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Gland cell cultures into 3D hyaluronan-based scaffolds

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In this study we report a preliminary investigation of the feasibility of non-woven/sponge fabrics of a hyaluronan derived biomaterials (benzyl ester of HA (HYAFF-11^{TM} FAB, Abano Terme, Italy) for the *in vitro* culture of rat hepatocytes and rat beta cells. Cell growth on hyaluronan derived biomaterials were tested in the presence of complete medium and in the presence of ECM (extracellular matrix) secreted by fibroblasts previously cultured into the scaffold. Hepatocytes and beta cells were extracted from rat liver/pancreas and seeded either on the HYAFF-11^{TM} scaffold alone, or on HYAFF-11^{TM} scaffold containing ECM. Direct assay of cell proliferation was performed with MTT test. For morphological observations samples were stained with hematoxylin and eosin. The results obtained by MTT test showed that hepatocytes cultivated in both the above described conditions were able to proliferate up to 14 days and Langerhans islet up to 21 days. After this time, cells started to undergo apoptosis. The morphological analyses showed cell aggregation in three-dimensional structures promoted by the fibers of the biomaterial.

Our results confirmed that HYAFF-11^{TM} meshes represent a suitable scaffold for hepatocyte adhesion/Langerhans islet organization and proliferation. In particular, the presence of a fibroblast secreted extracellular matrix improves the biological property of the scaffold.

Introduction

Tissue engineering can be defined as the tool for the development of biological substitutes (artificial organs or tissue) to restore, maintain or improve physiological functions. In the United States, nearly one person in 10 has an implanted medical device – a powerful statistic that indicates the importance of this technology. With the aging of our population and the improvements in technologies, these numbers will only increase [1].

In vitro reconstruction of living tissue is made possible by the creation of three-dimensional (3D) cell cultures in biomedical scaffolds devices. Cells obtained from biopsies are cultured inside a biomaterial matrix that supports 3D growth of cells and possibly, the synthesis of an extracellular matrix (ECM) typical of the tissue to be reconstructed. New and improved matrices and bioactive factors inevitably will play important roles in the evolution of tissue engineering. In recent years, significant advancements have been achieved in the research of biologically active biomaterials and in cell culture techniques of various tissues including dermis, epidermis, cartilage and bone. One promising biomaterial is a benzylic ester of hyaluronic acid, a ubiquitous molecule in animal connective tissues. The esterification of this polysaccharide renders a insoluble polymer that can be used to create various devices such as membranes, tubes, sponges, non-woven fabrics. The latter material has shown great promise for the culture of fibroblasts, which are able to proliferate and deposit extracellular matrix molecules into the interstices of the biomaterial, giving origin to a neo-dermal connective tissue [2].

In the present study, a 3D scaffold has been be utilized for the design of glandular tissues to be used as innovative liver support devices. Primary hepatocytes were utilized for this *in vitro* reconstruction of glandular parenchyma, the endocrine-secreting component of the pancreas. The technique may be used either for clinical purposes or for basic research such as *in vivo* and *in vitro* proliferation rate/cell function.

Materials and methods

Biomaterials

Biomaterials used in the present study were derived from the total esterification of hyaluronan (synthesized from 80–200 kDa sodium hyaluronate) with benzyl alcohol, and are referred to as HYAFF-11^{TM}. The final product is a non-crosslinked linear polymer with an undetermined molecular weight; it is insoluble in aqueous solution yet spontaneously hydrolyzes over time, releasing benzyl alcohol and hyaluronan. HYAFF-11^{TM} was used to create non-woven meshes (50 μm-thick fibers; specific weight of 100 g/m^2) and sponge (14 mm diameter, 3.5 mm thickness; specific weight of 0.05–0.09 g/cm^2;

porosity $> 96\%$; surface area 500–1000 m^2/g). These devices were obtained from Fidia Advanced Biopolymers (FAB, Abano Terme, Italy).

Cell cultures

Human dermal fibroblasts were prepared according to a modified version of the Rheinwald and Green protocol [3]. After epithelial sheet dispase removal, dermis was cut into small pieces (2–3 mm^2) and fibroblasts were isolated by sequential trypsin and collagenase digestion. These cells were then cultured with DMEM medium supplemented with 10% foetal bovine serum (FBS) (Bidachem) plus 2 mM L-glutamine and [100 U/mL]/[100 µg/mL] penicillin/streptomycin (cDMEM). Medium was changed twice a week and cells harvested by trypsin treatment.

Hepatocytes were harvested from 2- to 3-month-old male Wistar rats by a modified [4]. Animals were anesthetized in a chamber containing 1.5% isoflurane and 2 L of oxygen; 1 mL of pentothal 0.05 mg/mL was injected intraperitoneally. A midline laparotomy was performed, and after section of the vena cava, a silicon catheter was inserted in the portal vein. The liver was perfused for 9 min with 30 mL/min of calcium-free buffer solution (37 °C, pH 7.4) equilibrated with 2 L/min of O_2 30 min prior to utilization and continuing throughout the procedure. The excision of the liver was completed, with increasing the rate of the perfusion to 50 mL/min. The liver was subsequently perfused for 6 min with a buffer solution containing 0.05% collagenase and 5 mmol/L of calcium chloride equilibrated with O_2 at 50 mL/min. The perfusion was accelerated by 10 mL/min every minute for 9 min. Thereafter the swollen liver was gently stirred and the resulting cells resuspended in ice-cold wash medium. This suspension was filtered through a nylon mesh (grid size: 100 µm). The cell pellet was collected after centrifugation at 50 g for 4 min, and resuspended in 50 mL of wash medium. Further purification was carried out using a modified Kreamer procedure [5] to 12.5 mL of cell suspension, 12.5 mL of Percoll and 1.5 mL of 10 × HBS were added. The cell pellet was collected by centrifugation at 500 g during 5 min, resuspended, and washed twice.

Beta cells. Rats are anesthetized and a midline laparotomy was performed; the liver was retracted and the distal end of the common bile duct was occluded with a metal clip. Subsequently, cardiorespiratory arrest was induced by inflicting a pneumothorax. The proximal common duct is incised, cannulated with an 18 gauge polyethylene catheter, and slowly injected with 20 mL of Hank's solution containing 1 mg/mL of collagenase type V. Pancreatic islets are dispersed in collagen-coated culture flasks for one week in RPMI (+10 FCS and 5,5 mM D-glucose). Pancreatic islets are then dispersed in collagen-coated culture flasks for one week in RPMI medium supplemented with 10% FCS and 5,5 mM D-glucose. Alternatively, MEM supplemented with 0.2 mM L-glutamine, 20% FCS or RPMI FCS, 7.8 mM D-glucose can be used.

Cell cultures into HYAFF-11™ scaffolds

Pieces (1 × 1 cm) of the HYAFF-11™ non-woven/sponge material were fixed to culture plates with a fibrin clot. The following cells were then seeded:

Human dermal fibroblasts. After detachment from culture plates, fibroblasts were cultured in HYAFF-11™ scaffolds at a density of 3×10^4 cells/cm^2. These were cultured for three weeks in 10% FBS cDMEM with freshly added [10 ng/mL] bFGF and [50 µg/mL] ascorbic acid. After two weeks the dermal-like structures obtained were subjected to osmotic shock (1 h in sterile H_2O at 37 °C) to eliminate the cells yet maintain the ECM structure.

Hepatocytes and beta cells. Cell suspension was collected by centrifugation at 500 g during 4 min, and resuspended in 1 mL of DMEM and seeded onto non-woven HYAFF-11™ biomaterials decellularized at a density of 2×10^6 hepatocytes/cm^2; and onto HYAFF-11™ sponge at a density of 100 islets.

MTT assay

To determine the kinetics of cell growth both on plastic substrates and inside the HYAFF-11™ meshes, the MTT-based (Thiazolyl blue) cytotoxicity test was performed according to the method of Denizot and Lang [6] with minor modifications [6]. This assay is an indirect method for assessing cell growth and proliferation, since mitochondria oxidize the MTT solution, giving a typical blue–violet end-product. O.D. values of 540–620 nm can be quantified to cell number.

Briefly, after harvesting the culture medium from the collagen-coated dishes or biomaterials, the cells were incubated for 3 h at 37 °C with [50 µg/mL] MTT (Sigma) solution in PBS. After the removal of the MTT solution, samples were then incubated for 15 min at room temperature with an equal volume of extract solution (10% DMSO in isopropranol). The mesh scaffolds were discarded before reading of samples to avoid interference from the hyaluronan fibers (empty scaffolds were used as controls).

Histological analyses

Several specimens were collected from *in vitro* hepatocyte and β-cells culture onto HYAFF-11™ scaffold at 1–3 weeks after seeding. Specimens were fixed in formalin and paraffin-embedded for histochemistry and stained with hematoxylin and eosin.

Results

Decellularized matrix

Human fibroblasts attached to the HYAFF-11™ biomaterial fibers and deposited ECM molecules, such as type I, III and IV collagens, laminin and fibronectin inside the interstices of the scaffold [7]. After osmotic shock the fibroblasts are eliminate and the biomaterial maintains the ECM structure (data not shown).

Hepatocytes culture in mesh scaffolds

Hepatocytes from the above-described cultures were then seeded into 3D mesh scaffolds containing ECM. Fig. 1 illustrates the MTT values for hepatocytes grown in these 3D scaffolds. The cells did not proliferate in a time-dependent manner, but were maintained at approximately constant values.

As shown in Fig. 2, hepatocytes cultured onto HYAFF-11^{TM} meshes maintained their original phenotypes (i.e. double nuclei) after 14 days of growth.

Beta cells culture in HYAFF-11^{TM} sponges

Beta cells isolated with the above-described cultures were cultured into HYAFF-11^{TM} sponge for three weeks. Fig. 3 illustrates the cell growth measured with the MTT test. Pancreatic islets also maintained approximately constant growth values and did not proliferate in a time-dependent manner. Fig. 4 shows that beta cells cultured onto HYAFF-11^{TM} sponges maintained their original phenotype (i.e. ditizon positive labeling). Ditizon is a specific vital dye used for pancreatic islet staining.

Discussion

This study has shown that it is possible to reconstruct a glandular-like tissue by utilizing rat hepatocytes or Langerhans islets taken from adult organs and cultured into biocompatible 3D scaffolds enriched with ECM molecules. Morphological analyses showed that the biomaterial fiber matrix promoted cell aggregation into 3D structures.

These results confirmed that HYAFF-11^{TM} meshes are a suitable scaffold for hepatocyte adhesion/Langerhans islet organization and proliferation. In particular, the presence of a fibroblast secreted ECM improves the biological property of the scaffold. Assuming that a close relationship exists between cell morphology and function it can be concluded that the HYAFF-11^{TM} scaffolds are a good substrate for glandular cells growth.

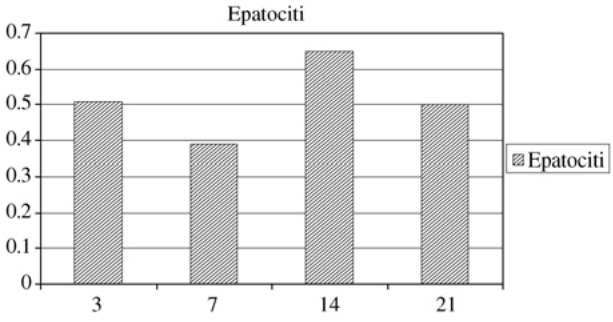

Figure 1 Proliferation rate of hepatocytes cultured at different days of culture. The graphs represent the mean of three different experiments.

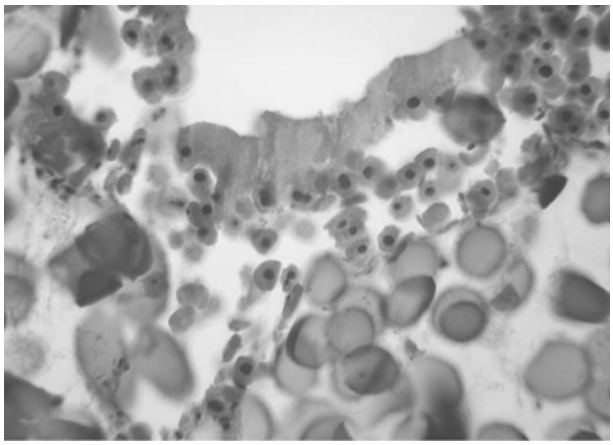

Figure 2 Hepatocytes onto HYAFF-11 non-woven.

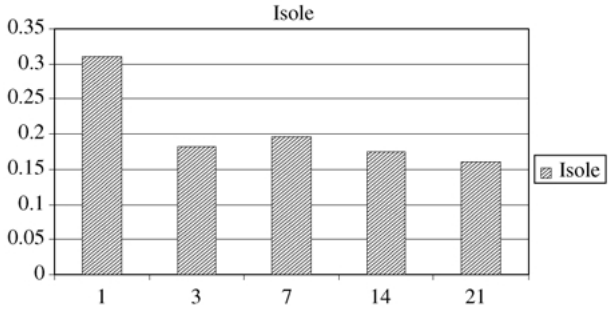

Figure 3 Proliferation rate of beta cells at different days of culture. The graphs represent the mean of three different experiments.

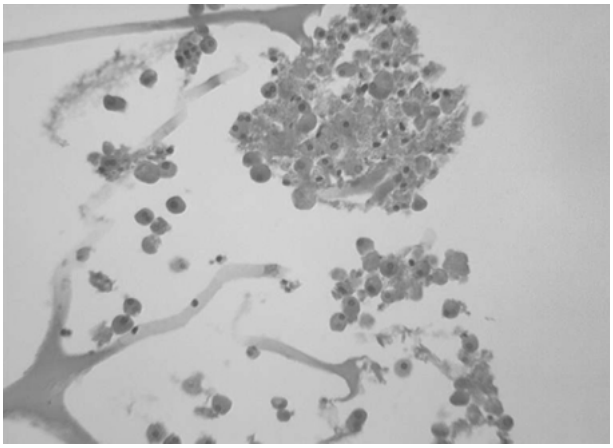

Figure 4 Beta cells onto HYAFF-11 sponge.

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