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May 31–June 2, 2006

Würzburg, Germany

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DEVELOPMENT OF A HIGH-RESOLUTION OCT SYSTEM FOR MONITORING AND CHARACTERIZATION OF ENGINEERED TISSUES *IN VITRO*

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Introduction: Optical coherence tomography (OCT) is a promising imaging tool for applications in cell and tissue biology. In the field of tissue engineering, in particular, there is a need for a non-invasive, non-destructive method for characterizing engineered tissues in real time during tissue growth and development. OCT uses low-coherence optical interferometry to obtain micron-scale-resolution tomographic images from backscattered or back-reflected light from tissues. As such, OCT has several advantages over other imaging modalities. However, limited depth penetration has prevented more widespread application of the technique. Here, we describe the development and evaluation of a new high-resolution OCT system for the purpose of monitoring tissue remodeling *in vitro*.

Materials & Methods: The OCT system we have developed utilizes a broadband light source (890 nm) which makes the μm-scale resolution possible. The delivery optics are designed to minimize attenuation and dispersion of the signal. The resolution of this system in monitoring the growth of human fibroblast cells in collagen-gel constructs in comparison with conventional OCT is investigated.

Results: Preliminary data have shown the potential of OCT to act as a monitoring tool and provide non-invasive information of cell growth, presence, and concentration in collagen-based constructs. The high optical scattering of cells in different concentrations within the constructs was observed with OCT imaging and confirmed by microscopy.

Conclusions: Current high-end OCT systems have resolutions capable of imaging at the cellular level, and the cost of such systems is small in comparison to existing three-dimensional techniques such as micro-CT. Moreover, the ability to step through the whole sample in real-time allows for the identification of structural defects that could be overlooked when examined using standard microscopy techniques. The OCT system described in this paper suggests that non-invasive monitoring of events at a cellular level and in real time is a realistic goal.

P-29

X-RAY MICROANALYSIS EVALUATION OF ORAL KERATINOCYTES AND FIBROBLAST CELLS FOR TISSUE ENGINEERING

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Introduction: Several scientists have tried to construct efficient substitutes of human oral mucosa by tissue engineering. In this context, it is essential to develop new methods leading to a more accurate evaluation of the viability of the cultured cells prior to their clinical use.

Materials & Methods: Ten small gingival biopsies were obtained from healthy humans undergoing extraction of impacted third molar teeth. To establish oral keratinocyte primary cultures, the biopsies were incubated at 37°C in 0.25 mg/ml trypsin. Detached keratinocytes were cultured in flasks containing a layer of 3T3 feeder cells inactivated by treatment with mitomycin C (10 mg/ml) for 2 h using a culture medium supplemented with different growth factors. To obtain primary cultures of human oral fibroblasts, the stroma of the oral biopsies was digested overnight in 2% *Clostridium histolyticum* collagenase I and incubated in DMEM culture medium supplemented with 10% fetal bovine serum. Evaluation of cell viability was carried out by trypan blue staining and by quantification of intracellular levels of Na, P, Mg, K, Cl, Ca, and S by electron-probe X-ray microanalysis associated with scanning electron microscopy. For that purpose, the cells were grown in gold grills, freeze-dried, and carbon-coated in a high-vacuum coating system. Cells were microanalyzed within the following hours in a Philips XL30 scanning electron microscope equipped with an EDAX DX-4 microanalytical system.

Results: Trypan blue staining showed that 96.5% of keratinocytes and 97% of fibroblasts were alive and viable. The analysis showed that the average ionic content of the cultured keratinocytes was as follows (expressed as mmol/kg of dry weight): Na: 36.87; P: 253.32; Mg: 20.49; K: 374.39; Cl: 172.05; Ca: 14.84; S: 44.63. The K/Na ratio was 10.15. For human fibroblasts, the average ionic content was: Na: 47.10; P: 308.49; Mg: 20.27; K: 398.19; Cl: 179.27; Ca: 13.61; S: 71.04.

Discussion: These results suggest that cultured oral fibroblasts and keratinocytes are viable and that the content of the analyzed elements is normal, suggesting that these cells are viable for clinical use. In comparison with trypan blue staining, the use of electron-probe X-ray microanalysis demonstrated to be an accurate technique for detection of cell viability and early cell death.

This work was supported by FIS 04/1306 and FIS 03/141.

P-30

CONFOCAL REFLECTION IMAGING OF 3D FIBRIN POLYMERS

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Introduction: The reconstruction of extracellular matrix (ECM) by confocal reflection microscopy is a physical approach for monitoring the ECM structure, cell-matrix interactions, and changes of ECM structure over time.

Materials & Methods: HFF+TERT were used as equivalent to normal regenerative fibroblasts and incorporated into nonpolymerized fibrin solution. After polymerization of the lattice, fibrin gels were transferred to a self-constructed chamber for staining and confocal microscopy. For simultaneous fluorescence and reflection microscopy, lattices were stained by type I collagen antibody.

Results: Reflection caused by fibrin fibrils was sufficient for high signal-to-noise ratio for detection of fiber location and network formation. At the interface between fibroblasts and fibrin matrix, the realignment of fibers toward the cell body was detected. Costaining of type I collagen showed that most fibrin reflection is devoid of immunoreactivity for type I collagen. However, a strong collagen signal was present in proximity to fibroblasts in a pericellular manner. After 7 d of culture, fibroblasts generated extensive collagen-positive tracks within the fibrin network and further induced increased reflection intensity, indicative of fibrin contraction and remodeling.

Discussion: We here show that confocal reflection imaging is useful in reconstructing the fibrillar architecture of 3D fibrin lattices and fibroblasts embedded therein at high resolution up to 250 nm. Together with confocal fluorescence microscopy of collagen deposited by fibroblasts, this technique allows the monitoring of fibrin remodeling by stromal cells. In conclusion, confocal reflection microscopy is a valuable technique for real-time monitoring of the remodeling of fibrin matrices, such as during thrombus formation, wound healing, and tumor formation.

P-31

TISSUE REGENERATION, GENOME REJUVENATION, AND CANCER: A NEW PROBIOTIC STRATEGY

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Successful tissue regeneration remains an important issue. Despite application of various regeneration-promoting drugs, in some cases (for example, in postoperative cancer patients) attempts to achieve an increased regeneration fail to succeed. In this connection, a methodological approach, as suggested by Russian Prof. Tokin a few decades ago, deserves attention. Disruption of regenerative processes in various tissues may result in the development of cancer, so regeneration and malignant growth are antagonistic events. Instead, promotion of tissue regeneration may theoretically be supportive in any organism facing a cancerous process.

Recently, we showed that oral administration of *B. oligonitrophilus* KU-1 resulted in suppression of cancer growth both in humans and animals; herewith, inhibition of metastatic development in animals was more pronounced. In special experiments with *Drosophila melanogaster* and various plants, the above-mentioned bacterial strain was found to be responsible for arrested development. Namely, monthly delays in florification and ruberization were found in American artichoke, gladiolus, and banewort due to soil treatment with *B. oligonitrophilus* KU-1 (titer 10^9 cells/ml, 1l per garden square meter). Broken barley seeds grown on the *B. oligonitrophilus* KU-1-treated soil were added to fly food, which resulted in a 2-d delay in nymphosis (taking into account the *D. melanogaster* 14-d life cycle, this delay can be considered as significant), thereby decelerating ageing and subsequent death.

We hypothesized that anticancer and other effects were mediated by the process of genome rejuvenation and by subsequent enhancement of organism regeneration events owing to DNA enrichment with silicon obtained from our *B. oligonitrophilus* KU-1, which was suggested to be a donor of the element. Although the precise role of silicon in chromosomes needs further investigation, application of a new probiotic strain with the ability to advance regeneration and promote rejuvenescent effect at the DNA level seems promising.

P-32

INFLUENCE OF SURFACE TOPOGRAPHY AND MECHANICAL STIMULATION ON CELL ARCHITECTURE AND OSTEOGENIC DIFFERENTIATION OF HUMAN BONE-RELATED CELLS

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Introduction: Human bone marrow cells (HBMC) are known to play an integral part in bone formation. Parameters which influence cell behavior range from queues that are present in the cell surrounding such as soluble factors and cell - cell contacts to substratum properties such as topography and chemistry, and further to this to mechanical stimulation. Bone is able to sense and respond to all such stimuli. This study investigates the effects of defined surface topographies (e.g. hemispheres with different diameters + spacing) on the adhesion, morphology, focal contacts as well as osteogenic differentiation of HBMC. In a second approach, mechanical stimulation of HBC in the form of isolated dynamic pressure was studied.

Material & Methods: Human bone cells (HBC) or HBMC were isolated from the bone marrow of patients receiving a total hip implant. Cells were either cultivated on substrates with defined surface topographies (i.e. flat, hemispheres of 30 µm diameter + 20 µm spacing, hemispheres of 50 µm + no spacing, polished and etched surface, and a surface with hemispheres of 30 µm + 20 µm spacing with additional secondary etched structure) or exposed to dynamic pressure application (30', 3 times a day for 10 d with max. pressure of 10 kPa, 20 Hz). Cells were fixed after 10 d of cultivation and stained for actin, vinculin, and nuclei. Osteogenic differentiation was analyzed by quantitative RT-PCR using the differentiation-specific markers osteocalcin, bone-specific alkaline phosphatase (ALP), and collagen I.

Results: Results from the study of cells cultivated on different topographies suggest that hemispheres with different diameter and spacing have distinct effects on cell morphology. Actin and vinculin staining reveal distinct rearrangements of cell architecture components on some surface structures. HBC kept in osteogenic medium and exposed to pressure stimulation have a very similar size and shape to the corresponding control cells, but actin filament expression of HBC after 10 d of treatment is changed. Also, the differentiation into osteoblasts is enhanced in cells stimulated by dynamically applied pressure, as observed by the quantification of osteocalcin, ALP, and collagen I mRNA.

Discussion: Different strategies can be chosen to obtain specific stimulatory effects on HBC and HBMC. Such strategies might be of value for the design of bone implants or therapeutic approaches with respect to bone healing/bone tissue engineering.

P-33

DEVELOPMENT OF BIOACTIVE GLASS FIBER REINFORCED STARCH-POLYCAPROLACTONE COMPOSITE

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Introduction: For bone regeneration and repair, combinations of different materials are often needed. In many applications, it is useful to combine biodegradable polymers with osteoconductive materials, such as bioactive glass. One other aim is to try to improve the mechanical properties of polymer matrix by means of its reinforcing. Thus, the aim of this work was to develop a bioactive glass fiber reinforced starch-polycaprolactone composite.

Materials & Methods: The composite was produced by extruding thick sheets from a starch-polycaprolactone (SPCL, 30/70 wt%) blend. Sheets were cut and heat-pressed in layers with bioactive glass fiber mats to form composite structures with different combinations: 6xSPCL + 5xBaG, 3xSPCL + 6xBaG, 3xSPCL + 4xBaG, and 3xSPCL + 2xBaG. 3xSPCL and 6xSPCL were used as non-reinforced controls. Thermal, mechanical, and degradation properties of the composite were studied. In addition, the real amount of glass in the composites was determined using simple burning tests.

Results: A strong endothermic peak indicating melting at about 56°C was observed from DSC analysis. TGA showed that thermal degradation of SPCL started at 300°C with degradation of starch and continued at 380°C with degradation of PCL. Mechanical properties of reinforced composites were considerably better than the properties of non-reinforced composites. Reinforcing increased shear strength by 50%, tensile strength by 52%, and bending strength by 67%. However, mechanical properties of the composites dropped during 2 weeks of hydrolysis to the same level of non-reinforced controls. The degradation time of SPCL, as expected, was long; during the 6 weeks of hydrolysis, the mass decreased by only about 5%. Degradation will of course occur faster at a later stage. The amount of glass in the composites remained the same for the 6-week period of hydrolysis.

Conclusion: It is possible to enhance initial mechanical properties of starch-polycaprolactone by reinforcing it with bioactive glass fibers. However, mechanical properties need to be further improved before allowing long-lasting bone applications.

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SCAFFOLDS BASED ON POLYSACCHARIDE FOR SOFT TISSUE ENGINEERING

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Introduction: Natural biodegradable polymers are some of the most suitable scaffold materials for articular cartilage implants. However, the ideal scaffold material for hyaline cartilage grafts has not yet been found. In order to improve tissue formation, we aimed to create a polysaccharide scaffold with a gradient of biochemical and biomechanical properties that can be adjusted to mimic the conditions in normal articular cartilage.

Materials & Methods: Chitin is a naturally derived polysaccharide that can be transformed by hydrolysis to chitosan, which possesses free amino groups. By crosslinking chitosan, a polymer network can be created. Amino groups react with aldehyde groups to form Schiff bases. Therefore, dialdehyde compounds can be used as crosslinkers. Here, either glutaraldehyde, as the smallest possible molecule, or dextran/starch was used for crosslinking. In the case of dextran and starch, the aldehyde groups were generated by oxidation with periodic acid. After mixing the solutions of chitosan (1- 4 wt%) and dialdehyde (0.13 - 3.5 wt% glutaraldehyde, 1 wt% oxidized starch/dextran), the complete solution was frozen at -32°C. The frozen water was then removed by lyophilization, producing a polysaccharide scaffold with interconnected pores.

Results: The total porosity, measured by water uptake capacity, is reduced from 99 to 95% when using 0.12 and 3.5 wt% glutaraldehyde, respectively. Different pore sizes are obtained, and pores vary between lamella and sponge form. In the same sample, both types can be found. Besides the geometry, the pore size also varies between 25 and 400 µm. Rheological measurements show an increase of shear stress with chitosan content, from about 500 Pa (2 wt% chitosan) to 5000 Pa (4 wt%). The scaffolds possess viscoelastic behavior; the elastic module is larger than the viscous module. In order to test the suitability of the constructs as scaffolds for osteochondral implants, they were seeded with chondrocytic mesenchymal cells and bovine chondrocytes, and incubated for several weeks. Cells adhered to the scaffolds and could be induced to differentiate along the chondrogenic pathway.

Discussion: By adding amino-functionalized hydroxyapatite to the solution, a composite material could be generated. A combination of regions with and without hydroxyapatite gives a gradient scaffold that could be used for the tissue engineering of the bone/cartilage interface. The integration of HA can give additional rigidity in the biomechanically critical transition zone.

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PATTERNED NANOSTRUCTURES AS SURFACE MODIFICATIONS FOR ENHANCED CELL ADHESION

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Introduction: Cell adhesion is the first step in the acceptance of an implant material by a tissue in contact. When cartilage is cultured on an artificial support such as a ceramic carrier material, special attention has to be given to the strength of the cartilage/carrier interface. Surfaces with enhanced cell adhesion strength are needed for chondroblastic cells, which display decreasing cell adhesion with advancing differentiation. Here, we attempt to evaluate the ability of fingerprint-nanostructured SiO_2/TiO_2 coatings to support firm attachment of chondroblastic cells.