Different Substitute Biomaterials as Potential Scaffolds in Tissue Engineering

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Purpose: *To find the optimal scaffold for tissue-engineered bone, one approach is to test existing biomaterials on their suitability as scaffolds. In this study, the suitability of different alloplastic and xenogenic biomaterials as scaffolds for ex vivo osteoblast cultivation was investigated.* ***Materials and Methods:*** *Normal human osteoblast cells were cultured on the surface of bovine collagenous materials, bovine hydroxyapatite, porcine gelatin, synthetic polymer, and collagen-containing bovine hydroxyapatite, and the investigation of proliferation was performed after 24, 72, and 120 hours. Measurement of the differentiation marker alkaline phosphatase and osteocalcin was made after 20 days of incubation.* ***Results:*** *The obtained data showed significantly higher proliferation and differentiation rates in cells cultivated on collagen-rich biomaterials in comparison to noncollagenous or collagen-poor biomaterials (*$P < .05$*).* ***Discussion:*** *In tissue engineering the scaffold should be biocompatible and serve as a proper matrix for the cells to produce the new structural environment of extracellular matrix ex vivo. Collagen supports initial cell attachment and cell proliferation, allowing immature osteogenic cells to differentiate into mature osteoblasts, but collagen may not be the only dominating factor for cell-matrix interaction during ex vivo bone formation.* ***Conclusion:*** *These data suggest that a 3-dimensional collagen matrix can provide a more favorable environment for the attachment, proliferation, and differentiation of in vitro osteoblastlike cells, at least until the initial stage of differentiation, than noncollagenous biomaterials.* (Basic Science) Int J Oral Maxillofac Implants 2006;21:225–231

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Autogenous bone transplants from intra- or extraoral donor sites are primarily used to regenerate bone defects in the craniofacial region and are currently accepted as the "gold standard." Bone substitutes are commonly used in orthopedic, oral, and maxillofacial surgery for various indications.[1,2] However, none of the biomaterials commercially available combines all characteristics defined as ideal for a bone grafting material.

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Hard tissue engineering may potentially provide alternative solutions with better properties than those current methods of bone grafting. Novel approaches in both tissue engineering and the generation of differentiated artificial tissues for biomedical applications are now emerging.[3] The reintroduction of osteoblasts to a matrix in a state that guarantees their differentiation into functional bone matrix–producing cells could be one possibility in the treatment of bony defects. This technique is the basis of the so-called "self-cell therapy," which has recently demonstrated clinical potential for the regeneration of bone tissue and the treatment of bony lesions.[4–8]

The essential elements of tissue engineering are stem or precursor cells, an appropriate biologic scaffold, and growth factors.[9] The suitability of a biologic scaffold for ex vivo engineering of vital bone-cell transplants is one of the central questions in the application of cell therapy for tissue engineering of new bone. The material used as a scaffold should permit the attachment of osteogenic cells, providing

Table 1 Overview of Collagen-Containing and Noncollagenous Biomaterials Used for Determination of Cell Proliferation and Differentiation

Group	Material	Origin
Biomaterials with collagen		
100% collagen		
A	Lyostypt	Bovine
B	Colloss	Bovine
HA + 25% collagen	Tutodent	Bovine
HA + 10% collagen	Bio-Oss Collagen	Bovine
Biomaterials without collagen		
HA	Bio-Oss Spongiosa	Bovine
Synthetic polymer	Ethisorb	PLGA and poly-p-diaxonon
Gelatin	Gelita	Porcine

HA = hydroxyapatite; PLGA = polyglactin 910.

an appropriate environment for their proliferation and differentiation, and it should be possible to process it into irregular 3-dimensional shapes.[10] Recently, in vitro osteoblast cell cultures have been demonstrated on a variety of matrices, such as poly(glycolic acid) meshes, collagen matrices, ceramics, calcium phosphate, and polymer constructs.[11–15] Alternatively, xenogenic bone materials such as denaturalized spongiosa of bovine origin and xenogenic mineral coated with collagen type I have been reported to facilitate new bone formation under certain conditions in vitro.[16,17]

In this study, commercially available biomaterials, including bovine collagenous materials, bovine hydroxyapatite, porcine gelatin, synthetic polymer, and collagen-containing bovine hydroxyapatite were analyzed for their ability to serve as scaffolds and to allow the proliferation of bone-forming cells as well as the differentiation of those cells into mature osteoblasts.

MATERIALS AND METHODS

The results from the analysis of cell growth and differentiation on different biomaterials (biomaterial group) were compared to those of cells cultured as a monolayer on the plastic culture plate without biomaterials (control group). Additionally, biomaterials without cells served as the negative control in each group (T_0), and the obtained data were subtracted from the results of biomaterial group.

Cell Culture

Commercially available normal human osteoblasts (NHOst; Cambex Bio Science, Verviers, Belgium) were seeded in T-150 culture flasks with fresh culture media (αMEM; Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (Gibco BRL, Paisley, Scotland, United Kingdom), 10^5 IU penicillin, and 100 mg/L streptomycin (Abbod, Wiesbaden, Germany). According to the manufacturer, the cells were alkaline phosphatase (ALP) -positive and formed von Kossa-positive nodules. Cultures of 90% confluent cells were impregnated with trypsin (2.5 g/L trypsin containing 1 mmol/L EDTA; Gibco), washed, and suspended in fresh media. Twenty thousand cells diluted in 100 mL of media were added to the surface of each biomaterial or to an empty well of tissue culture polystyrene (TCPS) and incubated for 30 minutes at 37°C in humidified 5% CO_2 conditions to allow the cells to adhere. One milliliter of culture medium was subsequently added to cover the biomaterials.

Biomaterials

All of the investigated biomaterials were commercially available, and their particular composition was defined by the manufacturer's protocol (Table 1). The biomaterials examined were

- **Lyostypt** (B. Braun/Aesculap, Tuttlingen, Germany), a hemostyptic composed of native absorbable collagen type I fibrils of bovine origin (group: 100% collagen [A]). One 10 × 10 × 5-mm sheet was used per well.[18]
- **Colloss** (Ossacur, Oberstenfeld, Germany), a natural extract from the cortical diaphyseal bovine bone. Its main component is the reconstituted collagen type I (group: 100% collagen [B]). One 5-mg sponge was used per well.[19]
- **Tutodent** (Tutogen Medical, West Patterson, NJ), a natural bovine material with associated acellular collagenous (group: 25% type I collagen) and noncollagenous bovine bone matrix (HA) (group: HA + 25% collagen). A 4 × 8 × 8-mm block was used per well.[17]
- **Bio-Oss Collagen** (Geistlich Pharma, Wolhusen, Switzerland), a combination of 100-mg spongiosa granules and 10% bovine collagen type I fibers (group: HA + 10% collagen) used in an 8 × 5 × 5-mm block per well.[20]
- **Bio-Oss Spongiosa Block** (Geistlich Pharma), a pure mineral HA (group: HA) derived from spongious bovine bone.[21] The crystalline dimensions come to approximately 400 × 10 nm, and the inner surface area of the material is approximately 100 m^2/g. The size used was 1 cm^3/well.
- **Ethisorb Dura Patch** (Johnson & Johnson/Ethicon, Somerville, NJ), a synthetic polymer composite consisting of Vicryl (PLGA; Ethicon) and PDS (poly-p-diaxonon) in a 90:10 ratio (group: synthetic polymer). A 10 × 10 × 2-mm sheet per well was used.[22]

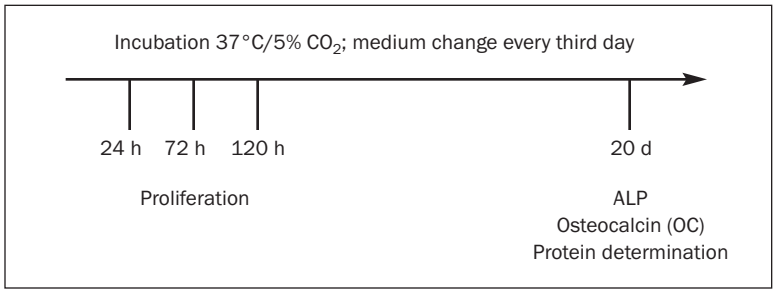

Fig 1 Flowchart of biochemical investigations of cell growth on different biomaterials.

- **Gelita tampon** (Aesculap), a spongy hemostyptic made from hardened gelatin of porcine origin (group: gelatin). One cm^3 per well, containing 10 mg of absorbable gelatin sponge, was used.[23]

Cell Proliferation Assay

Cell proliferation was assessed using a WST-1 colorimetric assay (Hoffman-LaRoche/Roche Diagnostics, Basel, Switzerland) specifically for the quantification of cell proliferation. The measurement of proliferation was performed after 24, 72, and 120 hours (Fig 1).

Following the manufacturer's protocol, the culture medium was removed, the cells were washed once with phosphate buffer solution (PBS), and 1 mL fresh culture medium containing 10% of the WST-1 labeling kit was added. The cells were incubated for 1 hour, and the proliferation rate was measured by monitoring light absorbance (450 to 650 nm; ELISA Vmax Molecular Devices, Sunnydale, CA).

ALP

Cells (2×10^4/well/biomaterial) were seeded on each biomaterial and incubated for 20 days (Fig 1). For the investigation of ALP activity, the adherent cells were washed twice with PBS and lysed for 10 minutes at room temperature (20°C) with 15 mmol/L Tris-HCl buffer (pH 7.4) containing 1 mmol/L zinc chloride ($ZnCl_2$), 1 mmol/L magnesium chloride ($MgCl_2$), and 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA). At the 20th day aliquots of cell lysates were mixed with assay buffer containing 7 mmol/L *p*-nitrophenol phosphate (Sigma, Roche, Mannheim, Germany), incubated for 20 minutes at room temperature (20°C), and then assayed for absorbance at 410 nm (Vmax, Molecular Devices), as described previously.[24] The results for the ALP assays were standardized by dividing the values obtained by total protein content in each sample (Bio-Rad Laboratories).

Osteocalcin Synthesis

Immunoassay of osteocalcin was performed using a Gla-type osteocalcin enzyme immunoassay (EIA) kit (Takara Shuzo, Shiga, Japan). Cells (2×10^4 /well/biomaterial) were seeded on each biomaterial and then cultured for 20 days (Fig 1). The reaction of each sample was measured by monitoring light absorbance at 450 nm, following the manufacturer's protocol. The results for the osteocalcin assays were standardized by dividing the values obtained by total protein content in each sample.

Statistical Analysis

All measurements of the independent experiments were performed at least 5 times and expressed as mean values. Single-factor analysis of variance (ANOVA) for multiple comparisons was employed to assess the statistical significance of the data. The statistical significance of differences between the biomaterial groups and the control group (cells without a biomaterial) was evaluated with the paired Student *t* test, and a *P* value of less than .05 was considered significant.

RESULTS

Osteoblast Proliferation

A significant difference in cell proliferation rate was observed between the control group and the biomaterial groups ($P < .05$). Among the experimental groups, 2 different growth tendencies could be seen. One group, composed of the collagen-rich biomaterials Lyostypt, Colloss, and Tutodent and the synthetic biomaterial Ethisorb showed a proliferation tendency comparable to the control group. The second group, which consisted collagen-poor or noncollagenous biomaterials (Bio-Oss Collagen and Bio-Oss Spongiosa), showed a significantly lower proliferation rate in comparison to the control group.

In comparison to results found in collagen-poor/noncollagenous group, a higher proliferation rate was demonstrated for the pure collagen groups (A and B) after 24, 72, and 120 hours of observation (Fig 2). The proliferation rate of osteoblasts cultured on Colloss and Lyostypt reached a maximum after 72 hours; this maximum was significantly higher (5% to 10%) than that of the control group ($P < .05$). Prolifer-

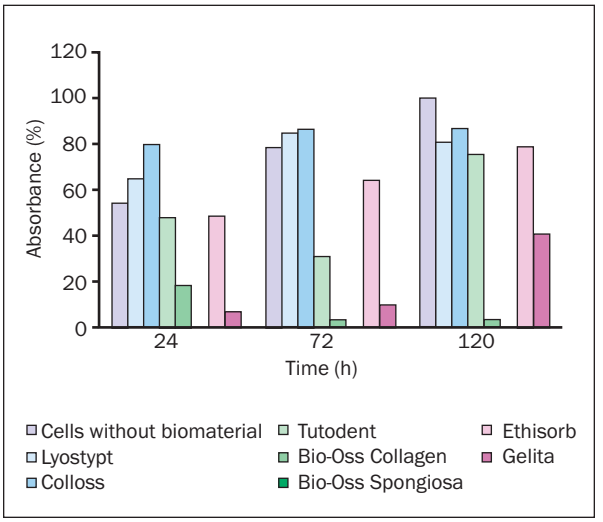

Fig 2 Proliferation of osteoblast cells expressed as the mean percent absorbance.

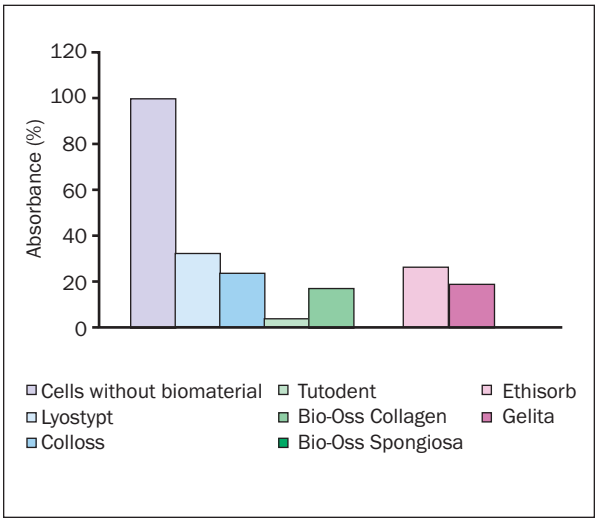

Fig 3 ALP activity expressed as the mean percent absorbance at 20 days.

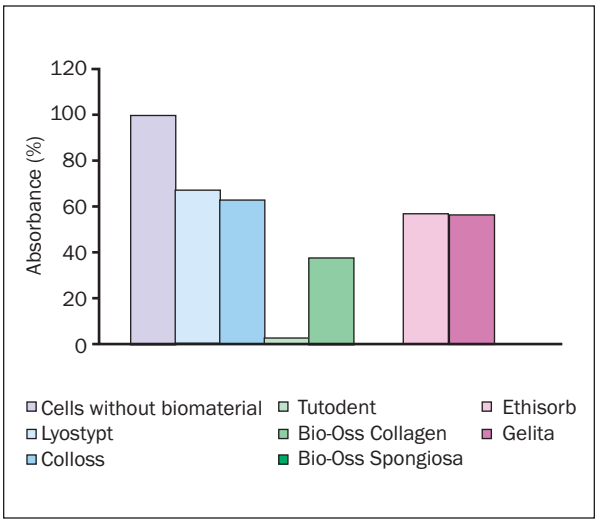

Fig 4 Osteocalcin activity expressed as the mean percent absorbance at 20 days.

ation in the Bio-Oss Collagen group decreased after 24 hours, and proliferation in the Ethisorb, Tutodent, and Gelita groups increased up to 120 hours after incubation. However, cell proliferation in the biomaterials groups was significantly lower than cell proliferation in the control group after 120 h of observation ($P < .05$) (Fig 2).

ALP

ALP activity, one of the markers of differentiated osteoblast function, was measured after 20 days of cultivation (Fig 3). The level of total ALP activity measured in osteoblasts cultured on the different biomaterials was comparatively lower than the level of activity measured in the control group ($P < .05$).

Of the biomaterial groups, the Lyostypt group showed the highest measurable ALP activity at day 20. Significantly lower ALP values were found in the Bio-Oss Collagen and Tutodent groups than in the Colloss, Gelita, Ethisorb, and Lyostypt groups ($P < .05$). No ALP activity was detectable in the Bio-Oss Spongiosa group after 20 days of incubation.

Osteocalcin Synthesis

The osteocalcin activity measured at the 20th day of cell proliferation was significantly lower in the observed biomaterial groups compared to the control group ($P < .05$) (Fig 4).

Osteoblasts grown on the biomaterials Lyostypt, Colloss, Gelita, and Ethisorb expressed significantly higher OC activity compared to the Bio-Oss Collagen and Tutodent groups ($P < .05$). No significant expression of OC synthesis was detected in the Bio-Oss Spongiosa group.

DISCUSSION

For the restitution of form and function of bony defects, tissue engineering, ie, the use of ex vivo cells cultivated on scaffolds to achieve vital bone tissue constructs, has been shown to be an attractive alternative to the use of allogenous or synthetic bone substitutes. For this purpose the scaffold should be biocompatible and serve as a proper matrix for the cells to produce the new structural environment of

extracellular matrix ex vivo. Several studies have evaluated the interaction of various matrices with cultured human osteoblastic cells in vitro.[25–30] Recently, it has been demonstrated that osteoblasts on synthetic polymer foams can form a calcified bonelike tissue.[31,32] Other biomaterials, such as natural bone mineral, have been shown to provide a favorable matrix for human osteoblastlike cells to attach, divide, and synthesize mature collagen.[25] All of these materials degrade slowly, supporting the growth and differentiation of osteoblasts and allowing the cells to expand and to produce new matrices.

This study was designed to evaluate commercially available biomaterials with varying collagen contents for their capacity to serve as scaffolding biomaterial in an ex vivo cell/scaffold culture model. All biomaterials were used as provided by the manufacturer and investigated under equal conditions without any prior biochemical preparation or treatment. The investigation showed a significantly higher rate of proliferation and differentiation in the control group (monolayer cell culture without biomaterial) compared to cells grown on the biomaterials in all biomaterial groups.

In the WST proliferation assay, which indicated the amount of viable cells adhered to the material, the collagen-rich biomaterials (collagen content ≥ 25%) Lyostypt, Colloss, and Tutodent showed a proliferation tendency similar to that observed in the control group. In particular, cells grown on biomaterials with collagen as a single component (Lyostypt and Colloss) showed higher proliferation rates after 24 and 72 hours than the control group or the other biomaterial groups. Furthermore, collagen-rich biomaterials provided superior differentiation activity of human osteoblastlike cells. The collagen-poor (≤ 10% collagen) and noncollagenous biomaterials, Bio-Oss Collagen, Gelita, and Bio-Oss Spongiosa, showed significantly lower rates of proliferation. As reported previously, this property may be related to the cell-matrix interaction of collagen type I.[25,33] The mechanisms through which collagen affects the expression of osteoblastic phenotypes are probably initiated through its interaction with heterodimeric integrin receptors.[34] Recent studies have demonstrated that the interaction of integrins with matrix proteins provokes various changes in cellular proliferation, differentiation, and functions via the activation of intracellular signal transduction pathways.[35] However, differentiation data obtained in the Tutodent group are not to be compared with the data of the other experimental groups. As previously described, it may not be possible to differentiate between the proteins (ALP and OC) synthesized by the human osteoblastlike cells cultured on Tutodent and the proteins that were present in the specimen prior to the experiment.[25]

The investigated synthetic polymer Ethisorb and the porcine gelatin Gelita do not contain any collagen components. Despite the low number of initially seeded osteoblastic cells, both biomaterials allowed cells to proliferate and differentiate; these materials showed comparable results to collagen-containing biomaterials. These data suggest that both the chemical properties of a biomaterial's surface, such as chemical composition, and its physical qualities affect cell-biomaterial interactions by influencing the attachment and subsequent modulation of the intracellular signals involved.[36–40]

Under the conditions of the present culture system, neither the chemical composition nor the structure of the biomaterial surface was able to modulate or support the cell proliferation or differentiation of cells grown on Bio-Oss Spongiosa. Direct comparisons between the present study and previous studies with Bio-Oss Spongiosa may be difficult because of differences in initial seeding density. Previous reports demonstrate that bone cells grown on HA increased ALP activity but decreased the proliferation compared with plastic surfaces in vitro.[41–43] Increased cell number is often associated with enhanced differentiation, and in the case of HA it appears that differentiation is enhanced by the ability of HA to support osteoblast attachment and promote bone formation within implants.[41] The findings of this study confirm that osteoblast growth is decreased on HA but cannot demonstrate that differentiation was enhanced in comparison with osteoblasts grown on plastic surfaces.

It seems important to initially seed and distribute a higher number of cells on scaffolding biomaterial to provide appropriate cellular attachment and allow the formation of an extracellular matrix.[25,44] Compared with the results of other working groups, the number of cells used in the present study might not have been sufficient for colonization.[25,45] Therefore, the density of cell seeding should be carefully estimated so that the number of cells exceeds that used in the present study ($2 \times 10^4/cm^2$).

The present data demonstrate that collagen supports initial cell attachment and cell proliferation, allowing immature osteogenic cells to differentiate into mature osteoblasts; however, collagen may not be the only dominating factor for cell-matrix interaction during ex vivo bone formation.

CONCLUSION

The present results demonstrate that a 3-dimensional collagen matrix can provide a favorable environment for the attachment, proliferation, and differ-

entiation of in vitro osteoblastlike cells, at least until the initial stage of differentiation. Collagenous biomaterials perform better in this respect than noncollagenous biomaterials do. The in vitro culture system used in the present study allowed the assessment of clinically useful biomaterials but also indicated the need for application of superior cell density for the elucidation of interaction and modulation of ex vivo bone growth by biomaterials.

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