Influence of Harvesting Technique and Donor Site Location on In Vitro Growth of Osteoblastlike Cells from Facial Bone

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Purpose: *Donor morbidity is minimized when tissue engineering is applied to produce osteogenic grafts by growing osteoblasts on biomaterials. However, limiting factors are the origin, proliferation, and differentiation of osteoblasts. Therefore, the aim of this study was to evaluate the efficacy of growing osteoblasts from different types of bone samples and to assess the influence of the donor site.* ***Materials and Methods:*** *From 28 patients 37 bone specimens were obtained during removal of third molars in the maxilla and mandible. Seventeen specimens were bone chips and 20 were bone sludge. After subculturing primary cultures, histochemical and immunhistochemical tests (EZ4U test, BrdU labeling, ALP histochemistry, type I collagen immunohistochemistry, osteocalcin ELISA) were performed to determine cell proliferation, viability, and differentiation.* ***Results:*** *Both bone chips and bone sludge from the mandible and maxilla are suitable for culturing human osteoblastlike cells. However, bone chips were superior to bone sludge with respect to ability to grow cells, and maxillary bone was superior to mandibular bone in this regard. Harvesting technique had only little influence on the expression of cell differentiation markers (ALP, type I collagen, osteocalcin).* ***Discussion and Conclusion:*** *Chips from human membrane bone, especially from the maxilla, are suitable for culturing high numbers of differentiated osteoblastlike cells. These cells may be used to tissue engineer bone grafts, which may be used to enhance the implant placement site.* Int J Oral Maxillofac Implants 2005;20:860–866

Key words: *alveolar ridge augmentation, cell culture, cell proliferation and differentiation, implant placement, tissue engineering*

Placement of dental implants may require augmentation of the atrophic alveolar ridge. Transplantation of autogenous bone is still the gold standard. The embryonic origin (membranous versus enchondral) and gross morphology (cortical versus cancellous, particulated versus bone block) of the graft tissue have been discussed as important factors for osseointegration and regeneration of the grafts.[1,2] Nonetheless, autogenous bone grafting can have disadvantages, such as limited graft material availability and donor site morbidity. To circumvent these limitations, adequate substitute biomaterials such as hydroxyapatites, α- and β-tricalcium phosphates, and demineralized bone matrices have been used clinically, with varying degrees of success.[3]

Tissue engineering is an alternative to further research into the use of biomaterials and their combination with osteoblasts and growth factors. In tissue engineering, bone cells are seeded on 3-dimensional bonelike scaffolds of natural or artificial origin.[4,5] Certain growth factors and proteins, eg, bone morphogenetic proteins (BMPs) can be added, as they play a crucial role in influencing the differentiation of osteoblasts.[6,7]

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In animal models 3-dimensional bonelike structures, even structures shaped like the jaw, have been developed using a combination of resorbable matrices and bone cells alone or bone and cartilage cells.[8,9] Implantation of xenogenic bone material loaded with BMPs in muscle resulted in the formation of bonelike structures.[10] Hutmacher and Lauer have reported the possibility of creating almost autogenous bone in humans using tissue engineering techniques.[4]

Although the isolation and culture of bone cells was reported more than 50 years ago,[11] the outgrowth of bone cells from biopsies needs to be reliably established before this method can be applied as a clinical routine. Different studies have evaluated the influence of embryonic origin, biopsy preparation, and cell isolation technique on the growth and differentiation of human osteoblastlike cells.[12–15] However, the harvest of biopsy material from facial bone has not yet been considered. Therefore, the aim of this study was to assess the influence of the biopsy harvesting technique on growth and differentiation of human osteoblastlike cells in vitro. The authors wished to investigate whether ground bone sludge is as useful as bone chips to grow primary osteoblast and whether there is a dependency on the location of the donor site.

MATERIALS AND METHODS

Cell Isolation and Culture Technique

For primary cultures, maxillary and mandibular bone obtained following the removal of third molars was used. The bone that was harvested would otherwise have been discarded. The study was approved by the Ethics Committee of Dresden University (15022002). The bone was received in 2 forms: as bone chips when a chisel and hammer or luer forceps were used to collect the bone, or as ground bone sludge collected using a bur and a bone collector (Fa Sulzer-Medica, Freiburg, Germany). Thirty-seven biopsy samples from 28 patients (13 women, 15 men) with an average age of 27.4 years (range, 14 to 57 years) were tested. In 17 cases the biopsy samples were bone chips, and in 20 cases they were ground bone sludge. From 9 patients both types of bone specimens were obtained.

The bone chips were briefly rinsed in 70% ethanol. They were subsequently rinsed 3 times in phosphate-buffered saline (PBS), broken into pieces about 2 × 1 mm in size, and cultured as explant culture.[16] The bone grinding sludge was removed from the bone collector, spread onto culture dishes, and carefully covered with a little culture medium.

The culture medium was Opti-minimal essential medium (Opti-MEM; Gibco, Karlsruhe, Germany) with 4% N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) (Gibco) with 10% fetal calf serum and 1% penicillin/streptomycin (Biochrom, Berlin, Germany). Primary cultures were kept in a humidified atmosphere of 5% carbon dioxide (CO_2) at 37°C (Heraeus Kulzer, Hanau, Germany). Cultures that did not show any cell growth after 5 weeks were discarded. After forming a subconfluent cell layer, cells were subcultured twice. After an adherence phase of 2 days in the second subculture, testing was performed.

Cell Proliferation Analysis

EZ4U Assay. The nonradioactive assay EZ4U (Biozol Diagnostica, Eching, Germany) was used for cell vitality and proliferation analysis. The test is based on the finding that an intracellular reduction system in mitochondria of living cells reduces slightly yellow-colored tetrazolium salts to intensely red-colored formazan derivatives.[17] These derivatives are excreted into the culture medium, and the amount absorbed is measured with a microplate reader. The amount of colored formazan derivatives correlates with the amount of living cells. The test was performed according to the manufacturer's instructions.

BrdU Labeling Technique. The bromodeoxyuridine (BrdU) labeling technique was used to determine the DNA synthesis rate—the labeling index—in cultured cells.[18] The cultures were kept in culture medium containing 20 mmol/L BrdU at 5% CO_2 under atmospheric pressure for 1 hour. Specimens were fixed in 70% ethanol, and prior to the immunohistology procedure (see type I collagen immunohistochemistry), the incorporated BrdU was exposed with 0.07 mol/L sodium hydroxide (NaOH) for 5 minutes.

Cell Differentiation Analysis

To assess the differentiation of osteoblasts in the subculture, a cascade of tests was used.

Alkaline Phosphatase Assay. For the staining of osteoblastlike cells, an alkaline phosphatase (ALP) assay kit (Sigma, Deisenhofen, Germany) was used. The culture dishes were air dried, fixed in a citrate-acetone-formaldehyde solution for 30 seconds, and rinsed gently with distilled water. They were incubated in the alkaline phosphatase staining solution for 15 minutes away from direct light, then washed with distilled water. The citrate-acetone-formaldehyde solution and the ALP staining solution were prepared according to the manufacturers' instructions. The slides were counterstained with neutral red for 5 minutes, rinsed with distilled water, and dry mounted with cover slips. The positive staining for ALP (red-violet) was identified by light microscopy and evaluated by morphometry using the computer program Analysis 3.1 (Soft Imaging System, Munster, Germany).

Table 1 Time Interval for Osteoblast Outgrowth in Primary Culture with Respect to Harvesting Technique

	n	Mean ± SD (d)	Median (d)	Maximum (d)	Minimum (d)
Bone chips	16	8.00 ± 1.00	8.00	10	6
Bone sludge	13	18.08 ± 2.43	19.00	21	14

Significant differences were observed between bone chips and bone sludge in mean number of days needed for cell outgrowth ($P < .025$).

Type I Collagen Immunocytochemistry. For the determination of type I collagen, the cells were washed in PBS for 5 minutes, fixed with 70% ethanol for 1 to 2 hours, washed in PBS for 5 minutes, allowed to air dry, and washed again in PBS for 5 minutes. After incubation in 0.3% H_20_2 in methanol for 30 minutes, unspecific immune reactions were blocked with 1% bovine serum albumin for 10 minutes before the anticollagen I antibody (Sigma) was administered for 1 hour. This was followed by incubation of the biotin-conjugated secondary antibody (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) for 45 minutes and by incubation of avidin mixed with biotin-conjugated peroxidase (Vectastain Elite Kit; Vector Laboratories) for 30 minutes. Sections were rinsed between each incubation step 3 times with PBS for 5 minutes each. The immune reaction was developed by diaminobenzidine solution (0.05 mg/L diaminobenzidine/0.05 mol/L Tris-HCI [pH 7.3]/ 0.01% H_20_2) at room temperature. The sections were counterstained with hematoxylin-eosin (H&E; Merck, Darmstadt, Germany) for 10 seconds and mounted in 40% glycerin (Merck) in PBS.

Assay for Osteocalcin. For the quantitative determination of osteocalcin in the cell culture supernatant of human osteoblastlike cells, the osteocalcin enzyme-linked immunosorbent assay test (ELISA) (DAKO, Glostrup, Denmark) was performed according to the manufacturer's instructions. In brief, the standards, the curve control, and the cell culture supernatants were premixed with biotinylated osteocalcin, incubated in microwells precoated with anti-osteocalcin for 1 hour, and washed and incubated with peroxidase-conjugated streptavidin for 15 minutes, which binds strongly to the biotinylated osteocalcin. After a further washing step, the chromogenic substrate was added and incubated for 30 minutes. The reaction was stopped by 2 mol/L H_2SO_4, and the absorbance at 450 nm was measured. Osteocalcin is exclusively synthesized by osteoblasts and is believed to prevent premature mineralization of newly formed disorganized bone matrix.[19]

Statistical Analysis

To compare the different harvesting techniques, mean values, variances, and standard deviations were calculated, and the Student *t* test was used.

RESULTS

Cell Growth in Primary Culture

In 16 of 17 bone-chip biopsies osteoblast outgrowth appeared, representing a success rate of 94%. No bacterial contamination was observed during the culture period. Of 20 bone sludge cultures, 3 had to be discarded because of bacterial contamination. In another 4, no osteoblast outgrowth was registered even after 5 weeks of culture, which represents a success rate of 65% of all bone-sludge cultures or 76.5% of the uncontaminated cultures.

On average, osteoblast outgrowth was observed after 8 days in the case of bone chips and after 18 days in the case of bone sludge (Table 1) . For bone chips the shortest period was 6 days, the longest 10 days (Fig 1). For bone sludge the minimal time to observe bone cell outgrowth was 14 days, the longest 21 days (Fig 1). Significant differences were observed between bone chips and bone sludge in mean number of days needed for cell outgrowth ($P < .025$).

Cell Proliferation and Vitality in Subculture

In the third subculture, the proliferation of osteoblastlike cells was correlated with the biopsy harvesting technique and location of the biopsy.

The results of the EZ4U test indicated that there was significantly more cell proliferation in bone chip cultures than in bone sludge cultures. The average measured obtained absorbance (OD) was 0.462 (median 0.462) in bone chip cultures and only 0.381 (median 0.490) in bone sludge cultures ($P < .025$) (Fig 2).

Using the BrdU labeling for cell proliferation, the labeling index was also higher in bone chip cultures than in bone sludge cultures (58.76% versus 53.14%; Fig 3). However, this difference was not statistically significant.

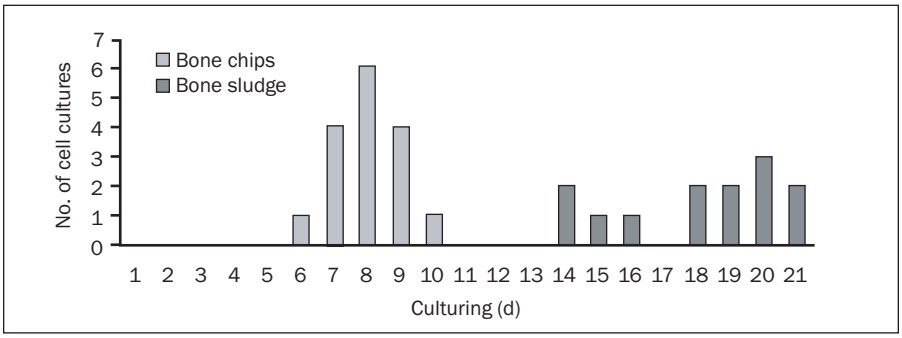

Fig 1 Cell outgrowth in primary cultures.

Cell proliferation was higher in cultures of maxillary bone than in cultures of mandibular bone. EZ4U testing showed an absorbance of 0.453 OD in biopsies from the maxilla versus 0.392 OD in biopsies from the mandible. The BrdU labeling index was also greater in the maxilla than in the mandible (57.80% versus 54.32%; Figs 4 and 5). No significant differences were found.

Cell Differentiation in Subculture

Harvesting technique and location of the biopsy had no influence on the expression of cell differentiation markers such as collagen I, ALP, and osteocalcin. In bone chip cultures, 69.66% of cells expressed collagen I, 41.19% of cells expressed ALP, and the osteocalcin content was 6.64 µg/L. In bone sludge cultures, these parameters were a little lower (Table 2).

In cell cultures of the maxilla, 68.63% of cells expressed collagen I, 41.52% of the cells expressed ALP, and the osteocalcin content was 6.83 µg/L. In cell cultures of the mandible, collagen I expression was higher, with 69.66% of cells, but ALP-positive cells (37.82%) and osteocalcin content (5.75 µg/L) were lower (Table 3).

DISCUSSION

In this study, both harvesting technique and location within the facial skeleton were found to have an influence on the efficacy of bone biopsies to grow osteoblastlike cells in culture. These features persisted even after subculturing. The culturing of osteoblasts from chips of the maxilla has been described previously.[16,20] Other studies have mainly used iliac bone cells and have focused on particle size[15] or cell isolation technique.[14] However, the present study showed that it is possible to establish sufficient numbers of cultures from facial bones. The finding that cell outgrowth was more efficient from bone chips than from bone sludge confirms previous findings regarding iliac cells.[15]

Bacterial contamination was only observed when using bone sludge. The reason may be the longer processing chain when using the bone collector and subsequently a higher risk for contamination. Although the bone collector was used only during the osteotomy, it still contained a mixture of saliva, irrigation fluid, and grinding sludge. It is likely that this mixture carries a higher load of pathogens than bone chips taken directly from the sterile wound site. Further, a brief rinse with ethanol, the routine used when culturing cells from bone chips or from mucosa,[21] was more difficult to facilitate because of the consistency of bone grinding sludge. To minimize the risk of infection the culture medium contained penicillin G, which is sensitive to most oral pathogens.[22]

When comparing sterile cultures only, the efficacy of setting up primary bone cultures increased to 75% for bone sludge but could not reach that of bone chips (94%). Because of their small weight, bone sludge particles tend to float when suspended in more culture medium. As contact with the culture dish allows for a better outgrowth of cells,[23] very little culture medium was used, and the fixation of particles to the culture dish was achieved by adhesion forces.

Other studies on setting up primary osteoblast cultures have compared neither different donor sites nor different harvesting techniques for facial bone, both interesting issues for the implant-oriented oral and maxillofacial surgeon. The surgeon is familiar with this type of harvesting and may use the bone chips and the bone sludge directly as augmentation material around dental implants.[24] In regard to the tissue engineering of bone grafts, Voegele and coworkers observed for iliac osteoblasts an earlier confluency in culture after enzymatic isolation of cells in contrast to spontanous cell outgrowths from explants.[14] However, after subculturing explant cells, a growth spurt occurred, and the cell counts

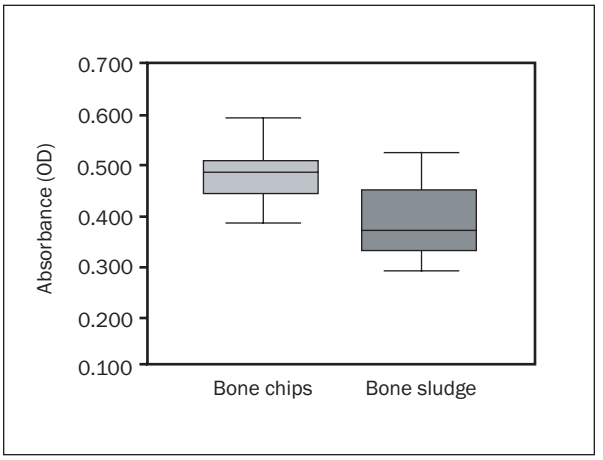

Fig 2 Cell proliferation measured by EZ4U test with respect to the harvesting technique (bone chips versus bone sludge).

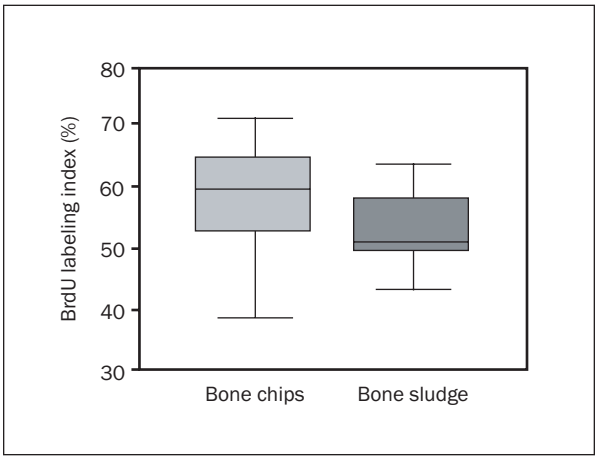

Fig 3 Cell proliferation measured by BrdU labeling technique with respect to the harvesting technique (bone chips versus bone sludge).

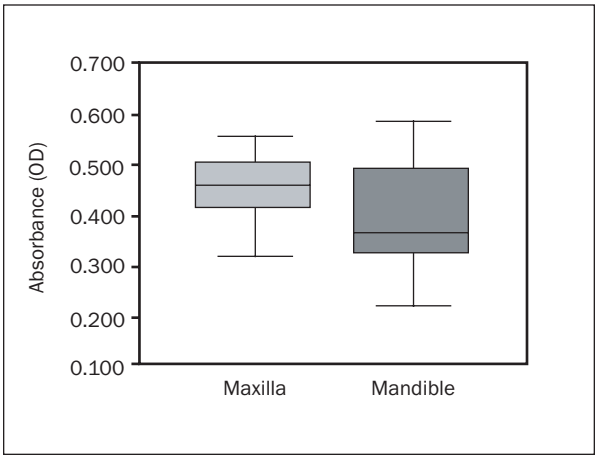

Fig 4 Cell proliferation measured by EZ4U test with respect to the location of the donor site (maxilla versus mandible).

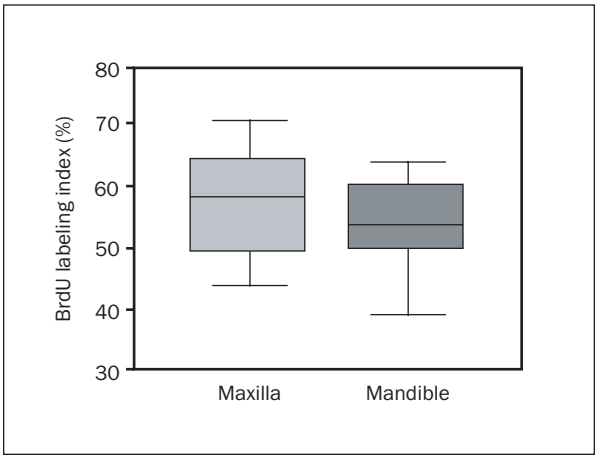

Fig 5 Cell proliferation measured by BrdU labeling technique with respect to the location of the donor site (maxilla versus mandible).

Table 2 Cell Differentiation with Respect to Harvesting Technique

Test/harvesting technique	n	Mean ± SD	Median	Maximum	Minimum
Collagen I–positive cells (%)					
Bone chips	16	69.66 ± 14.29	67.49	92.23	41.82
Bone sludge	13	68.39 ± 13.29	67.81	88.32	49.19
ALP-positive cells (%)					
Bone chips	16	41.19 ± 10.30	42.23	55.10	20.36
Bone sludge	13	38.22 ± 9.64	33.94	52.62	25.69
Osteocalcin (μg/L)					
Bone chips	16	6.64 ± 2.47	5.75	11.50	2.40
Bone sludge	13	5.98 ± 2.13	5.20	10.40	3.90

No significant differences were observed between bone chips and bone sludge.

Table 3 Cell Differentiation with Respect to Location of Bone

Test/ location	n	Mean ± SD	Median	Maximum	Minimum
Collagen I–positive cells (%)					
Maxilla	16	68.63 ± 12.52	67.22	92.23	49.19
Mandible	13	69.66 ± 15.36	70.68	88.32	41.82
ALP-positive cells (%)					
Maxilla	16	41.52 ± 9.58	42.23	55.10	25.69
Mandible	13	37.82 ± 10.38	35.02	53.37	20.36
Osteocalcin (μg/L)					
Maxilla	16	6.83 ± 2.40	6.10	11.50	3.80
Mandible	13	5.75 ± 2.13	5.10	10.40	2.40

No significant differences were observed between maxillary and mandibular bone.

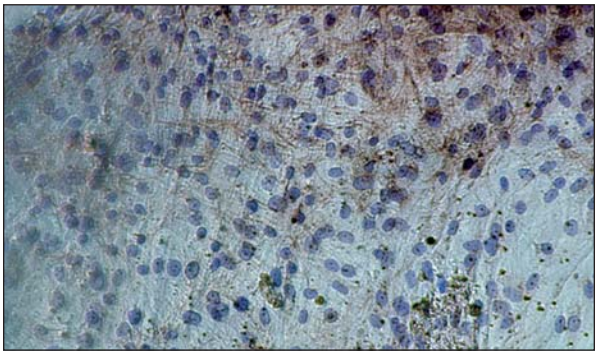

Fig 6 Immunohistologic staining for collagen I. Typical section used for morphometric assessment (H&E counterstaining; original magnification ×100).

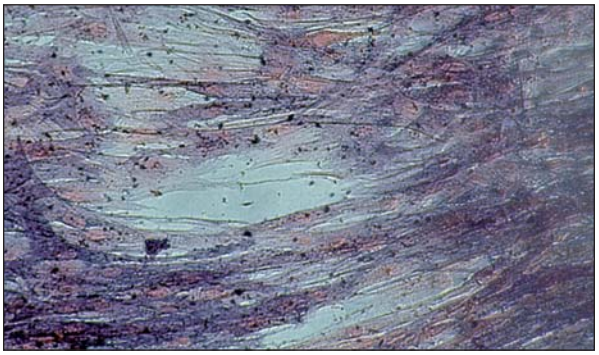

Fig 7 Histochemical alkaline phosphatase staining of osteoblastlike cells in secondary culture. Typical section used for histomorphometric assessment (H&E counterstaining; original magnification ×500).

"caught up"; there was no longer a significant difference between the cultured cells and those that grew from the explants.[14] Springer and associates compared drill sludge from mandible bone and different preparations of iliac bone with respect to their ability to grow cells after 4 weeks of primary culture.[15] The present findings confirm their finding that grinding sludge was the least effective in setting up primary cultures. However, as an internal comparison they used bone chips from the hip, and there was no statement about the cell behavior when further subcultured.

To further assess whether different proliferations are related to harvesting trauma or to intrinsic differences between cell population, the second subculture was used to investigate cell viability and differentiation. In the second subculture there were standardized conditions with identical numbers of seeded cells and an identical amount of culture media. The cell proliferation alone (BrdU) and cell viability and proliferation (EZ4U) were significantly better in those cells grown from bone chips. The different cell differentiation markers were also more strongly expressed in cells from bone chips. However, there was considerable variation in measurements, an observation which has been reported previously for similar assessments in cultures of human bone cells and other human cells.[21,25]

Kasperk and colleagues investigated osteoblast growth and differentiation from different embryonic origins, comparing iliac and mandible bone.[13] They found a higher proliferation for mandibular osteoblasts, but a lower expression of differentiation markers such as ALP and osteocalcin. This trend persisted even after several subcultures. This is in contrast to the findings of Springer and coworkers; they gained higher cell counts from iliac bone.[15] However, they did not assess differentiation and proliferation in subculture. In a previous study it was shown that the expression of differentiation markers peaks in the second subculture and vanishes afterward.[16] By the second and third subculture the cells have multiplied enough to be used for tissue engineering and grafting, while still retaining good differentiation. From the sixth culture onward, the cells lose the capability to attach to the petri dish.

The difference in cell outgrowth between mandible and maxilla might be related to their different bone structures and textures. It is known that cortical bone (similar to mandibular bone) contains

30 times more osteocalcin than trabecular bone (similar to maxillary bone), which in turn contains 21 to 47 times more osteonectin.[26] Differences in proliferation and differentiation were also present in the subcultures; thus, the cells may have different intrinsic programs, as suggested by Kasperk and colleagues[13] and Marie and de Vernejoul.[27] They showed that in vitro bone culture differences correlate well with in vivo bone cell activity and that osteoblast phenotype can vary depending on the origin of the cells.[13]

CONCLUSION

Cancellous bone chips from the maxilla have good propensity for growing osteoblastlike cells in vitro. The maxilla may be an ideal source for bone cells for culturing and for tissue-engineered grafts. Grafts may be used in oral and maxillofacial surgery, eg, for periimplant augmentation. The authors have started to use this harvesting technique and this bone source to tissue engineer autogenous bone grafts for filling dental cystic cavities.

ACKNOWLEDGMENT

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REFERENCES

1. Pallesen L, Schou S, Aaboe M, Hjorting-Hansen E, Nattestad A, Melsen F. Influence of particle size of autogenous bone grafts on the early stages of bone regeneration: A histologic and stereologic study in rabbit calvarium. Int J Oral Maxillofac Implants 2002;17:498–506.
2. Matsumoto MA, Filho HN, Francischone E, Consolaro A. Microscopic analysis of reconstructed maxillary alveolar ridges using autogenous bone grafts from the chin and iliac crest. Int J Oral Maxillofac Implants 2002;17:507–516.
3. Kübler NR. Osteoinduktion und -reparation. Mund Kiefer Gesichtschir 1997;1:2–25.
4. Hutmacher DW, Lauer G. Grundlagen und aktuelle Anwendungen des Tissue Engineering in der Mund-, Kiefer- und Gesichtschirurgie. Implantologie 2002;10:143–156.
5. Ripamonti U, Crooks J, Rueger DC. Induction of bone formation by recombinant human osteogenic protein-1 and sintered porous hydroxyapatite in adult primates. Plast Reconstr Surg 2001;107:977–988.
6. Seto I, Asahina I, Oda M, Enomoto S. Reconstruction of the primate mandible with a combination graft of recombinant human bone morphogenetic protein-2 and bone marrow. J Oral Maxillofac Surg 2001;59:53–61.
7. Pereira RC, Economides AN, Canalis E. Bone morphogenetic proteins induce gremlin, a protein that limits their activity in osteoblasts. Endocrinology 2000;141:4558–4563.
8. Ishaug SL, Crane GM, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. J Biomed Mater Res 1997;36:17–28.
9. Weng Y, Cao Y, Silva CA, Vacanti MP, Vacanti CA. Tissue-engineered composites of bone and cartilage for mandible condylar reconstruction. J Oral Maxillofac Surg 2001;59:185–190.
10. Terheyden H, Jepsen S, Rueger DR. Mandibular reconstruction in miniature pigs with prefabricated vascularized bone grafts using recombinant human osteogenic protein-1: A preliminary study. Int J Oral Maxillofac Surg 1999;28:461–463.
11. Doyle A, Griffiths JB, Newell DG (eds). Cell and Tissue Culture: Laboratory Procedures. Chichester, England: Wiley, 1993.
12. Doglioli P, Scortecci G. Characterization of endosteal osteoblasts isolated from human maxilla and mandible: An experimental system for biocompatibility tests. Cytotechnology 1991;7:39–48.
13. Kasperk C, Wergedal J, Strong D, et al. Human bone cell phenotypes differ depending on their skeletal site of origin. J Clin Endocrinol Metab 1995;80:2511–2517.
14. Voegele TJ, Voegele-Kadletz M, Esposito V, et al. The effect of different isolation techniques on human osteoblast-like cell growth. Anticancer Res 2000;20:3575–3581.
15. Springer IN, Terheyden H, Geiss S, Harle F, Hedderich J, Acil Y. Particulated bone grafts—Effectiveness of bone cell supply. Clin Oral Implants Res 2004;15:205–212.
16. Lauer G, Drews M, Hübner U. Isolierung und Kultivierung humaner Osteoblasten aus cortico-lamellären Knochen der Maxilla. Osteologie 1999;8:179–185.
17. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res 1987;47:936–942.
18. Patterson MK Jr. Measurement of growth and viability of cells in culture. Methods Enzymol 1979;58:141–152.
19. Hyldstrup L, Clemmensen I, Jensen BA, Transbol I. Non-invasive evaluation of bone formation: Measurements of serum alkaline phosphatase, whole body retention of diphosphanate and serum osteocalcin in metabolic bone disorders and thyroid disease. Scand J Clin Lab Invest 1988;48:611–619.
20. Mailhot JM, Borke JL. An isolation and in vitro culturing method for human intraoral bone cells derived from dental implant preparation sites. Clin Oral Implants Res 1998;9:43–50.
21. Lauer G, Siegmund C, Hübner U. Influence of donor age and culture conditions on tissue engineering of mucosa autografts. Int J Oral Maxillofac Surg 2003;32:305–312.
22. Otten JE, Drews M, Pelz K, Lauer G. Odontogene Infektionen—ein systemisches Risiko? Dtsch Zahnärztl Z 1998;53:85–88.
23. Stenn KS, Dvoretzky I. Human serum and epithelial spread in tissue culture. Arch Dermatol Res 1979;264:3–15.
24. Lauer G, Schilli W. Collected implant cavity borings used as peri-implant osseous augmentation material. Int J Oral Maxillofac Implants 1994;9:437–443.
25. Morike M, Schulz M, Nerlich A, et al. Expression of osteoblastic markers in cultured human bone and fracture callus cells. J Mol Med 1995;73:571–575.
26. Ninomiya JT, Tracy RP, Calore JD, Gendreau MA, Kelm RJ, Mann KG. Heterogeneity of human bone. J Bone Miner Res 1990;5:933–938.
27. Marie PJ, de Vernejoul MC. Proliferation of bone surface-derived osteoblastic cells and control of bone formation. Bone 1993;14:463–468.