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Osteoproperties of polyethylene glycol hydrogel material

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ABSTRACT

Aim The aim of the present study was to test the osteogenic potential of a synthetic hydrogel made of polyethylene glycol (PEG), loaded with adult mesenchymal stem cells, used as a biodegradable membrane for guided bone regeneration (GBR).
Materials and methods Adult mesenchymal stem cells derived from adipose tissue (ADSCs) were isolated, characterized, and seeded on the hydrogel. After 15 days of culture, the scaffolds were analyzed with scanning electron microscopy (SEM) and real-time PCR to assess osteogenesis, and by means array CGH (Comparative Genomic Hybridization) to test their safety.
Results The *in vitro* results confirmed that the ADSCs were able to attach to the hydrogel and differentiate towards the osteogenic phenotype. Furthermore, array CGH analysis detected no chromosomal abnormalities, confirming the safety of the 3D cultures.
Conclusion The PEG hydrogel, loaded with adult mesenchymal stem cells, seems to have an osteogenic potential and therefore could be successfully used as a membrane in the treatment of bone defects.

KEYWORDS Adipose derived stem cells, Guided bone regeneration, Hydrogel, Osteogenesis, Polyethylene glycol.

INTRODUCTION

The treatment of bone defects represents a great challenge for orthopedic and cranio-maxillofacial surgery, as well as in dentistry. Although several methods for bone reconstruction exist, all of them have specific indications and limitations. The concept of using barrier membranes for the restoration of defective bones has been developed to simplify their treatment by offering a single-stage procedure. The established methods are distraction osteogenesis and bone transport or bone grafting, performed with autologous bone grafts, bone marrow aspirates, allografts, or bone substitutes supplied with growth factors (1-4). Furthermore, the concept of an induced membrane represents another strategy for bone regeneration. This method involves a two-stage procedure in which a 'biological' membrane is placed after the application of a cement spacer in the first stage, acting as a 'chamber' for the insertion of a bone graft in the second stage (1-3).
It has been shown that this membrane possesses osteoinductive, osteogenic and angiogenic properties, and several clinical studies have reported satisfactory results (1-4). Finally, the procedure of guided bone regeneration (GBR) using a bioabsorbable or non-resorbable membrane, that acts as a barrier to prevent soft tissue invasion into the defect and forms a 'chamber' to 'guide' the bone regeneration process (5-7), has also been used for bone reconstruction. In dental implants, for example, cell- or tissue-occlusive membranes help to restore the functional osseous tissue by allocating space for bone growth and preventing the competition between bone regeneration and soft tissue in-growth.
The materials used for GBR membranes have to meet specific criteria, such as biocompatibility, cell or tissue occlusion, space provision, and tissue integration. Membranes made of several different materials are commercially available and are either non-resorbable (e.g., expanded polytetrafluoroethylene) or resorbable (e.g., polylactic acid or collagen). The advantage of the resorbable materials is that they do not require a second surgery for their removal.
The vast majority of membranes available on the market today have to be trimmed to the desired shape. A liquid-applicable, in situ-formed biodegradable membrane would offer many advantages, especially for complicated

shapes (e.g., when used around a dental implant) or sites that are difficult to reach (8). In this regard, a novel polyethylene glycol (PEG) hydrogel was recently developed due to its biocompatibility and is already used in several medical devices (9). In a recent animal study, the barrier function of PEG was examined after subcutaneous placement in rats (10). Histological analysis revealed that it prevented cellular penetration in the membrane group for up to 4 months. To date, no information regarding the use of this material for osteogenesis induction is available. The present study was performed to test the osteogenetic properties of a PEG-based in situ applicable hydrogel. Adult mesenchymal stem cells derived from adipose tissue (ADSCs) were isolated and seeded on PEG-based scaffolds. The biocompatibility and osteogenic properties of the scaffolds were tested by quantification of DNA content, morphologic analysis (electron microscopy), and gene expression. Moreover, the genetic safety of the scaffolds (i.e., ability to induce tumorigenesis) was assessed with array CGH.

MATERIALS AND METHODS

Biomaterial

PEG hydrogels (Institut Straumann AG, Basel, Switzerland) were used as scaffolds for cell cultures. The hydrogels were composed of two PEG molecules. The single molecules, dissolved in its buffer, were mixed and then solidified within 20-50 s at room temperature.

Cell cultures

ADSCs were extracted from the adipose tissue of 5 healthy women and 5 healthy men (age: 21-36; BMI: 30-38) undergoing cosmetic surgery procedures according to the guidelines of the Plastic Surgery Clinic at the University of Padua. The adipose tissues were digested and the cells isolated, expanded, and seeded following our previous protocol (11).

Proliferation test

The cell proliferation was assessed by measuring DNA content in the cell cultures after 3, 7 and 15 days from seeding in medium without any differentiation factor (standard medium). The DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) also suitable for purification of total DNA from cells. The DNA concentration was detected by measuring the absorbance at 260 nm in a spectrophotometer. The number of cells was then determined from a standard curve (micrograms of DNA versus cell number) generated by DNA extracted from counted cells. The standard curve was linear over the tested range of 5-80 µg DNA (r=0.99).

Scanning Electron Microscopy (SEM)

Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h before processing with either hexamethyldisilazane or critical-point drying followed by gold-palladium coating. All micrographs were obtained at 20 kV on a JEOL 6360LV SEM microscope (JEOL, Tokyo, Japan). The SEM analysis was carried out at the Interdepartmental Service Center C.U.G.A.S. (University of Padua).

Real-time PCR

For the first-strand cDNA synthesis, 800 ng of total RNA of each sample was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Human primers were selected for each target gene with Primer 3 software (Table 1). Real-time PCRs were carried out using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions were as follows: 15 min denaturation at 95°C; followed by 40 cycles of 15 s denaturation at 95°C; annealing for 30 s at 60°C; and 20 s elongation at 72°C. Values were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal reference, whose abundance did not change under our experimental conditions. Experiments were performed with 3 different cell preparations and repeated at least 3 times.

GENE	FOR (5′ - 3′)	REV (5′ - 3′)	PRODUCT LENGTH (BP)
Osteonectin	TGCATGTGTCTTAGTCTTAGTCACC	GCTAACTTAGTGCTTACAGGAACCA	186
Osteopontin	TGGAAAGCGAGGAGTTGAATGG	GCTCATTGCTCTCATCATTGGC	192
Collagen type I	TGAGCCAGCAGATCGAGA	ACCAGTCTCCATGTTGCAGA	178
CD31	TCCAGCCAACTTCACCATCC	TGGGAGAGCATTTCACATACGA	171
von Willebrand Factor	GCTTCACTTACGTTCTGCATGA	CCTTCACTCGGACACACTCATTG	174
GAPDH	TCAACAGCGACACCCAC	GGGTCTCTCTCTTCCTCTTGTG	203

TABLE 1 Human primer sequences.

Array CGH (Comparative Genomic Hybridization)

Array CGH was conducted using the Agilent Human Genome CGH Microarray 4x44K Kit (Agilent Technologies, Palo Alto, CA, USA) with a median resolution of 43 kb. Labeling and hybridization were performed following the Agilent protocols. The graphical overview was obtained using the CGH analytics software (v3.1) (Agilent Technologies, Santa Clara, CA, USA).

RESULTS

Cell proliferation and morphology

ADSCs were able to proliferate on the top of the scaffold, increasing their number, as demonstrated by the proliferation test performed at 3, 7 and 15 days of culture in standard medium (Fig. 1).

The SEM images acquired 3 hours after seeding revealed that the cells started to attach to the substrate (Fig. 2A). After 15 days of in vitro culturing without differentiation medium, ADSCs adhered to the scaffolds, forming a continuous cell monolayer characterized by a typical osteoblastic (star-like) phenotype (Fig. 2B).

Gene expression

Real-time PCR was performed on 3D ADSCs cultures in the presence of osteoinductive factors, vasculogenic factors or standard medium. ADSCs cultured on PEG hydrogel without differentiation medium showed a well-defined osteoblastic expression profile (Fig. 3, green bars). Indeed, the expression of osteonectin, osteocalcin, and collagen type I was observed, with values comparable to those obtained with osteogenic medium (Fig. 3, blue bars). These results confirm that

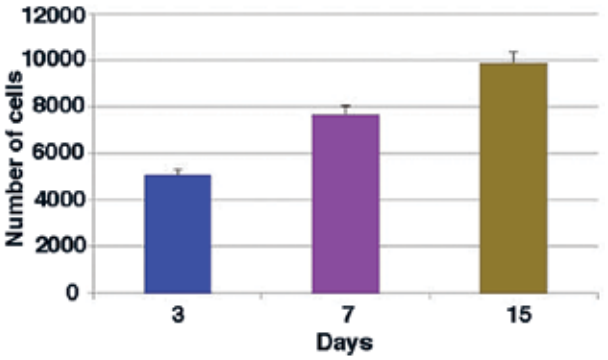


FIG. 1 Proliferation test of ADSCs seeded on PEG hydrogels. Cell proliferation was assessed by measuring DNA content in the cell cultures after 3, 7 and 15 days from seeding in standard medium.

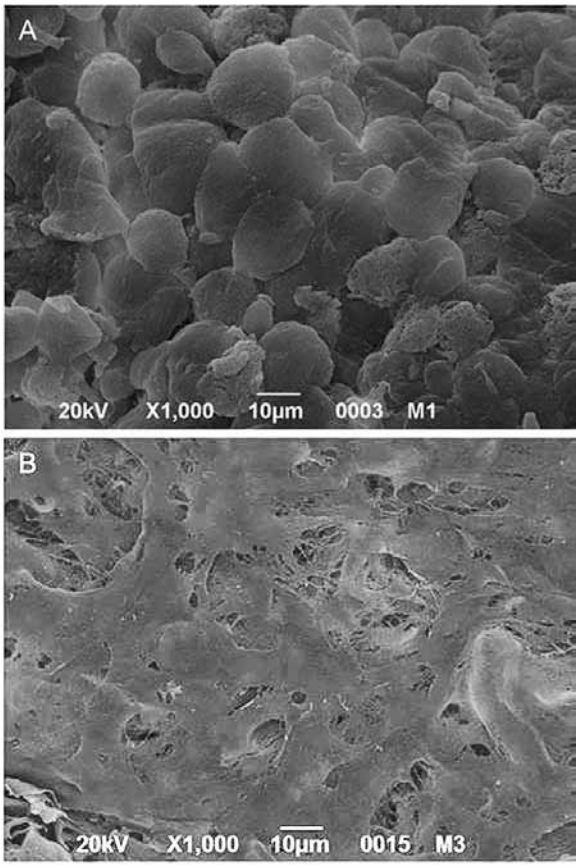
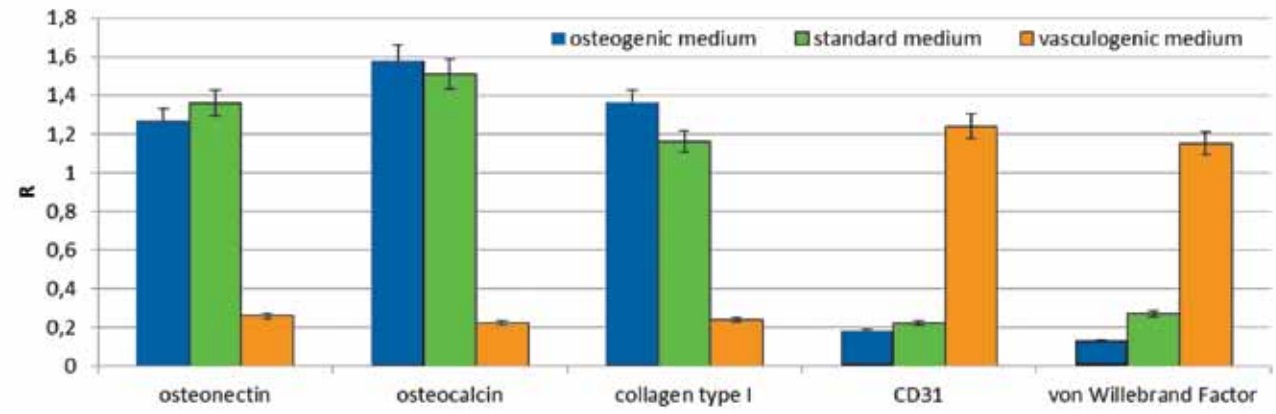


FIG. 2 SEM analysis of ADSCs on PEG hydrogel after 3 hours (A) and 15 days (B) of culture in standard medium.

FIG. 3 Real time PCR analysis of ADSCs cultured on PEG hydrogel for 15 days in osteogenic medium (blue bars), standard medium (green bars) or vasculogenic medium (orange bars). Expression profile of osteogenic markers (osteonectin, osteocalcin and collagen type I) and vasculogenic markers (CD31 and von Willebrand Factor).



the presence of the hydrogel alone was enough to induce bone commitment of the stem cells. ADSCs cultured on PEG hydrogel with vasculogenic medium (Fig. 3, orange bars) expressed markers of vascular cell phenotype (CD31 and von Willebrand Factor). On the contrary, no vascular markers expression was detected in ADSCs cultured in standard medium.

Array CGH analysis

In order to identify genomic alterations, the DNA was extracted from cells derived from two different donors and seeded on PEG hydrogel material for up to 15 days. As shown in Figure 4, no chromosomal imbalances (duplications or deletions of DNA regions) were detectable in either the donor cell populations, confirming that neither long-term cultures nor the use of growth factors induced structural alterations of the DNA.

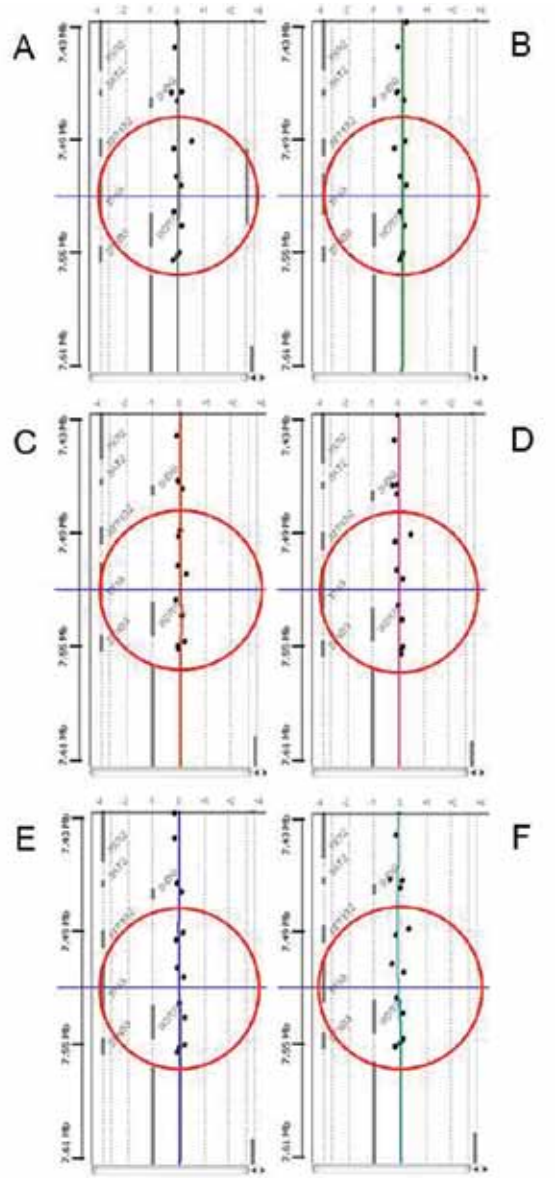


FIG. 4 Array CGH analysis of ADSCs cultured on PEG hydrogel for 15 days. Cells isolated from two different donors and 3D cultured in osteogenic medium (A, B), standard medium (C, D) or vasculogenic medium (E, F). The figure shows a representative region of the whole genome analyzed, in particular the region of the p53 gene (indicated by the red ovals).

DISCUSSION AND CONCLUSION

Today, implant therapy is regarded as an extremely reliable approach to replace missing teeth. The introduction of osseointegrated implants in dentistry represented a turning point in dental clinical practice. A patient's expectations for prosthetic rehabilitation are increasingly high, especially with regard to quality of life and functionality. The introduction of dental implants has led to a turning point in the rehabilitation of partially or totally edentulous patients. However, the placement of standard-length dental implants is not always possible or feasible in the first instance (12-14).

As a general principle for implant surgery, the implant surfaces should be surrounded by alveolar bone. Sometimes, due to the prosthetic or anatomical limitations of the alveolar ridge, it is not possible to appropriately insert the implants in bone. Several methods are used for the reconstruction of destroyed alveolar bone, including GBR. In most cases of GBR, the membranes are supported by protective materials consisting of allografts, synthetic materials or xenografts (15). In this context, a novel *in situ* gelling hydrogel composed of two PEG components was recently suggested as a new material for GBR procedures. In particular, PEG components have been proven to be highly biocompatible, cell occlusive, and biodegradable, thus meeting the important criteria required for serving as a barrier membrane (16).

In light of these considerations, the present study aimed to assess the osteogenic properties of PEG hydrogel loaded with adult stem cells derived from human adipose tissue.

Proliferation test based on DNA quantification confirmed that the ADSCs seeded onto PEG hydrogel increased in number and generated a vital tissue. The SEM morphological analysis showed that the cells were able to adhere to the niches of the biomaterial, forming a thin monolayer. In the context of tissue engineering and regenerative medicine, it is increasingly recognized that it is important for different cell types to co-exist in a 3D environment to generate structures with greater functionality and engraftment capacity (11).

In this study, adipose tissue was used as a readily available source of cells for the generation of a 3D structure with both osteogenic and vasculogenic properties. The gene expression analysis for osteogenic markers strongly supported the commitment of cells towards the osteogenic phenotype. Real-time PCR confirmed the

presence of extracellular matrix components, such as osteonectin and osteocalcin, which play a fundamental role in the interaction of cells with the bone matrix and in matrix mineralization, after 15 days of 3D culture. The expression of collagen type I, which is essential to the formation and maturation of hydroxyapatite crystals, was also clearly detectable, confirming the correct extracellular matrix composition. In parallel cultures containing vasculogenic factors, ADSCs were committed to mature endothelial cells, as confirmed by the expression of surface markers, such as CD31, and specific endothelial soluble factors, such as von Willebrand Factor. Surprisingly, expression of osteogenic markers was also detectable when the ADSCs were cultured on the hydrogel without any differentiation factors.

Detailed cytogenetic analyses were performed to validate the safety of the PEG hydrogel loaded with ADSCs. The safety of cell-based products need to be guaranteed before use in *in vivo* implantations, for example through the validation of chromosomal stability. Chromosome analysis remains one of the most commonly performed diagnostic genetic tests and it is suitable for a wide variety of indications in oncology, gynecology and pediatrics. At the cytological level, banded human chromosomes show a consistent and similar pattern in clinically healthy individuals. Hence, balanced and unbalanced chromosomal aberrations can serve as informative markers for a clinical phenotype, such as the dangerous transformation of a normal genotype into a tumor one. Array CGH represents an innovative molecular cytogenetic assay to test the chromosome stability. This technique allows the screening of the DNA content at high resolution, revealing DNA copy number changes (gains/losses), even if DNA is extracted from 3D cell cultures (11). In this way it is possible to establish whether prolonged in vitro cultures can give rise to chromosomal anomalies that might be implicated in the etiology of diseases or disorders (17).

The aim of the present study was to test the chromosomal stability of ADSCs expanded in vitro on PEG hydrogels. The results of the array CGH showed no DNA alterations, thus confirming that the cells were able to differentiate while maintaining genomic stability.

In conclusion, PEG hydrogel behaves as a good scaffold to induce the osteogenic commitment of adult stem cells, commonly used for tissue engineered bone products, and at the same time it seems to be genetically safe.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that there are no competing interests.

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