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Proliferation rate of human osteoblast-like cells on alloplastic biomaterials and their clinical application for the transnasal duraplasty procedure

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Abstract

The possibility of transmission of slow virus infection (HIV) and Creutzfeld-Jakob disease by cadaveric dura implants makes it necessary to find synthetic, absorbable materials for the reconstruction of the dura mater. Various procedures with autologous or alloplastic material are described. Four commercially available biomaterials were choosen to study the proliferation rate and the biocompatibility of human osteoblast-like cells (HOB-like cells) on 2-dimensional material by biochemical analysis. With a proliferation assay, the viability and the proliferation capacity of osteoblast-like cells were evaluated. A clinical trial was added to study resorbable fleece as one of the previously tested biomaterial in a small patient group (8 patients) to close anterior cranial fossa dura defects. The results of the proliferation assay showed the highest proliferation rate of HOB-like cells on resorbable fleece. All patients in our clinical trial with anterior cranial fossa dura defects were successfully treated with resorbable fleece. There was no evidence for persisting cerebrospinal fluid rhinorrhea or foreign body reaction after the period of wound healing. The present study demonstrated an excellent biocompatibility of resorbable fleece. The vicryl fleece is an alternative alloplastic material for endonasal closure of defined substantial defects of the dura with cerebrospinal fluid.

Keywords: biocompatibility • biomaterials • human osteoblast-like cells • proliferation assay • duraplasty • vicryl-PDS fleece • absorbable implants

Introduction

Various procedures have been described for transnasal closure of anterior cranial fossa dura defects using autologous or alloplastic materials [1–9]. Due to foreign-body reactions, autologous transplantates like nasal septum cartilage, paraumbilical fatty tissue, fascia lata or mucosa are preferred [2, 7, 10, 11]. However, sufficient autologous material is not always available or its excision creates a second surgical wound with additional scarring and risk of infection and needs longer operating time. The advantage of

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alloplastic materials is the time-independent and the immediate ubiquitous availability. Nevertheless, all available implants lacked two of the most critical characteristics of living tissues: the ability to self repair and ability to modify their structure and properties in response to environmental factors such as brain pressure due to the anterior cranial fossa bone defect. had only osteoconductive property. Different compositions and implant surfaces of commercially available replacement products or defect covering materials had effects on osteoblast behavior [12–14]. Therefore, investigating methods to study cell-biomaterial interactions were a crucial prerequisite for the development of optimal biomaterials, which could elicit specific, timely, and desirable responses from surrounding tissues [15]. The tendency of tissue engineering is to enhance the utility of biomaterials for closure of anterior cranial fossa bone defects by incorporation of an osteogenic cell source into the framework followed by the *in vitro* promotion of osteogenic differentiation before host implantation [16]. Due to the limited manipulation space for transnasal duraplasty, the alloplastic material must comply with certain conditions from the rhinosurgeon's point of view. Among these, the material must be easy to handle and fit while guaranteeing adequate stability against the cerebrospinal column pressure. Additional required properties are biocompatibility with cerebrospinal fluid and tissue, impermeability and complete resorbability in total substitution by endogenous tissue with formation of a stable scar plate or its irritation-free integration.

Materials and methods

Basic research

The following four different biomaterials were used for the cultivation of human osteoblast-like cells and for the subsequent examinations: Ethisorb® Pledget/Patch Type 6 (Johnson & Johnson, Intl. Ethicon GmbH & Co. KG, Norderstedt, Germany); PDS (Poy-P-Dioxannon) foil (Johnson & Johnson, Intl. Ethicon GmbH & Co. KG, Norderstedt, Germany); Beriplast® P (Aventis Behring GmbH, Marburg, Germany), MacroSorb FX® (MacroPore, San Diego, USA).

The medium for culturing human osteoblast–like cells consisted of opti-minimal essential medium (Opti-MEM, Gibco Laboratories Life Technologies Inc, Grand Island, NY, USA) added with 10% fetal calf serum (FCS, PAA Laboratories GmBH, Linz, Austria), 2% HEPES buffer (Gibco Life technologies, Paisley, Scotland), 5 ml of the antibiotics penicillin (100,000 U/L, Biochrom AG, Berlin, Germany) and streptomycin (100 mg/L, Biochrom AG, Berlin, Germany).

Isolation and cultivation of human osteoblast-like cells (HOB-cells)

For the cultivation of HOB-cells, pieces of human corticospongiosa bone like craniofacial bone, pelvic bone and fibula from twelve patients were used. The pieces were taken from 17 to 73 year old male and female patients. The bone-specimens were crumbled into explants (size 2 mm x 2 mm) and seeded in culture flasks (25 cm^2, Greiner, Frickenhausen, Germany) using Opti-MEM, pH 7.2 with 10% FCS and kept in humidified atmosphere of 5% CO2 at 37ºC (Heraeus, Hanau, Germany). The osteoblast–like cells, which migrated to the buttom of the culture flask formed a confluent layer after 4–5 weeks and the first passage was used for the growth experiments on the various biomaterials.

Seeding the cells onto biomaterials

The confluent osteoblast cultures were detached from the culture flask by incubation with 0.5% trypsin (Gibco Life Technologies, Paisley, Scotland) in phosphate buffered saline (PBS). The cell solution was filtered through a 100 µm cell-strainer (Falcon, Heidelberg, Germany), centrifugated (Biofuge Strato, Heraeus, Hanau, Germany) and resuspended in 1 ml culture medium. The cells were transfered in a 75 cm^2 culture flask (Greiner, Frickenhausen, Germany), filled up with 25 ml culture medium. After 14 days the cells of the first passage were detached again from the culture flask with 0.5% trypsin, centrifugated and resuspended in 1 ml culture medium. After staining with trypanblue the vital cells were counted by light microscope (Zeiss Axiovert, Jena, Germany) in a counting chamber. As seeding concentration 1 x 10^6 cells/ml was used.

Proliferation assay for comparing growth rate of human osteoblast-like cells (HOB-like cells)

To compare the proliferation rate each material was prepared to have a surface area about 16 mm^2. 96 well microplates (Corning, NY, USA) were used. The probes were analysed in triplicate. 20 µl of 10^6 cells/ml (2×10^4 cells) were seeded onto the surface of the four different materials. The cells were incubated for two hours at 37ºC in 5% CO_2 atmosphere. 200 µl culture medium was added and an incubation for two days followed. For cell proliferation analysis the commercially available kit EZ4U (Biomedica, GmbH, Wien, Austria), a non radioactive cell proliferation assay, was used. This method was based on the fact, that living cells were capable of reducing slightly yellow coloured tetrazolium salts to intensively red coloured formazan derivatives. An intracellular reduction system, most likely located in the mitochondria, did this function in viable cells. This water soluble formazan is secreted into the culture medium. The amount of colour developed correlates with the amount of living cells in the sample. The kit was designed for 96 well microplates and permits easy quantification using a standard microtiterplate reader (Spectophotometry, Anthos Labtec Instruments, Salzburg, Austria). In brief, one vial of substrate was dissolved in 2.5 ml activator solution. This procedure yielded a straw coloured solution. 30 µl of substrate was added to each well containing biomaterial with proliferating HOB-cells as well as 200 µl culture medium. Incubation at 37ºC for 3 hrs followed. 100 µl medium from each well was transfered to another well into a new microplate. The absorbance was measured at 450 nm, with 620 nm as reference. Additionally, 24 well plates with $1x10^5$ cells from each specimen were incubated for one week at 37ºC at 5% CO_2 atmosphere for the detection of alkaline phosphatase (ALP) and of type I collagen, markers for HOB-cells.

Alkaline phosphatase assay

For the staining of HOB-like cells an alkaline phosphatase assay kit (Sigma, Deisenhofen, Germany) was used. The culture dish was air dried, fixed in citrate-acetone formaldehyde solution for 30 sec and rinsed gently with Aqua dest.. An incubation with the alkaline phosphatase staining solution for 15 minutes protected from direct light and washing with Aqua dest. followed. The citrate-acetone formaldehyde solution as well as the alkaline phoasphatase staining solution were prepared according to the manufacturer`s instructions. The culture dish was counterstained with neutralred for 5 minutes. After rinsing with Aqua dest. and air drying, the culture dish was cut in a superficial part to leave just a basal plate with staining cell layer. The positive staining for alkaline phosphatase (red-violet) was identified by light microscope and evaluated by the computer program Analysis 3.1 (Soft Imaging System, Muenster, Germany).

Type I collagen immunocytochemistry

For the determination of type I collagen the cells were washed in phosphate buffered saline (PBS) for 5 min, fixed with 70% ethanol for 1–2 h, washed in PBS for 5 min, allowed to air dry and washed again in PBS for 5 min. After an incubation with 0.3% H_2O_2 in methanol for 30 min, unspecific immune reactions were blocked with 1% bovine serum albumin for 10 min before the anti-collagen I antibody (Sigma, Deisenhofen, Germany) was administered for 1 h in refrigerator followed by incubation of the biotin-conjugated secondary antibody (Vectastain Elite Kit, Vector Laboratories, Burlingame, USA) for 45 min and by incubation of avidin mixed with biotin-conjugated peroxidase (Vectastain Elite Kit, Vector Laboratories, Burlingame, USA) for 30 min. Sections were rinsed after each incubation step three times with PBS for 5 min. The immunoreaction was developed by diaminobenzidine solution (0.05 mg/L DAB / 0.05 M Tris-HCl pH 7.3 / 0.01% H_2O_2) at room temperature. The sections were counterstained with hematoxylin (Haemalaun, Merck, Darmstadt, Germany) for 2 min and rinsed with cool tap water. The evaluation was done by light microscope and the computer program Analysis 3.1 (Soft Imaging System, Muenster, Germany).

Determination of osteocalcin

For the quantitative determination of osteocalcin in the cell culture supernatant of HOB-cells, the osteocalcin ELISA (DAKO, Glostrup, Denmark) was performed according to the manufacturer's instructions. The standards, the curve control and the cell culture supernatants were premixed with biotinylated osteocalcin, incubated in microwells precoated with anti-osteocalcin for 1 h, washed and incubated with peroxidase-conjugated streptavidin for 15 min, which bound strongly to the biotinylated osteocalcin. After a further washing step the chromogenic substrate was added and incubated for 30 min.

Table 1 Patients in a clinical trial with an idiopathic, traumatic or tumor-related rhinogenic dura fistula with the previously tested resorbable fleece

patient/age/sex	aetiopathology	donor region of autologous material	nasal package	liquor drainage	Follow-up time
K.H-P./52/m	tumor progress	inferior turbinate	2 days	yes	34 months
M.F./59/f	traumatically, after paranasal operation	inferior turbinate	2 days	yes	38 months
K.I./66/f	traumatically, after paranasal operation	inferior turbinate	2 days	no	44 months
U.H./35/f	traumatically, after septorrhinoplasty	inferior turbinate	no	no	55 months
N.A./30/m	traumatically, after paranasal operation	inferior turbinate	3 days	no	43 months
L.H./43/m	traumatically, after paranasal operation	middle turbinate	2 days	yes	32 months
D.U./68/f	idiopathic liquor fistula	inferior turbinate	1 day	yes	24 months
T.M./21/m	traumatically, after paranasal operation	inferior turbinate	2 days	no	6 months

Data of patients: f = female, m = male, age of patients at the treatment

The reaction was stopped by 2 M H_2SO_4 and the absorbance at 450 nm was measured. Osteocalcin was exclusively synthesized by osteoblasts and was believed to prevent premature mineralization of newly formed, but yet disorganized bone matrix.

Statistics were performed by Friedman test, followed by repeated measure analysis of variance (ANOVA) to determine the significance of the proliferation rate of HOB-like cells between the tested biomaterials.

Clinical application

We treated 8 patients in a clinical trial with an idiopathic, traumatic or tumor-related rhinogenic dura fistula with the previously tested resorbable fleece (Ethisorb®). The localisation of the dura defect of the rhinobase was determined preoperatively endoscopically and by high-resolution computer tomography. In one case, diagnostic procedure was supplemented by using computer assisted surgery, in another case by using transthecal-applied fluorescein. The patient age at the time of admission was between 21 and 68 years, 4 patients were women and 4 men (Table 1). All patients were treated with antibiotics (2nd generation Cephalosporin) for at least 3 days starting on the day of surgery. All patients were monitored for the development of signs of meningitis. The surgical procedures were performed under microscopic or endoscopic control. The vicryl fleece was placed between the dura and the bony skull base in underlay technique to seal and hold the cerebrospinal pressure column. In addition, another appropriately shaped implant was placed endonasally to the exposed dura-/bone cleft space below the bone line as overlay. Finally, we sealed the defect

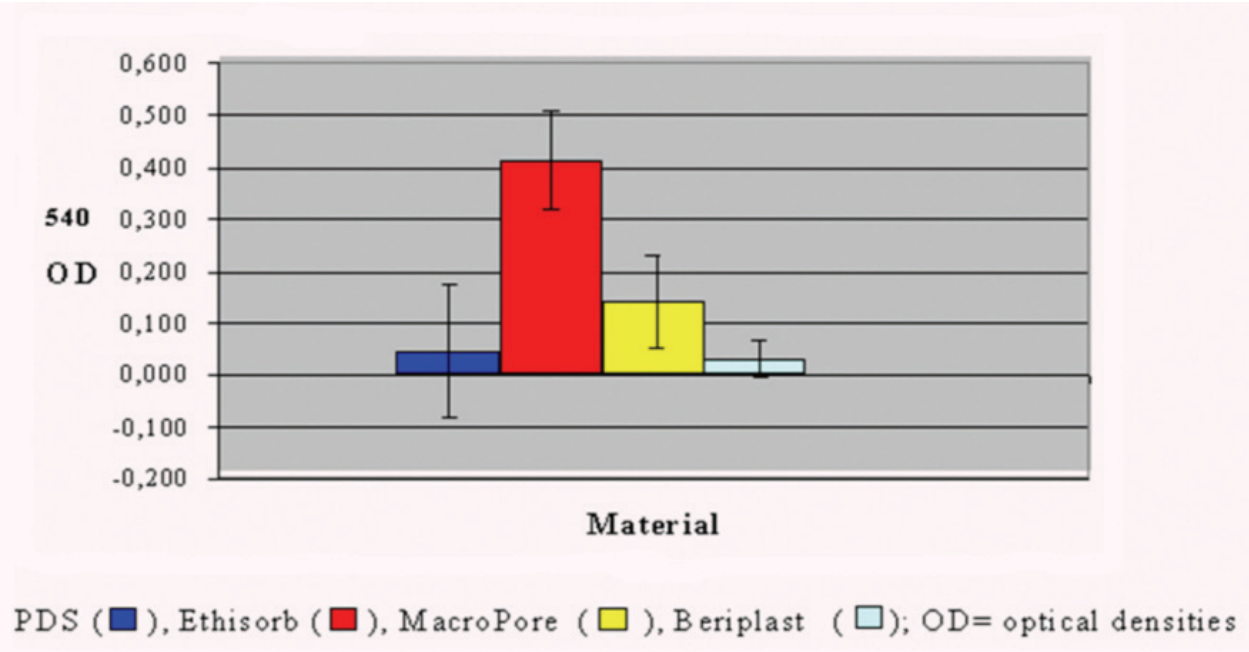

Fig. 1 Cell proliferation assay (EZ4U) of human osteoblast-like cells cultivated on four biomaterials. The standard deviation is indicated as error bar.

using mucosa from the inferior or middle turbinate as autologous material and fibrin glue. For compression and hemostasis in the mucosa donor areas, we inserted a nasal package in the lower part of the nasal cavity for a maximum of 3 days after surgery. The defect-closure area remained free, so that any leakage around the defect closure could be immediately recognized after removal of the nasal package. Control examinations for possible postoperative renewed cerebrospinal fluid leak was made using blood-sugar sticks and endoscopic inspection of the surgical area. Since clinical examination brought no evidence of recurrent cerebrospinal fluid, no additional diagnostic measures, such as a fluorescein test or cisternography were needed up to now.

Results

Basic research

HOB-like cells were characterized according to well-established parameters of osteoblastic phenotype: determination of the amount of osteocalcin, the alkaline phosphatase activity and the presence of cells expressing type one collagen. The average amount of osteocalcin was 10.42 ng/ml. The alkaline staining of these cells was typically intensively positive (about 58.70%). Fig. 2 shows micrographs of alkaline phosphatase staining. The alkaline phosphatase positive cells were blue- stained. Immunocytochemistry of the fixed cells showed the presence of type I collagen in about 31.18% of the cells. In Fig. 3 photographs of type I collagen positively stained cells were shown. All these parameters were consistent with osteoblastic phenotype, therefore, these cells, which were seeded on the four different biomaterials, can be characterized as osteoblasts. Results represented the mean values of all experiments. For studying the viability and the proliferation capacity of the osteoblast-like cells from twelve patients seeded onto four different biomaterials, the EZ4U test was performed. The measured optical densities (ODs) or absorbance values of colorimetric assay correlated with the number of cells. The average of the measured absorbance of each material was shown in Fig. 1. Standard deviations were between 0.035–0.130. The highest proliferation rate resulted in the group of Ethisorb® (OD_{540} = 0.413). The lowest proliferation rate was measured in the group of Beriplast® (OD_{540} = 0.031). Statistical analysis showed that the prolifer-

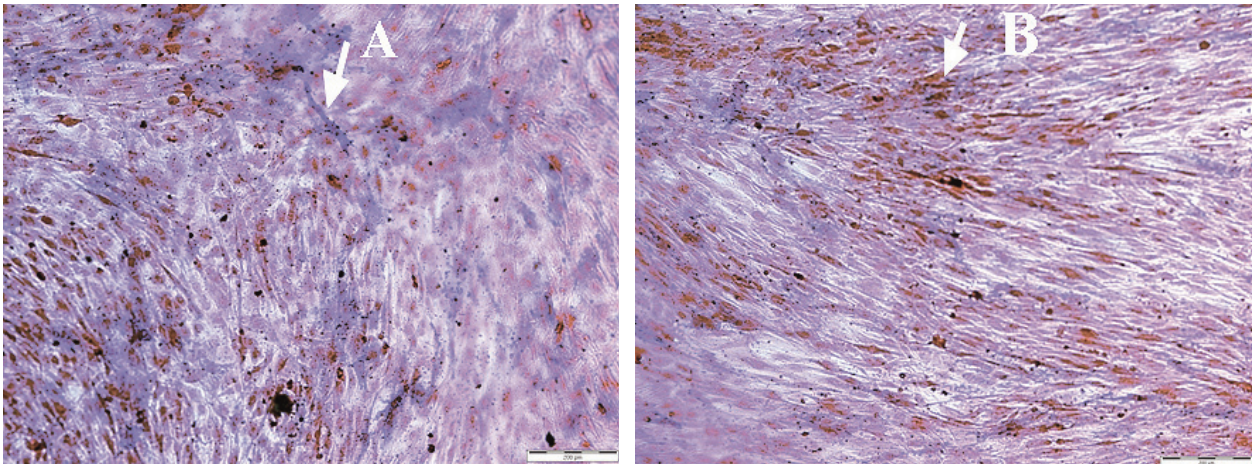

Fig. 2 Alkaline phosphatase staining of human osteoblast-like cells. Positively stained cells (blue colour, **A**) and round- or oval shaped nucleuses (red colour, **B**).

ation rate of Ethisorb® was significantly higher, compared to the other tested biomaterials (Friedman test, $p < 0.0000$). There was statistical significance of the proliferation rate between PDS®, MacroPore® and Beriplast® ($p < 0.0006$).

Clinical application

In a quality-assurance analysis, we followed the clinical course of 8 patients, treated for a dura defect in the rhinobase area using resorbable fleece. The follow up ranged from 3 to 46 months. The frontobasal dura defect could be safely closed in all patients after completion of wound healing with no evidence of persistent cerebrospinal fluid. In one patient there was renewed endoscopically-visible cerebrospinal fluid flow in the defect area which had originally been expanded by tumor infiltration following a valsalva-procedure on the second day after surgery (Table 1). The leakage stopped after placement of lumbar fluid drainage (100–150ml flow/24 hrs) and 2-day bed-rest. The lumbar drainage was clamped off after 6 days and removed after 7 days without any signs of further fluid leakage. A lumbar drainage had already been placed in another three patients preoperatively, in one case for the application of fluorescein and in two other cases as a cautionary measure to reduce fluid pressure following trauma. There were no clinical signs of meningeal irritation or local infection in any patient during the postsurgical in-patient course or after discharge. At the outpatient clinical control examination, as part of the quality-assurance analysis, endoscopic examination revealed non-irritative, smooth mucosal conditions around the former defect area with no evidence of foreign body granulation. Intraoperatively, the alloplastic material could be adapted easily to the corresponding defect size in all patients and was easily molded in situ under the limited manipulation space, by transnasal procedure. Due to the relatively short observation period of 6 to 55 months, the course of the results presented here must be supplemented by additional control examinations.

Discussion

The discovery of previously unknown infection paths of slow virus diseases like Jakob-Creutzfeld or transmission of the HI-virus by heterologous grafts like lyodura or by local superinfections from harvested autologous materials, brought an increasing number of alloplastic materials as alternatives for the closure of dura defects into the focus of interest. Concerning the high demand of replacement materials to reconstruct defects, numerous biomaterials consisting of different chemical substances were developed for the application in the field of tissue engineering [18]. In the present study, four commercially available biomaterials were choosen to study the proliferation rate of HOB-like cells on 2-dimen-

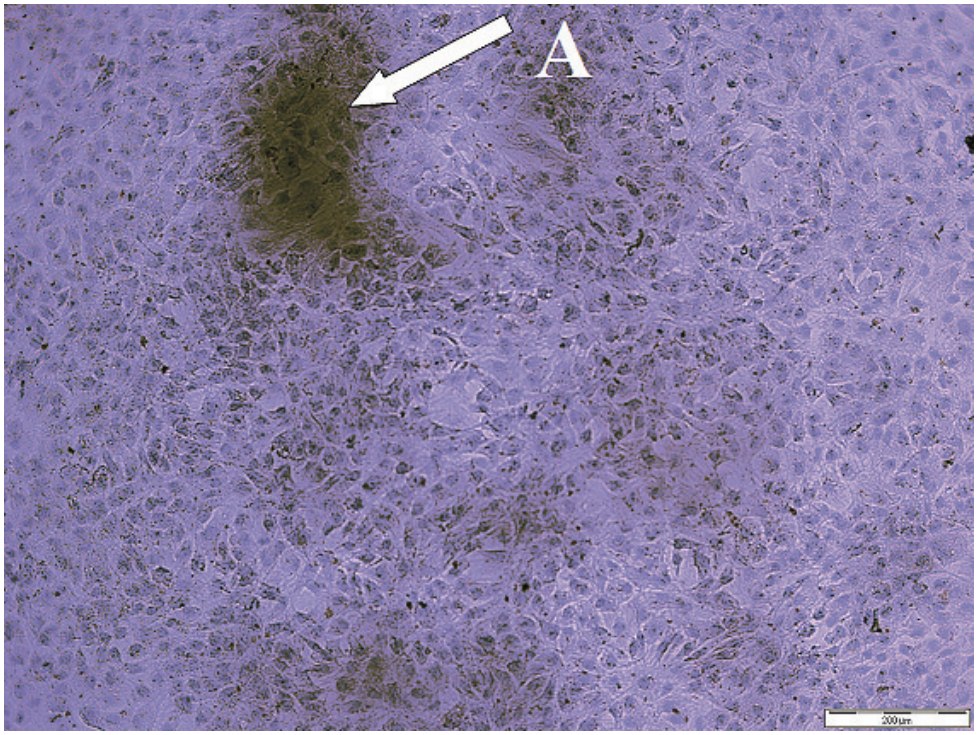

Fig. 2 Type I collagen staining of human osteoblast-like cells. Type I collagen positively stained cells (brown colour, **A**).

sional material by biochemical analysis. Primary HOB-like cells were used as they were representative of the cell type in contact with the material *in vivo*. The source of the cell for cultivation *in vitro* was of great importance for the development of transplanted substitutes. A cell based therapy for regeneration of tissue should utilize autologous cells, because ingrowth of immunocompetent cells of the host organism might destroy allografts or xenografts [19]. In this study, we attempted to verify the cells used for cultivation onto the biomaterials as osteoblast-like cells. Because bone chips were not treated with collagenase before cell cultivation, multipotent cells could have been present as well. Thus, the colonization onto the biomaterial surface could have taken place by committed osteoblasts and also by osteoprogenitor cells [20]. HOB-like cells used for the seeding procedure were characterized as osteoblasts by the determination of osteocalcin, of type I collagen as well as by the enzymatic activity of alkaline phosphatase. Osteocalcin, an extracellular noncollagenous matrix protein, was a very specific marker for osteoblasts. The quantitative determination of osteocalcin in the cell culture supernatant resulted positive in all of our seeded cells. The average amount of osteocalcin was 10.42 µg/l. Both alkaline phosphatase and type I collagen were early markers of osteoblastic differentiation and type I collagen was the primary component of the extracellular matrix. Type I collagen was expressed in approximately 31% of the cells. It was generally accepted that increased specific activity of alkaline phosphatase in a population of bone cells reflected a shift to a more differentiated state. Furthermore, it appeared that alkaline phosphatase had a crucial role in the initiation of matrix mineralization as the expression of this enzyme is down regulated after mineralization start [21]. The positive alkaline phosphatase staining in this study was equal to 58.70%. By analyzing the synthesis of osteocalcin, type I collagen and alkaline phosphatase, we were able to confirm that the cells possessed an osteoblastic phenotype. Regarding the proliferation assay, the viability and the proliferation capacity of HOB-like cells were evaluated by the EZ4U test to close the bone defect at the rhinobase. The results showed the highest proliferation rate of HOB-like cells on Ethisorb®. On MacroPore® only a moderate rate of proliferation was measured. PDS® had just about one-third proliferation rate of MacroPore®. The lowest proliferation rate was found on Beriplast®. Fibrin glue (Beriplast®) was widely and successfully used in tissue engineering of articular cartilage, as well as in tissue engineering of skin and mucosa [22–24]. In this study, the lowest proliferation rate of HOB-like cells on Beriplast® supported,

that fibrin glue has a biocompatibility to osteoblasts but does not have a osteoconductive property. Fibrin glue can be considered as cell-embedding material, whereas resorbable polymers provided a more mechanical stable scaffold [8, 25]. In conclusion, the present examination not only demonstrated an excellent biocompatibility of resorbable fleece, but also indicated its potential for tissue-engineered growth of human bone in a three-dimensional scaffold using bone cell autografts. Further experiments should be performed to create three dimensional biomaterial scaffolds with an appropriate surface topography (porosity, surface roughness) to compare them again *in vitro* and *in vivo*, respectively. This would provide a complete characterization of osteogenetic and osteointegration properties of the potential bone subtitute material.

We used resorbable fleece in patients with rhinobase dura defects of various genesis. Clinical criteria for evaluation of alloplastic materials are the availability, danger of contamination, modeling characteristics like elasticity and flexibility, impermeability and biocompatibility. The vicryl fleece is a completely resorbable synthetic material for temporary bridging of defects of the dura mater. The porous structure of the vicryl fleece permits an immigration of endogenous tissue, the undyed PDS-thread is intended to support fluid impermeability and minimize the formation of cortical adhesions. Futher studies [26, 27] in animals and humans analysed the biocompatibility of vicryl fleece in six month follow up examinations and it was generally characterized by low-grade granulomatous inflammation and initial adhesions to the brain surface. The three-dimensional structure of this implant acted as a scaffold to guide the development and integration of a 'replacement' dura mater. The absorption of the material was associated with complete resolution of the inflammatory reaction, a lack of cerebral adhesions, and restoration of the normal architecture of this region. The observation of a recurrent cerebrospinal fluid rhinorrhea after excision of a malignant tumour in our study with an extensive dura defect (Table 1) indicates, that the procedure described here with resorbable fleece, is appropriate only for small to medium defects up to about maximal 6 cm^2. Covering larger defects with avital alloplastic materials in the area of the dura may lead to the problem that the implant is not completely replaced or enclosed by scar tissue, due to the lack of biological activity. For larger defects, we would prefer in any case autologous grafts of cartilage to stabilize the defect and fascia with respect to possible tissue revitalization. In our study, the PDS-layer in the resorbable fleece has demonstrated an excellent biocompatibility and the highest proliferation rate of HOB-like cells on vicryl fleece. It must be remarked here, however, that there is no experience about late meningitis associated with this alloplastic material in our study. Based on our results, resorbable fleece can be recommended as alloplastic tissue material, especially for small to medium dura defects of the rhinobase due to the immediate intraoperative availability and guaranteed sterility and to its favorable modeling and fitting capacity in endonasal defect closures of the frontobasal dura. It remains to be seen whether the most recent research results on *in-vitro*-cultivated cell transplantations (tissue engineering) of autologous cerebral membrane cells will show, that these can be used for dura defect closure [28–30]. In conclusion, each material has its own physical and biochemical properties. Concerning the clinical application, anatomical and physiological conditions such as load-bearing area in lower extremity, moisture from tissue fluid or bleeding, brain pulsation etc. affect the properties of biomaterials. Nowadays there is no single material available that has all ideal properties for clinical applications in every location of the human body.

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