Alginate based microbeads containing Wharton's Jelly Mesenchymal Stem Cells

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INTRODUCTION

Mesenchymal Stem Cells (MSCs), that have largely been investigated, are functionally defined as non-hematopoietic multipotential cells. These cells are able to self-renew and possess a high proliferative capacity (Minguell, J.J. et al. 2001).
Many studies suggest that MSCs may differentiate towards cells of different lineages, including chondrocytes, adipocytes, osteocytes, miocytes, neurons and tenocytes.3-10 Because of their peculiar characteristics, MSCs are considered to be very important for several applications in the field of regenerative medicine, including the development of cell-based therapies and tissue repair procedures (Uccelli, A. et al. 2008).
Different adult human tissues have been considered as MSCs sources, including bone marrow, trabecular bone, adipose tissue, peripheral blood, synovium, skeletal muscle, dental pulp and periodontal ligament.4,11-16 Although bone marrow still represents the main and most investigated source of adult MSCs, the isolation and use of these cells still present some drawbacks. For instance, the number of MSCs, found in bone marrow, decrease progressively starting at age 17 and the harvesting techniques are invasive, often causing severe infections, bleeding and chronic pain for donors.
Looking for alternative MSCs sources, fetal tissues, extra-embryonic tissues such as placenta and amniotic fluid, umbilical cord blood and stroma have recently been considered (Parekkadan, B.et al.2007). Umbilical cord, due to the unique morphological properties, represents an interesting alternative source for MSCs, especially if compared to umbilical cord blood. Umbilical cord, usually weighing 40 g and spanning between 60 and 65 cm in length, contains a special primitive connective tissue called Wharton's Jelly. This jelly act as a protective tissue for vessels and contains into its stromal compartment cells with specific mesenchymal characteristics, called Wharton's Jelly Mesenchymal Stem Cells (WJMSCs).30. Unfortunately, to the best of our knowledge, in literature there is limited data available about the qualitative and quantitative characterization of factors secreted by WJMSCs. The ability to self-renew and to differentiate into several cell types makes the use MSCs particularly attractive for the development of innovative therapeutic strategies aimed at repairing and replacing damaged tissues and makes them a very promising cell source for tissue engineering applications.
Nevertheless, several factors still hurdle the extensive clinical use of cell based therapy. The protection of implanted cells from the host's immune response is of primary importance. To solve this problem, one of the most promising approaches is represented by cell's encapsulation within semipermeable capsular systems (Luca, G et al. 2007)
With the aim of proposing WJMSCs for tissue engineering application, the current paper describes (a) the isolation of human WJMSCs, (b) the production of barium alginate microbeads by a vibrating nozzle approach for WJMSCs encapsulation, and finally (c) in vitro characterization of WJMSCs encapsulated into alginate microbeads, including viability, proliferation and secretive profile.

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MATERIALS AND METHODS

Wharton's Jelly Mesenchymal Stem Cells: isolation procedure and culture conditions: Human umbilical cords (all from natural deliveries) were collected after mothers' consent and approval of the "Ethical committee of University of Ferrara and S.Anna Hospital ". Harvesting procedures of Wharton's Jelly from umbilical cord were conducted in full accordance with the "Declaration of Helsinki" as adopted by the 18th World Medical Assembly in 1964 successively revised in Edinburgh (2000) and the Good Clinical Practice guidelines.
Cords were processed within 4 hours, and until that moment had been stored at 4°C in sterile saline. Typically, the cord was rinsed several times with sterile PBS before processing and was cut into pieces, 2-4 cm in length. Blood and clots were drained from vessels with PBS, to avoid any contamination. Single pieces were dissected, first separating the epithelium of each section along its length, to expose the underlying Wharton's Jelly. Later cord vessels (the two arteries and the vein) were pulled away without opening them. The soft gel tissue was then finely chopped.
The same tissue (2-3 mm2 pieces) were placed directly into 75-cm2 flask for culture expansion in 10% FCS (Euroclone S.p.A., Milan, Italy) D-MEM Low Glucose media supplemented with antibiotics (penicillin 100 mg/mL, streptomycin 10 mg/mL) at 37°C in a humidified atmosphere of 5% CO2. After 5-7 days, the culture medium was removed and thereafter changed twice a week. At a ~70-80% confluence, cells were scraped off by 0.05% trypsin/EDTA (Gibco, Grandisland, USA) (2 min), washed, counted by hemocytometric analysis, assayed for viability and thereafter used for further in vitro experiments or for encapsulation procedures.

Encapsulation of Wharton's Jelly Mesenchymal Stem Cells: Monodisperse alginate beads containing WJMSCs, were prepared using an encapsulation device which is based on a vibrating-nozzle (Encapsulator Research Inotech, Dottikon Switzerland) according to the experimental procedure previously described (Luca, G et al. 2007). The encapsulator is composed by a 2-liter glass reaction vessel with stainless steel top and bottom plates. The top plate contains a feed-line connected to a syringe and a vibrating nozzle. A nozzle with an internal diameter of 300 µm was used. The flow of alginate to the nozzle is achieved by a precision syringe pump. The production of WJMSCs-filled alginate microcapsules was optimized by changing the following experimental parameters: the vibrational frequency ("freq"), the vibrational amplitude ("amp"), the alginate pumping rate ("pump") and the distance between the nozzle and the surface of the gelling bath ("height") (see Table 1). Before encapsulation, WJMSCs were suspended in a 1.5% (w/v) aqueous solution of highly purified sodium alginate (Na-AG) (Stern Italia, Milano, Italy) at a concentration of 8-12 x 106 cells/mL. The generated microdroplets were dropped into an isotonic barium chloride solution (1.2%; w/v), after gelation (3 minutes), the microbeads were washed twice with saline and cultured in 10% FCS (Euroclone S.p.A., Milan, Italy) D-MEM Low Glucose media supplemented with antibiotics (penicillin 100 mg/mL, streptomycin 10 mg/mL) at 37°C in an humidified atmosphere of 5% CO2.

Dimensional and morphological characterization of microbeads: The morphology of Ba-alginate microbeads was evaluated by optical stereomicroscopy (Nikon SMZ 1500 stereo microscope, Tokio, Japan). Microbead size and size distribution were determined by photomicrograph analyses (Eclipsenet version 1.16.5, Laboratory Imaging s.r.o. for Nikon B.V.). Microbead samples, immediately after preparation and at intervals after storage under different conditions, were deposited onto a microscope slide and examined microscopically. A sample of 100–300 beads was examined, and the mean size was determined.

Induction and evaluation of osteogenic differentiation: For the analysis of osteogenic differentiation, both free WJMSCs (growing in monolayer) and microbeads entrapped cells, were incubated in hMSC Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza, Walkersvill, MD USA) for 21 days.
The extracellular matrix composition was analyzed by FTIR spectroscopy. Cell layers were collected in ammoniated water (50 mM ammonium bicarbonate, pH 8.0), lyophilized and analyzed by FT-IR Spectrometer Perkin Elmer, Spectrum 100 (Perkin Elmer, MA, USA). Absorption spectra were collected from 4000 to 600 cm-1. After 21 days of incubation in standard and osteogenic conditions, microbeads were assayed for alkaline phosphatase (ALP) activity. Microbeads were incubated at 37°C in a 50 mM EDTA-solution (pH 7.00) for 2 min, in order to dissolve the Ba-alginate microbeads and obtain the free cells. The cells were lysed with 300 µl of 0.2 % Triton X-100. ALP activity was assayed by measuring, after 30 min of incubation at 37°C, the conversion p-nitrophenylphosphate (PNPP) to p-nitrophenol, by using an ELISA-reader at 405 nm wavelength. ALP activity was normalized to total cellular proteins, determined by the Bradford protein assay protocol and expressed as U/mg of protein. One unit was defined as the amount of enzyme which hydrolyzes 1 µMol/min of PNPP. For Alizarin Red S staining, free WJMSCs (growing in monolayer) and microbeads entrapped cells, were fixed and then stained with 40 mM Alizarin Red S solution, pH 4.2 at room temperature for 10 min. Samples were then rinsed 5 times with distilled water and washed 3 times in PBS on an orbital shaker at 40 rpm for 5 min each, to reduce non-specific binding. The stained matrixes were microphotographed by an optical microscope (Nikon, Optiphot-2, Nikon Corporation, Japan). For RT-PCR analysis, Ba-alginate microbeads were dissolved as previously reported, and total RNA was isolated from the WJMSCs free cells using Total RNA Isolation system (Promega, WI, USA). Two micrograms of total RNA was reverse transcribed with the Improm-II RT System (Promega, WI, USA). mRNA of target genes was quantified by real-time PCR using the ABI Prism 7700 system and TaqMan probes 5'AACCCAGAAGGCACAGACAGAAGCT3' for RUNX-2 (Applied Biosystems, CA, USA). PCR was carried out in a final volume of 25 µl. After a 10 min pre-incubation at 95°C (denaturation), 1 min at 60°C (annealing/elongation). The mRNA levels were corrected for GAPDH mRNA levels (reference gene) and normalized to a calibrator sample (control cells).

RESULTS AND DISCUSSION

Isolation and characterization of Wharton's Jelly MSCs (WJMSCs): As source of human MSCs, solely Wharton's Jelly was used, instead of the whole cord (easier and faster to treat); this choice was made with the aim of isolating a relatively homogeneous cell population, possibly avoiding any epithelial cell contamination. The primary cells, isolated from Warthon's Jelly, when in vitro cultured, display a MSC-like phenotype (data not shown), after 3 days in culture, the WJMSCs grow in adherent colonies, reaching confluence in 10-14 days.

Encapsulation of WJMSCs in alginate microbeads: WJMSCs were embedded into alginate microbeads by an encapsulation device based on a vibrating-nozzle (see scheme in Fig. 1A). The encapsulation procedure was relatively simple and consisted of a limited number of steps. In order to achieve complete biocompatibility, essential for mammalian cells, the encapsulation procedure was conducted at room temperature under physiologic pH and tonicity using a pyrogen-free alginate solution. The resulting barium alginate microbeads were elastic and transparent facilitating the microscopic observation of the WJMSCs viability and morphology during the in vitro studies. The hardening of alginate solution was accomplished by an ionic gelation procedure based on barium chloride. The use of barium ions (instead of more often used calcium ions) resulted in the formation

of mechanically stable microbeads with an extremely high biocompatibility, preserving the in vitro and in vivo viability of the embedded cells.

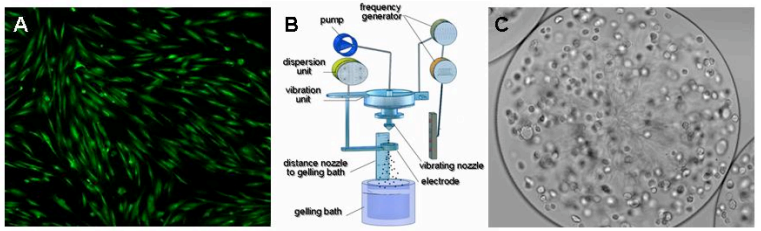

Fig 1. Representative fluorescence photomicrograph of WJMSCs monolayer after Calcein-AM staining (magnification: 20x)(A), schematic representation of the encapsulation device based on a vibrating nozzle (B) and optical photomicrographs alginate microbeads containing WJMSCs (C).

Viability, proliferation and osteoblastic differentiation of encapsulated WJMSCs: The viability of WJMSCs encapsulated in alginate beads was determined by the live/dead test. Beads were incubated with Calcein-AM (a marker of living cells, fluorescent signal was monitored using 485 nm excitation wavelength and 530 nm emission wavelength) and with propidium iodide (a marker of cell death, excitation, 535 nm; emission, >610 nm), as described in the experimental section. The observation of the fluorescent images recorded immediately after the encapsulation procedure at the typical excitation wavelengths, indicated that the cells were highly viable (>95%). In order to strengthen these data, the cell viability was determined after different lengths of culture time (up to 9 days) by using two alternative procedures, namely, the double staining with a Calcein-AM cell viability assay kit.

The ability of the WJMSCs entrapped in alginate to differentiate in osteoblasts was assessed at day 21 of osteogenic induction (WJMSCs/alg/ost) by a number of classical criteria and compared to WJMSCs entrapped in alginate in absence of osteogenic medium (WJMSCs/alg). The WJMSCs/alg/ost cells showed an appreciable increase of ALP activity, an early marker for osteoblast differentiation. The WJMSCs/alg/ost cells showed also an increase of the expression of a bone-specific gene such as Runx2 analyzed by quantitative RT-PCR. Similarly, mineralization was present in WJMSCs/alg/ost cells. All together, these results suggest that the cells were undergoing osteogenic differentiation despite encapsulation.

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