ABSTRACT: In the present study, based on a biomimetic approach, novel 3D nanofibrous hybrid scaffolds consisting of poly(ε-caprolactone), poly(vinyl alcohol), and chitosan were developed via a multi-jet electrospinning method. The influence of chemical, physical, and structural properties of the scaffolds on the differentiation of mesenchymal stem cells into osteoblasts, and the proliferation of the differentiated cells were investigated. Osteogenically induced cultures revealed that cells were well-attached, penetrated into the construct and were uniformly distributed. The expression of early and late phenotypic markers of osteoblastic differentiation was upregulated in the constructs cultured in osteogenic medium. (Int J Artif Organs 2007; 30: 204-11)

KEY WORDS: Bone tissue engineering, Stem cell, Nanofiber, Chitosan, Hybrid scaffold

INTRODUCTION

Bone defects resulting from tumors, diseases, infections, trauma, biochemical disorders, and abnormal skeletal development raise significant health problems (1, 2). Traditional biological procedures such as autografts, allografts, and grafts with nondegradable materials have been used to overcome bone defect problems. Bone transplantation by these treatment methods, however, is often limited by donor scarcity and is highly associated with the risk of rejection and disease transfer (3, 4).

In recent years, tissue engineering, as a new discipline integrating surgical techniques with the concepts of the life sciences, such as biology, chemistry, and engineering, has been able to develop approaches for the regeneration of skeletal tissues (5, 6). It is believed that appropriate strategies for orthopedic tissue engineering should ideally contain osteoprogenitor stem cells, osteoinductive growth factors, and biodegradable osteoconductive scaffolds (7). A successful strategy involves in vitro seeding and attachment of human osteoprogenitor stem cells onto a scaffold. These cells then proliferate, migrate, and differentiate into the osteoblasts that secrete the mineral extracellular matrix required for the creation of the bone. It is evident that the choice of the most appropriate scaffolding material is crucial to enable the cells to behave in the manner required in order to produce bone of the desired shape and size (8-10).

Over the last few decades, a vast majority of natural and synthetic scaffolding materials have been studied (11-14). Despite their good formability and suitable mechanical properties, most synthetic polymers lack cell-recognition signals, and have a hydrophobic nature which prevents uniform seeding of cells and incorporation of growth factors in three dimensions. In contrast, natural biopolymers have the potential advantages of specific
cell interactions and hydrophilicity. However, they have poor mechanical strength (15). From the point of view of engineering and material science, however, no single biomaterial provides all acceptable chemical, physical, mechanical, and biological properties, especially, osteogenesis, osteoinduction, and osteoconduction (16, 17). Therefore, the concept of hybridization of synthetic polymers with biopolymers and/or bioresorbable bioceramics seems very promising in combining their advantages in order to provide ideal 3D porous biomaterials for tissue engineering (18).

Among synthetic polymers, poly(α-hydroxy acids), especially poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), and a range of their copolymers have a relatively long history of use as biodegradable scaffolds for cell transplantation. As a clinically approved material, PCL is a semi-crystalline polymer with rubbery properties which is degraded by hydrolysis of its ester linkages in physiological conditions; it has therefore received a great deal of attention regarding its use as an implantable biomaterial. Owing to its degradation, which is even slower than that of PLLA, it is particularly interesting for the preparation of long-term implantable devices. It has been used in the human body as a drug delivery device, for example, as suture, and as an adhesion barrier, and has been investigated as a scaffold for tissue repair via tissue engineering.

Chitosan, on the other hand, has been investigated as a useful and functional biopolymer for a variety of tissue engineering applications because of (i) its structural similarity to naturally occurring glycosaminoglycans (GAGs); and (ii) its enzymatic degradation by lysozyme in the human body to absorbable oligosaccharides (19-22). It is a linear polysaccharide of (1-4)-linked D-glucosamine and N-acetyl-D-glucosamine residues derived from chitin, which is found in arthropod exoskeletons. It has excellent biocompatibility, bacteriostatic and hemostatic properties. Furthermore, it can form insoluble complexes with common connective tissue components such as collagen and glycosaminoglycans, and can be easily fabricated into bulk porous scaffolds.

Finally, poly(vinyl alcohol) (PVA) is a semi-crystalline hydrophilic polymer with good chemical and thermal stability. PVA is highly biocompatible and non-toxic. These properties have led to the use of PVA in a wide range of applications in the medical, cosmetic, food, pharmaceutical and packaging industries.

In natural tissues, from a morphological point of view, cells are surrounded by extracellular matrices (ECMs), which have physical structures ranging from the nanometer to the micrometer scale (23). Recent studies have shown that cells recognize nanometric topologies of fibrous or microporous structures, and that such nanoscale features directly impact different cellular behaviors (24-26). Therefore, the design and fabrication of submicron to nanoscale structural architectures have received much attention in scaffolding technology. Among the different fabrication techniques available, electrospinning represents an attractive approach to the fabrication of nanofibrous scaffolds for tissue engineering purposes (27-32). Electrospinning is a fiber spinning technique driven by a high-voltage electrostatic field, using a polymeric solution or melt that produces polymeric fibers with diameters ranging from several micrometers down to one hundred nanometers or less (33).

In this study, based on a biomimetic approach, novel 3D nanofibrous hybrid scaffolds consisting of PCL, PVA, and chitosan were developed via a multi-jet electrospinning method for bone tissue engineering, especially for non-load bearing applications. Moreover, the influence of chemical, physical, and structural properties of the scaffolds on the differentiation of mesenchymal stem cells (MSCs) into osteoblasts, and the proliferation of the differentiated cells were the main focuses of the present study.

MATERIALS AND METHODS

Materials

Chitosan powder with a viscosity average molecular weight of 2×10⁵ g mol⁻¹ and a deacetylation degree of 85% was obtained from Fluka Biochemika (Buchs, Switzerland). PCL with a number average molecular weight of 80000 g mol⁻¹ was purchased from Aldrich Chemical Co. (Steinheim, Germany) and PVA (98% hydrolyzed, weight average molecular weight of 98000 g mol⁻¹) was purchased from Merck (Germany). Chloroform and N,N-dimethylformamide (DMF) were obtained from Sigma Chemicals (Steinheim, Germany). Glacial acetic acid and methanol were purchased from Merck. All other reagents were of analytical grade and used as received from the manufacturer.
PCL/PVA/chitosan nanofibrous scaffolds

The electrospinning setup utilized in this study consisted of an adjustable high DC voltage power supply, two syringe pumps (SP-500, JMS, Tokyo, Japan), and a ground electrode (a stainless steel drum with an external diameter of 50 mm, length of 10 cm, and variable rotating speed). A schematic representation of the multi-jet electrospinning setup is shown in Figure 1. For the fabrication of PCL, PVA/chitosan hybrid scaffolds, a 5% wt/wt chitosan solution in 0.5 M acetic acid aqueous solution was mixed with a 10% wt/wt aqueous PVA solution at a mass ratio of 20:80 under agitation for 12 hours at 25°C. PCL was dissolved in chloroform under gentle stirring to obtain a 10% wt/wt solution. The desired amount of DMF, chloroform/DMF ratio of 10/1 (v/v), was added directly to the PCL/chloroform solution. Utilizing two separate syringe pumps, PCL and PVA/chitosan solutions were simultaneously delivered to metal capillaries (an internal diameter of 0.8 mm and length of 2.5 mm) with the constant mass flow rate of 1.5 and 0.6 ml/h, respectively. The distance between the tips and the ground electrode was 7.5 and 10 cm for PCL and PVA/chitosan solutions respectively, while the positive voltage applied to the polymer solutions was 25 kV. Finally, it should be mentioned that the drum was continuously rotated at 250 rpm throughout the course of electrospinning.

Treatment of the nanofibrous mats with methanol for 12 hours stabilized the PVA/chitosan nanofibers against disintegration while in contact with culture medium. Afterwards, the nanofibrous scaffolds were rinsed with 1 M aqueous sodium hydroxide solution and then washed with deionized water. The nano-structural hybrid scaffolds were then freeze-dried at 0.02 mbar and -10°C for 48 hours and stored in a desiccator at room temperature until use. Finally, for in vitro assays, the electrosynap nanofibrous scaffolds, approximately 500 µm thick, were cut into 1x1 cm² squares and sterilized by decreasing concentration of ethanol for 1 to 2 days.

Culture methods

Bone marrow mesenchymal stem cells (MSCs) were isolated from the femurs of young adult wistar rats, as described by Maniatopoulos et al (34), and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, GrandIsland, NY, USA) supplemented with 15% fetal bovine serum (FBS; Gibco). After determining the proliferation potentiality of the MSCs, they were cultured in a humidified atmosphere composed of 95% air with 5% CO₂ at 37°C, extensively propagated, and passaged from 15 to 20 times in vitro. The adherent MSCs, with a passage number of 3 to 5, were detached from tissue culture flask with 0.05% trypsin containing 1 mM EDTA (Gibco) and suspended to 3x10⁵ cells/mL in culture medium. The cell suspension was applied to seed on sterilized nanofibrous scaffolds placed in 12-well tissue culture plates at a density of 1.5x10⁵ cells/scaffold. Following the incubation of the cell seeded scaffolds at 37°C for 3 to 4 hours, 2 mL of culture medium was then added to each well. MSCs were treated with osteogenic medium containing DMEM supplemented with 15% FBS, 10 nM dexamethasone (Sigma-Aldrich, (St. Louis, MO, USA), 10 mM β-glycerophosphate (Merck), and 0.28 mM ascorbic acid two-phosphate magnesium salt n-hydrate (Sigma-Aldrich). The culture media were changed three times a week during the two-week differentiation treatment.

Scanning electron microscopy

The morphology of the electrosynap PCL/PVA/chitosan nonwoven mats, with and without cells, was observed by means of scanning electron microscopy (SEM,
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Vega©Tescan, Cranberry Twp., PA, USA) at an accelerating voltage of 20 kV. Before the observation, samples of cell-scaffold constructs were fixed in 2.5% glutaraldehyde, dehydrated through a graded series of ethanol, and vacuum-dried. All samples were coated with gold using a sputter coater.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

For the RT-PCR analysis, total cellular RNA was extracted using TRI-reagent (Sigma T-9424) according to the manufacturer's protocol. Synthesis of cDNA was

**TABLE I - RT-PCR PRIMERS FOR BONE-SPECIFIC GENE EXPRESSION ANALYSIS**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (Sense: top, antisense: bottom)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-2-microglobulin (β2M)</td>
<td>TGAAAAGAGATACCAAATATCGA GATGATGAGCTCCATAGAGCT</td>
<td>201</td>
</tr>
<tr>
<td>Bone-specific genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>TTAGGGCAGCTACACCAC GATGGCTGATGTCTCTGAG</td>
<td>401</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP)</td>
<td>CGCTACTTTTTATCCTCCTG GTCACCTGCTAGGTCCTTGA</td>
<td>795</td>
</tr>
<tr>
<td>Osteocalcin (OC)</td>
<td>CGCTGACGATGCTAGGAGAAGC GCTTCGAGGCGAGAGAGAAG</td>
<td>404</td>
</tr>
<tr>
<td>Osteopontin (OP)</td>
<td>AGAGGAGAAGGCCGATTACA GCACTGGGATGACCTTGAT</td>
<td>497</td>
</tr>
</tbody>
</table>

Fig. 2 - SEM micrographs of the electrospun PCL/PVA/chitosan nanofibrous structure composed of randomly oriented Ultra-fine PCL and PVA/chitosan fibers: (a) low magnification view (700 X); (b) high magnification view (3500 X).
PCL/PVA/chitosan nanofibrous scaffolds

Fig. 3 - SEM micrographs of MSCs seeded and differentiated on nanofibrous scaffolds: (a) attachment of anchorage-dependent mesenchymal stem cell on pore structure of nanofibrous scaffold; (b) low magnification view of the cell-polymer constructs after 2 weeks of culture in differentiation medium. The scaffold surface is partially covered by a layer of attached cells; (c) and (d) high magnification view of the cell-polymer constructs after a 2-week differentiation treatment.
carried out with M-MuLV reverse transcriptase (RT) and random hexamer as primer, according to the manufacturer’s instructions (Fermentas Inc., Hanover, MD, USA). PCR amplification was performed using a standard procedure with Taq DNA Polymerase (Fermentas Inc., Hanover, MD, USA) with denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. Depending on the abundance of the particular mRNA, the number of cycles varied between 25 and 30. The primers and product lengths are listed in Table I.

RESULTS AND DISCUSSION

The natural ECM is a complex mixture of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue-specific 3D ultrastructure. Structural proteins such as collagen, the most abundant protein in the ECM, and GAGs are especially important in mechanically supporting tissue reconstruction and providing attachment sites for cell surface receptors. GAGs play important roles in binding growth factors, water retention, and the gel properties of the ECM. It is worth mentioning that chitosan has a chemical structure similar to a repeating unit of GAGs. Hence, PCL and chitosan were chosen as the substitutes for collagen and GAGs in the ECM.

In electrospinning, a solid fiber is generated as the electrified jet (composed of a highly viscous polymer solution) is continuously stretched due to the electrostatic repulsions between the surface charges and the evaporation of solvent. There are some problems in electrospinning of polyelectrolytes. In the case of chitosan, for instance, the repulsive interaction among the polycations prevents sufficient chain entanglement, necessary for fiber formation. In the present study, PVA was added to the chitosan solutions to moderate the repelling interaction between polycationic chitosan molecules and to enhance the molecular entanglement. Scanning electron micrographs obtained prior to cell seeding revealed a three-dimensional scaffold of nonwoven, randomly oriented nanofibers. Figures 2a and 2b show typical SEM micrographs of the hybrid matrix composed of PCL and PVA/chitosan nanofibers. As shown in this figure, PVA/chitosan nanofibers were distributed uniformly in the PCL nanofibrous structure. The region of distribution of PVA/chitosan nanofibers ranged from 100 to 400 nm and the majority was in the range of 100 to 200 nm. However, the diameters of pure PCL nanofibers were broadly distributed in the range of 200 to 1500 nm. Furthermore, the hybrid matrices produced were approximately 500 µm thick, which can be changed by increasing the volume and electrospinning time. It is also worth mentioning that the nano-structural matrices prepared in this study are easily compressible.

In this work, osteogenic differentiation of adult rat bone marrow-derived MSCs was investigated on the nanofibrous hybrid scaffolds. Cell-scaffold constructs cultured for two weeks in osteogenic medium containing β-glycerophosphate, ascorbic acid and dexamethasone were analyzed using SEM (Fig. 3). Osteogenically induced cultures revealed that cells were well-attached, penetrated into the constructs, and uniformly distributed. In constructs cultured in

Fig. 4 - RT-PCR analysis of osteogenic differentiation of rMSCs seeded in nanofibrous scaffolds. The addition of osteogenic supplements to cellular constructs led to increased expression of ALP, OC, OP and BSP compared to control cultures.
PCL/PVA/chitosan nanofibrous scaffolds

the presence of osteogenic medium, the formation of mineralized nodules in the MSC-derived osteoblast-like cells was observed. As can be seen in Figure 3, the high surface-area-to-volume ratio and the high porosity of 3D nanofibrous scaffolds support attachment and migration of anchorage-dependent MSCs inside the scaffolds with interconnected pores as well as their differentiation into osteoblast-like cells. In contrast with tissue culture plates, the cells grow and develop more extensively with appropriate interactions inside the 3D medium of randomly oriented nanofibers. This is mainly due to their chemical and morphological similarity to natural extracellular matrices.

To confirm the osteogenic differentiation of MSCs on the nanofibrous scaffolds, RT-PCR analysis was utilized to detect the expression of mRNA transcripts of bone-specific molecules by the second week of culture (Fig. 4). The expression of markers of osteoblastic differentiation, including alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OP), and bone sialoprotein (BSP) was upregulated in constructs cultured in osteogenic medium. ALP is a protein localized on the cellular membrane of the osteoblasts and has been used as a marker for early osteogenic differentiation cascade. Osteocalcin is a bone-specific protein which has been used as a late marker of osteogenic differentiation. This protein is exclusively produced by osteoblasts and accumulated in the bone matrix, which is also produced by the osteoblasts. OP and BSP are other late phenotypic markers of osteoblastic differentiation. By contrast, untreated control cultures failed to express either gene by the second week.

CONCLUSION

The capability of MSCs to expand and differentiate into different lineages makes them a highly promising cell source for tissue engineering and regenerative medicine. Specific cellular differentiation and expression of cellular phenotypes in cells seeded within the scaffolds is required for its application in tissue engineering. In the present study, nanofibrous PCL/PVA/chitosan hybrid structures were obtained by multi-jet electrospinning. The results obtained suggest that these nanofibrous scaffolds are fully capable of supporting attachment and osteogenic differentiation of rat MSCs. The ability of cultured stem cells to self-renew and differentiate into lineage-committed osteoblast-like cells on nanofibrous scaffolds may be beneficial for tissue engineering.

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