluorescence spectrophotometer (Fluoroskan Ascent FL; Thermo Scientific, Zürich, Switzerland) using 560 ex nm/590 em nm. The controls were osteoblast medium without alamarBlue™ and osteoblast medium with 10 % alamarBlue™, both without bone chip construct (see ESM for further details of the metabolic test).

Immunohistochemistry

The samples used for immunohistochemistry were decalcified overnight using Serva Decal (no. 18140, Serva (Promega AG), Dübendorf, Switzerland) and diluted 1:5 with double distilled water. Specimens were dehydrated with a gradient ethanol series, embedded in paraffin (H-Plast Gewebeeinbettparaffin 56°, Haslab GmbH, Ostermundigen, Switzerland) and cut into 4-μm sections. Collagen protein expression was analysed using monoclonal antibodies against type I (no. AF 5610-1 Acris, Herford, FRG) and type IV collagen (no. K1500 peroxidase, Dianova, Hamburg, FRG), respectively. Bound antibodies were detected using Dako REAL™ Detection System (no. K5005, Glostrup, Denmark). Antibody to collagen type I was labelled using biotinylated goat anti-mouse and anti-rabbit immunoglobulins followed by streptavidin conjugated to alkaline phosphatase and detected using Chromogen Red; antibody for collagen type IV was conjugated to horseradish peroxidase and detected using DAB according to the manufacturer's protocol (Dako REAL™ Detection System).

Protein detection in the supernatants

In order to analyse pro-angiogenic as well as other bone growth factors such as TGFβ1 and osteocalcin, bone chip constructs were washed twice in 1× PBS; resuspended in DMEM/F12 medium, with or without 20 % FCS; and incubated at 37 °C, 5 % O_2 and 5 % CO_2. After 24 h of incubation, the supernatants were harvested, diluted 1:100 with protease inhibitor (no. P8340; Sigma-Aldrich) and stored at −20 °C. No other incubation periods were tested.

von Willebrand factor ELISA

von Willebrand factor (vWF) protein expression was determined in bone chip construct supernatants using a vWF BioAssay™ ELISA Kit (no. US Biologics V2700-09; LucernaChem, Luzern, Switzerland) according to the manufacturer's instructions. Control sera and the calibration sera were used for standards and positive and negative controls (nos. 5450212 and 5450210, respectively; technocloneTC, Heidelberg, FRG). Absorbance was measured at 450 nm; values were calculated with a four-parameter calibration fitting algorithm (see ESM for further details).

Suspension bead assay

To screen for pro-angiogenic factors in the supernatant of the bone chip constructs, the 9plex Bio Plex ProTM angiogenesis assay (nos. 171-A4011M and 171-304001; Bio-Rad, Reinach, Switzerland) was performed according to the manufacturer's instructions. Analysis was performed with the luminex-100 machine and the Bioplex 3.0 software (Bio-Rad; see ESM for further details).

TGFβ1 ELISA

TGFβ1 secretion was measured in serum-free 24-h supernatants using the TGFβ1 Emax ImmunoAssay System (no. G7590; Promega AG). Supernatants were analysed for total TGFβ1 as well as for biologically active TGFβ1. ELISA was performed according to the manufacturer's instructions. Absorbance was measured at 450 nm, and values were calculated with a linear regression algorithm (see ESM for further details).

Polymerase chain reaction

Total RNA extraction and reverse transcription into cDNA were performed with the RNeasy® Plus Mini Kit (Qiagen, Hombrechtikon, Switzerland) and the RevertAidTM H Minus First Strand Synthesis Kit (Fermentas, St. Leon-Rot, FRG) according to the manufacturer's instructions (see ESM for further details).

Polymerase chain reactions (PCR) were performed using the PCR Core Kit from Roche (Basel, Switzerland). The primers used were specific for GAPDH, bone alkaline phosphatase, osteocalcin, osteonectin, osteopontin, bone sialoprotein, or collagen type I (Entelechon GmbH, Regensburg, FRG; see ESM for further details).

Collagen type I expression

To measure collagen type I expression, supernatants were screened for C-terminal peptide using the Metra® CICP I EIA kit (no. Quidel 8003; Tecomedical, Sissach, Switzerland) according to the manufacturer's instructions. Optical density was determined within 15 min at

a wavelength of 405 nm. Data were calculated using a four-parameter calibration curve fitting algorithm (see ESM for further details).

Embedding of samples and Toluidine Blue staining

After 3 weeks of culture, the samples were fixed in 4 % buffered formaldehyde solution (no. HT5014; Sigma-Aldrich) for 24 h at room temperature. The samples were then rinsed in tap water, dehydrated in alcohol and embedded in methylmethacrylate. The samples were cut into 600-μm-thick sections, which were glued to section holders; milled to a final thickness of 100–150 μm using a polycut-E microtome (Leica, Vienna, Austria); and surface-polished with fine-grained sandpaper. After surface staining with Toluidine Blue O according to the procedure of Schenk et al. [13], specimens were examined using an Olympus BX61 microscope equipped with a digital camera using the Cell^P software (Olympus, Munich, FRG).

Results

Vitality of bone chips

Ten bone chip samples were harvested as per the initial method (group A). All samples were found to be non-vital, as indicated by the results of the alamarBlue™ test. The harvesting method was changed, and a further 32 samples were harvested as per the revised method (groups B and C). Four samples from group B were found to be microbiologically contaminated either with bacteria or fungi. Two-dimensional culture and metabolic testing was performed on the remaining 12 samples from group B. Two of these samples were metabolically inactive. The remaining ten samples were metabolically active at day 1 and metabolic activity increased until day 5, as shown in the alamarBlue™ assay (data not shown). Cell outgrowth was also detected microscopically (Fig. 1).

Establishment and characterisation of 3D fibrin bone chip organ culture

All 16 bone chip samples in group C were cultured in 3D fibrin bone chip organ culture. After 3 weeks of incubation, all but one of the 3D cultures were found to be metabolically active (Table 1).

Histology and immunohistological assessment of 3D fibrin bone chip organ culture

In order to determine whether the 3D culture was successful, histology of bone chips prior to 3D culture was performed, giving a basis of comparison between the pre- and post-3D culture structures. Pre-3D culture histology revealed a mixture of differently sized bone chips with classical osteon and vascular structures (Fig. 2a).

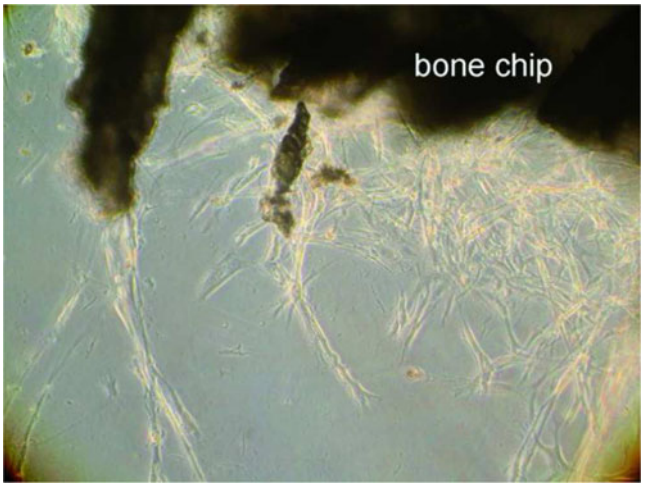

Fig. 1 Intra-orally harvested bone chips were found to be vital; microscopically visible cellular outgrowth was observed (magnification, ×100)

Intact cell nuclei were found throughout the chips (Fig. 2b). Histology after 3 weeks of 3-D culture revealed bone chips embedded in a matrix consisting of fibrin remnants and fibrous-appearing extracellular matrix (Fig. 2c). Nuclei within the bone chips were rarely seen; however, a small amount of cells could be seen on the surface of the bone chip particles (Fig. 2c). In addition, a large number of spindle-shaped cells could be seen bridging the bone chip particles (Fig. 2c).

Immunohistochemistry performed after the 3D culture revealed the presence of collagen type I in the bone chips (Fig. 2d (i)). Although less prominent, collagen type I deposition was also observed in the fibrous matrix (Fig. 2d (i)). Immunohistochemistry showed collagen type IV-positive labelling within the fibrous matrix in close vicinity of the bone chips (Fig. 2e (i)).

Histological signs for the formation of new bone tissue (osteoid or woven bone) or calcification of the matrix were not evident. The absence of matrix calcification was confirmed via Alizarin Red S staining that was found to stain only the original bone chips (data not shown).

Determination of pro-angiogenic factors

Analysis of 24-h cell culture supernatant of the 15 metabolically active organ cultures showed the presence of vWF in 10 out of the 15 tested cases (Table 1), indicating the presence of vital endothelial cells in the cultures.

Table 1 Metabolic activity of 3D culture samples (group C) and the concentrations of vWF, TGFβ1 and C-terminal collagen type I peptide in the 24-h supernatants

Donor	Metabolic activity		vWF		$TGFβ_1$ acid-activated (pg/mL)	SD	$TGFβ_1$ not acid-activated (pg/mL)	SD	C-terminal peptide of collagen type I (ng/mL)	SD
	Fluorescence	SD	Concentration (IU/mL)	SD						
27	591	15	0.29	0.03	39	1	<15.6[c]		<2[d]	
28	2,187	40	0.82	0.03	26	2	<15.6[c]		38	0.9
29	1,661	26	0.26	0.01	95	7	<15.6[c]		94	1.2
30	1,573	40	0.46	0.02	307	26	<15.6[c]		30	1.7
31	1,375	36	0.42	0.01	<15.6[c]		<15.6[c]		34	1.5
32	1,488	58	>1.6[a]		<15.6[c]		<15.6[c]		56	2.2
33	1,229	21	0.35	0.01	549	182	<15.6[c]		19	1.8
34	638	22	0.52	0.02	346	14	<15.6[c]		31	1.1
35	10	0	n.d.		n.d.		n.d.		n.d.	
36	3,143	58	0.61	0.03	67	7	<15.6[c]		6	0.3
37	1,837	41	0.98	0.02	n.d.		n.d.		n.d.	
38	4,814	137	<0.15[b]		1156	249	<15.6[c]		7	0.2
39	394	15	<0.15[b]		<15.6[c]		<15.6[c]		8	0.2
40	3,183	3	<0.15[b]		135	1	40	7	13	0.1
41	12,047	55	<0.15[b]		349	9	<15.6[c]		11	0.8
42	4,813	19	<0.15[b]		324	104	<15.6[c]		10	0.1

n.d. not done, *SD* standard deviation

[a] Measured amount more than the highest standard of 1.6 IU/mL

[b] Measured amount less than the lowest standard of 0.15 IU/mL

[c] Measured amount less than the lowest standard of 15.6 pg/mL

[d] Measured amount less than the lowest standard of 2 ng/mL

Since vWF was found to be present in the 24-h supernatant, we additionally investigated the secretion of other pro-angiogenic factors. Five 24-h cell culture supernatants were analysed with the (pro-)angiogenic suspension bead assay (Table 2). Hepatocyte growth factor (HGF), interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) could be detected in all five samples. Granulocyte colony stimulation factor (G-CSF) and platelet endothelial cell adhesion molecule (PECAM) were detectable in four out of five; leptin in three out of five; and angiopoetin-2, follistatin and platelet-derived growth factor (PDGF-BB) in two out of five samples.

Determination of TGFβ1, collagen type I and osteocalcin (osteoblast-specific marker)

Determination of TGFβ1

There was no evidence from the histological data that bone formation took place under the culture conditions. To examine whether this might be due to missing production and/or activation of TGFβ1, supernatants were tested for the presence of TGFβ1 and found positive in 11 out of 15 cases. However, in only 1 of these 11 cases could we demonstrate active TGFβ1 (Table 1).

Determination of collagen type I

Immunohistochemistry revealed the presence of collagen type I in the spaces between the bone chips. This was confirmed via detection of the C-terminal peptide of collagen type I in the supernatants (Table 1).

Fifteen supernatants were tested using the osteocalcin-specific assay from Quidel and found to be negative (data not shown). These findings are in accordance with the missing histological signs of calcification and the absence of active TGFβ1 in the supernatants (Table 1).

Determination of osteoblast-specific mRNA

To further investigate the possible presence of functional osteoblasts in our 3D culture, bone-specific gene expression was assessed. All of the transcripts tested for were detected, including the transcripts for bone alkaline phosphatase, osteonectin and osteopontin. Most notably, the presence of osteocalcin and

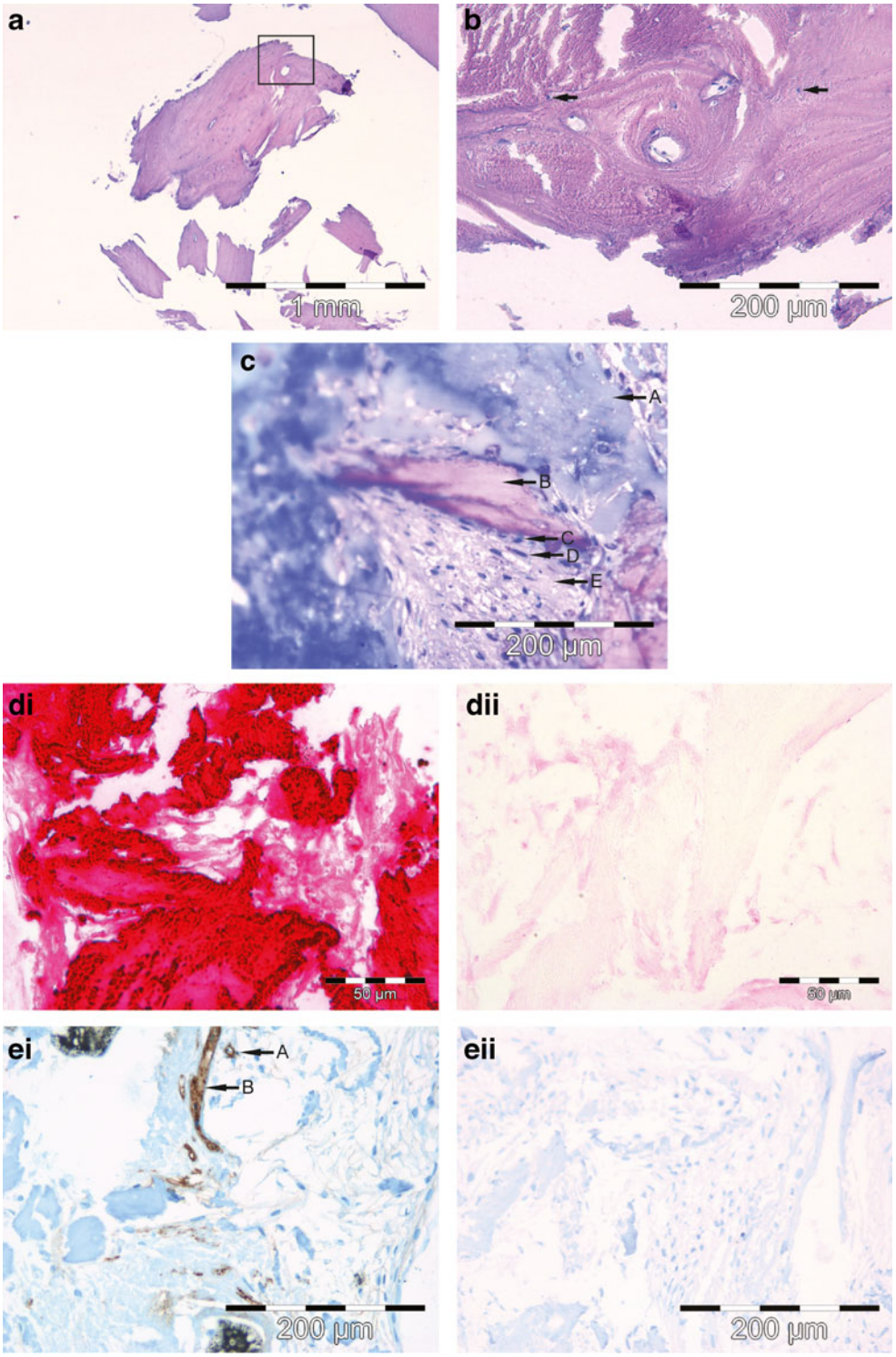

Fig. 2 **a** Haematoxylin and eosin stain of bone chip particles prior to culture (*overview*); the area enlarged in (**b**) is marked with a rectangle. **b** Haematoxylin and eosin stain of bone particles prior to culture (*arrows* indicate osteocyte nuclei). **c** Toluidine Blue staining of 3D bone chip organ culture after 3 weeks. *A* Fibrin. *B* Bone chip. *C* Cells on the surface of the bone chips. *D* Cells. *E* Newly formed fibrous matrix. **d** (*i*) Anti-collagen type I staining (bone chips are intensively stained in *red*; the interspaces show fibrous, newly formed tissue). **d** (*ii*) PBS control for collagen type I staining. **e** (*i*) Anti-collagen type IV staining. *A* This structure appears to have the form of a vessel with a lumen-like structure. *B* Brown staining indicates collagen type-IV positive structures. **e** (*ii*) PBS control for collagen type IV staining

bone sialoprotein transcripts, both transcripts characteristically not found in gingival fibroblasts or periodontal ligament cells [14], indicated the presence of vital osteoblasts. Furthermore, collagen type I mRNA was found, thereby complementing our collagen type I protein data.

Discussion

We have successfully developed a 3D organ culture using bone chips harvested during surgery of the mandible or maxilla. Under our 3D fibrin bone chip organ culture conditions, a

Table 2 Pro-angiogenic factors found in 24-h supernatants of 3D bone chip cultures

	No. of positive tested supernatants out of 5	Average of measured picograms cytokine per millilitre supernatant	SD
Angiopoetin-2	2	23	40
Follistatin	2	234	488
G-CSF	4	2	1
HGF	5	249	140
IL-8	5	264	327
Leptin	3	90	11
PDGF-BB	2	8	2
PECAM	4	115	138
VEGF	5	558	405

SD standard deviation

high number of cells were seen to populate and bridge the spaces between the bone chips, as well as covering the bone chips. The fibrin matrix was partially replaced with newly formed, non-calcified, collagen type I-containing extracellular matrix, and collagen type IV-positive structures were identified. Furthermore, growth factors indicative of a pro-angiogenic environment were secreted. Although typical ossification protein markers, e.g. osteocalcin, were not expressed, specific mRNA was detected.

Bone chip cultures have been shown to be a successful method for growing and isolating osteoblasts [15, 16]. In the presence of fibrin glue (fibrinogen and thrombin), bone chip cultures have been shown to be even more successful [11]. In vitro data as to how bone chips could augment in vivo bone formation are scarce and usually lack the aspect of 3D culture conditions, with the exception of one study reported by Richards et al. [17]. In their study, by using a bioreactor, a 3D load-providing culture system enabling cancellous bone explants to be studied ex vivo was characterised.

Bone chips (foetal mouse) have also been used for the isolation of endothelial cells, as described by Deckers et al. [18]. Furthermore, in a study by van Hinsbergh et al. [11], a 3D fibrin matrix was used to successfully study the outgrowth of human microvascular endothelial cells in capillary-like tubular structures.

Our study aimed to develop a 3D bone chip culture which would allow the behaviour of bone chips as well as bone chip-derived cells to be observed in vitro. During the course of this study, we realised that the bone chips harvested using the original harvesting method were not vital. After analysing the harvesting procedure, we found several possible reasons. Bone chips were harvested using a round bur, which induced considerable mechanical damage. The bone chips were then cooled with sterile water and allowed to dry in the bone collector. Furthermore, the bone chips were rinsed with 0.1 % chlorhexidine, a known cytotoxic agent [19] and pharmacon used to reduce bacterial contamination of bone samples collected for use in grafting procedures [20]. The vitality of bone chips has been shown to be influenced via the harvesting technology, whereby the main issue seems to be the extent of mechanical damage [21]. The more the bone tissue has disintegrated, the less bone chips will be vital. Bone chips harvested with a twist drill or piezosurgery appear to be equally vital [22] and are more vital when compared to chips harvested with a round bur. In an attempt to achieve vital bone chips, we altered our harvesting method: instead of a round bur, a twist drill was used, cooling was performed using physiological saline, and the bone chips were regularly rinsed with physiological saline, rather than water, preventing them from drying out. Furthermore, we omitted the use of 0.1 % chlorhexidine.

After enhancing our bone harvesting method, we produced successful 3D bone chip cultures, as would be expected based on previous literature [21, 23]. We demonstrated that the bone chip cultures were metabolically active, although variability in the metabolic activity of individual samples was observed. This was possibly due to the unrestricted choice of the patients for our study. Patient inherent factors including age, gender, smoking habits, underlying diseases such as periodontitis and concomitant medication may explain the observed variability. Another factor could be the composition of the harvested bone chips, namely the unknown distribution of cortical and cancellous bone in individual samples. Finally, cancellous bone explants vary in trabecular density and volume [24], which could also contribute to the observed variability.

Furthermore, our results imply that at least some cells of the bone chips survive in vivo under hypoxic conditions. To confirm these results, we are currently running a combined retrospective/prospective clinical study comparing patients augmented with bone chips harvested with our revised method (to gain vital bone chips) against patients augmented with bone chips harvested with the old method (gaining avital bone chips) [20].

In this study, we demonstrated the presence of collagen type IV-containing structures. We also positively identified vWF, which suggests the presence of endothelial cells. Since collagen type IV is found in basal lamina, we hypothesise that these two findings are suggestive of vessel-like structures. Furthermore, the growth factor pattern observed ($IL\text{-}8^{+}$, HGF^{+}, $VEGF^{+}$ and active $TGF\beta1^{-}$) is indicative of a pro-angiogenic environment [25–29]. However, to prove that bone chips promote angiogenesis under our 3D culture conditions, further investigations are required. These investigations should examine whether growth factors are indeed produced by vital cells or are primarily released from the bone chip matrix in the course of matrix degradation.

After a 3-week 3D culture period, we observed very few cell nuclei within the bone chips. This is indicative of a loss of osteocytes from the bone chips, possibly due to cell death. A small number of cells, however, could be seen on the surface of the bone chips, which were suggestive of osteoblasts. However, since we were unable to confirm this purely by histology, we tested for osteoblast-specific mRNA. We detected osteocalcin and bone sialoprotein transcripts, which, taken together with our histological findings, imply the presence of vital bone cells in our 3D cultures [14]. Since we could not demonstrate osteoblast differentiation into osteocytes either with histology (no osteoid or woven bone) or with testing of the 24-h supernatant (for osteocalcin), we hypothesise that our culture conditions prompted both the outgrowth of osteoblasts and their maintenance in the pre-differentiated status. However, to further investigate this, we are currently working on a bone differentiating culture regime that could potentially induce osteoid and woven bone formation in our 3D cultures. This includes stimulation protocols with vitamin D3 or glycerol/vitamin C [30]. If this is successful, our culture system could become a versatile in vitro test system enabling the concurrent analysis of the angiogenic and osteogenic processes essential for effective bone regeneration [31]. Our 3D culture is, therefore, a physiologically relevant model for oral bone regeneration [14].

This culture method adds to the already existing co-culture systems previously reported in the literature [32–35]. In addition, it opens up an avenue for testing the influence of different bone donor localities (enchondral versus intramembranous bone chips) as well as the effect of drugs, metabolic disorders (diabetes) or biomaterials on bone repair mechanisms. Since bone augmentation using autogenous bone is a widespread technique for aesthetic and reconstructive procedures [1, 3], having an in vitro 3D culture method which recapitulates the in vivo environment is a vital tool for investigating the behaviour of autogenous bone chips when used for grafting purposes.

Conclusion

We have established a 3D bone chip culture model that allows investigations of the behaviour of bone chips, potential vessel outgrowth and the generation of osteoblasts, and further differentiation processes. This model resembles in vivo conditions and could be used as a tool for analysing the influence of pharmaceuticals, biomaterials, mechanical stress, pro-inflammatory conditions and pathophysiological conditions such as hyperglycaemia on the vascularisation process during early bone healing.

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