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Research Paper

Bioactive Long-Term Release from Biodegradable Microspheres Preserves Implanted ALG-PLO-ALG Microcapsules from *In Vivo* Response to Purified Alginate

Stefano Giovagnoli,[1,4] Paolo Blasi,[1] Giovanni Luca,[2] Francesca Fallarino,[3] Mario Calvitti,[3] Francesca Mancuso,[3] Maurizio Ricci,[1] Giuseppe Basta,[2] Ennio Becchetti,[3] Carlo Rossi,[1] and Riccardo Calafiore[2]

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Purpose. To assess whether prevention of unexpected *in vivo* adverse inflammatory and immune responses to biohybrid organ grafts for the treatment of Type I Diabetes Mellitus (T1DM) is possible by superoxide dismutase and ketoprofen controlled release.
Methods. Superoxide dismutase and ketoprofen-loaded polyester microspheres were prepared by W/O/W and O/W methods, embodied into purified alginate-poly-L-ornithine-alginate microcapsules and intraperitoneally implanted into CD1 mice. The microspheres were characterized for morphology, size, encapsulation efficiency, enzyme activity and *in vitro* release. Purified alginate contaminants were assayed, and the obtained microcapsules were investigated for size and morphology before and after implantation over 30 days. Cell pericapsular overgrowth and expression were evaluated by optical microscopy and flow cytometry.
Results. Superoxide dismutase and ketoprofen sustained release reduced cell pericapsular overgrowth in comparison to the control. Superoxide dismutase release allowed preserving the microcapsules over 30 days. Ketoprofen-loaded microspheres showed some effect in the immediate post-grafting period. A higher macrophage and T-cell expression was observed for the control group.
Conclusions. Microspheres containing superoxide dismutase and ketoprofen may represent novel tools to limit or prevent unpredictable adverse *in vivo* response to alginate, thus contributing to improve cell transplantation success rates in T1DM treatment.

KEY WORDS: alginate microcapsules; biodegradable microspheres; diabetes; post-grafting response; superoxide dismutase and ketoprofen.

INTRODUCTION

Exogenous insulin administration, while enabling rapid and timely control of hyperglycemia, cannot eliminate the risk of developing chronic secondary complications associated with Type 1 diabetes mellitus (T1DM) (1) and, as recently observed, might be associated with serious safety concerns (2). In this regard, pancreatic islet transplantation, according to and already established by the "Edmonton Protocol" (3,4), could represent an effective strategy to treat T1DM. Nevertheless, two major obstacles still persist, namely the restricted availability of cadaveric human donor pancreata and the need for life-long recipient's general immunosuppression.

General pharmacological immunosuppression could be obviated by entrapping islets into alginate-poly L-ornithine-alginate (ALG-PLO-ALG) microcapsules (MC) (5,6). Such an immunoprotective shield has proved to be effective in preserving the grafted islets from both immune rejection and autoimmune recurrence of disease, thereby enhancing success rate of grafts (7,8). These important evidences supported the approval of the first pilot phase-I clinical trial of microencapsulated human islet allografts in 10 non-immunosuppressed patients (file no. 19382, PRE 805, 5 September 2003) (9,10).

On the other hand, porcine islets, harvested from neonatal piglet pancreata, could be used for human substitutes and, at least partially, solve the problem posed by the restricted availability of human tissues (11,12). Such strategy showed promising results in ongoing phase-I/IIa and Phase II clinical trials on microencapsulated porcine islet xenografts in T1DM patients (R.B. Elliot. Submission of an application of a clinical trial of xenotransplantation to the health authorities. IPITA-IXA joint meeting, October 12–16, 2009, Venice, Italy) in Russia and New Zealand, respectively (13,14).

Moreover, ALG-PLO-ALG MC can be supplied with several other systems enabling delivery of a wide variety of

[1] Dipartimento di Chimica e Tecnologia del Farmaco, Faculty of Pharmacy, University of Perugia, Via del Liceo 1, Perugia 06123, Italy.
[2] Dipartimento di Medicina Interna (Di.M.I.), Sezione di Medicina Interna e Scienze Metaboliche ed Endocrine, School of Medicine, University of Perugia, Via E. Dal Pozzo, Perugia 06126, Italy.
[3] Dipartimento di Medicina Sperimentale e Scienze Biochimiche, University of Perugia, Via del Giochetto, Perugia 06123, Italy.
[4] To whom correspondence should be addressed. (e-mail: eureka@unipg.it)

agents, proven very helpful to cell survival and functional competence retention over time (15–20). Such multicompartmental systems can protect and support cells over a long period of time after transplantation. In this context, controlled release of anti-inflammatory drugs, such as ketoprofen (KET), within implanted ALG-PLO-ALG MC, has been shown to prevent post-transplant inflammatory response and protect the MC *in vivo* over the first week after grafting (15). Furthermore, the use of antioxidants, such as vitamins and enzymes, and growth factors can prolong β-cell viability while improving morphological integrity and physiological competence (16–20). In particular, long-term release of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, allowed for establishing a new protocol for neonatal porcine islets maturation (21).

Sustained release of these actives from multicompartmental ALG-PLO-ALG MC could be used to treat post-transplant adverse events as well. In fact, albeit strong efforts have been devoted to assess and prevent host reactivity to MC implantation (22–24), unexpected adverse reactions continue to be the subject of great concern. MC biocompatibility depends not only on the purity and sterility of the ALG and the other constituent polymers, but also on MC size, shape, surface charge, smoothness, and membrane integrity (25–27).

Non-specific inflammatory and/or immune responses generally result in pericapsular cell overgrowth with increased risks for graft failure (28–30). This can provoke changes of membrane porosity and permeability to oxygen, nutrients, metabolic products, and insulin, eventually leading to function loss and cell death. Besides MC properties, endotoxins, protein and polyphenol contaminants have been identified as major causes for ALG MC transplant failures (31–33). In this regard, in spite of optimal MC features and contaminant levels (34,35), past experiences seem to suggest that host response, albeit poorly addressed and not necessarily related to ALG MC rejection, can still unpredictably occur and cannot be completely prevented (36).

Therefore, this work was aimed at determining whether previously developed drug delivery systems (16,37,38) could represent new tools to limit or avoid undesired random post-transplant events, leading to graft failure. This approach was intended as proof-of-concept about the possible use of such systems to improve graft success in encapsulated cell transplantation.

In previous works, SOD was employed only to improve maturation of neonatal porcine cell clusters *in vitro*, while the anti-inflammatory effect of KET was investigated over 6 days in MC fabricated with non-clinical grade ALG (16,21,38).

For this purpose, SOD- and KET were loaded into poly (D, L-lactide-co-glycolide) (PLGA) and poly(D, L-lactide) (PLA) microspheres (MS), respectively, and encapsulated into ALG-PLO-ALG MC prepared with purified ALG, which resulted in a clear, while yet unexplained, *in vivo* host response upon intraperitoneal implantation into CD1 mice (Fig. 1). SOD and KET-loaded PLGA and PLA MS were prepared by water-in-oil-in-water (W/O/W) and oil-in-water (O/W) solvent extraction/evaporation methods, respectively. The MS were characterized in terms of drug content, size and morphology, *in vitro* release and activity retention of the entrapped enzyme (21,37). The *in vivo* effects of SOD and KET release were evaluated over 30 days post implantation of multicompartmental ALG-PLO-ALG MC in CD1 mice. MC integrity and host response were assessed by optical microscopy and flow cytometry.

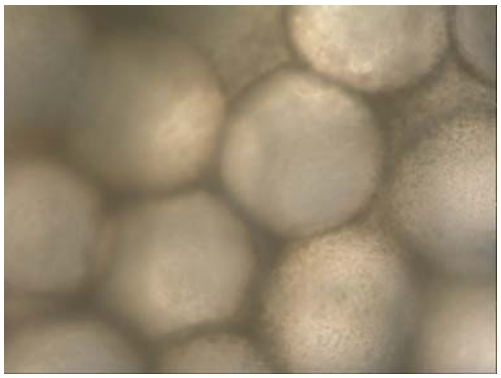

Fig. 1. Picture of recovered blank MC made with purified ALG showing cell overgrowth upon intraperitoneal grafting in CD1 mice.

MATERIALS AND METHODS

MS Preparation

SOD-loaded PLGA (Resomer® RG504H 50:50, MW 70 kD, Boehringer Ingelheim, Bidachem, Italy) MS were prepared by a W/O/W solvent extraction/evaporation double emulsion method as previously reported (37). All preparation steps were conducted under aseptic conditions to prevent possible contamination. Briefly, an amount of bovine Cu, Zn-SOD (Sigma, Milan, Italy) corresponding to 10% theoretical loading was dissolved in 150 μL of 88 mM polyethylene glycol (PEG) 400 (Sigma, Milan, Italy) aqueous solution. The protein solution was added upon stirring to a small volume of 20% polymer methylene chloride (J.T. Baker, Milan, Italy) solution to form a W/O emulsion. After proper emulsification, the mixture was injected into 50 mL of 6% polyvinyl alcohol (PVA) (Sigma, Milan, Italy) aqueous solution under stirring (1,000 rpm, at 4°C) to form a W/O/W double emulsion. The double emulsion was then poured in 500 mL of deionized water and kept at 4°C for 30 min. In order to evaporate the organic solvent, the temperature was slowly increased up to 20°C over 2 h. The hardened MS were filtered through a 5 μm nitrocellulose filter (Millipore, Milan, Italy), washed with deionized water and freeze-dried overnight.

KET-loaded PLA (Mw 106 kD, Sigma, Milan, Italy) MS were prepared according to an O/W solvent extraction/evaporation method previously described (16,38). Briefly, 250 mg of polymer and 75 mg of KET were dissolved into 20 ml of methylene chloride and slowly added to 60 ml of 2.4% w/v hydroxypropylmethyl cellulose (Sigma, Milan, Italy) solution stirred at 800 rpm. The solvent was evaporated under stirring for about 3 h. The hardened MS were recovered by centrifugation (refrigerated centrifuge ALC, RCF Meter, 4233R International Equipment Company, Chicago, IL) (3,000 rpm, 5 min, 4°C), washed thrice with deionized water and freeze-dried overnight.

Assessment of Active Encapsulation

To extract the protein, SOD-loaded MS (10 mg) were hydrolyzed in 0.5 mL of 0.5 M NaOH under overnight rotation. The solution was neutralized with an equal volume of 0.5 M HCl, and the protein content was determined by Micro-BCA protein assay (Sigma, Milan, Italy) by using an 8453 Agilent UV/VIS spectrophotometer (Agilent Technologies, Germany) (39).

KET content was determined by measuring the absorbance at 254 nm after dissolving the MS in methylene chloride. The encapsulation efficiency was calculated as actual/theoretical loading ratio.

All data are the result of three measurements, and the error was expressed as S.D..

Size Distribution and Morphology

MS size distribution was determined by an Accusizer ™ 770 Optical Particle Sizer (PSS Inc., Santa Barbara, CA, USA). Morphology was investigated by scanning electron microscopy (SEM) using a Philips XL30 microscope (Philips, the Netherlands) and by optical microscopy by a Nikon E80 digital microscope (Nikon, Italy).

SOD *In Vitro* Activity

The entrapped SOD activity retention was evaluated *in vitro* according to the pyrogallol (Sigma, Milan, Italy) autoxidation inhibition assay (40). The buffer for analysis (pH 8.2) consisted of 50 mM cacodylic acid (Sigma, Milan, Italy) and 1 mM diethylenetriaminepentaacetic acid (DTPA) (Sigma, Milan, Italy). Before running each experiment, all samples and standards were equilibrated at 25°C in an open vessel to allow contact with air, as the oxygen content influences the rate of the reaction. The pyrogallol solution was kept under nitrogen in an ice bath to slow down the autoxidation process. Standard and sample analyses were performed at 25°C in the 20–5,000 ng/mL SOD concentration range by reading pyrogallol absorption decrease at 320 nm in the presence of the enzyme. A control consisting of pyrogallol without SOD was also run to establish the actual rate of autoxidation. SOD extraction was carried out by suspending 10 mg of MS in 0.1 M phosphate buffer and by incubation for 4 h at 37°C. An aliquot of the pyrogallol water solution was added to the SOD solution, and data were recorded over 120 s. The measurement was started after 30 s of induction time. Analyses were conducted in triplicate, and the error was calculated as S.D.. One unit of active enzyme corresponds to the concentration needed to produce 50% inhibition (40).

Alginate Analysis

To evaluate the purification efficiency and possible contamination of the ALG employed, analyses were performed in order to assess content of endotoxins, proteins, and metals. The ALG was purified according to a well-established protocol (41). Protein analyses were performed by the Bradford protein assay (42), while endotoxins were assayed by the LAL (limulus amebocyte lysate) test (43) at both the University of Perugia (Italy) and at Living Cell Technologies laboratories (SOP Q209, Belgium). Metal contamination was tested at Sereco Biotest s.n.c. (Perugia, Italy).

Fabrication of Multicompartmental ALG/PLO/ALG MC

MS microencapsulation into ALG/PLO/ALG MC was carried out according to a previously optimized method (25). Briefly, SOD- and KET-loaded MS (2 and 0.8 mg/mL respectively) were suspended in 1.7% aqueous solution of purified sodium ALG (mannuronic acid, 61%; guluronic acid, 39%, Stern Italia, Milan, Italy). The ALG/MS suspension was continuously aspirated by a peristaltic pump at a flow rate of 12–14 ml/min, and thereafter extruded through a mono air-jet device (air flow rate, 4.3 L/min) under sterile conditions to form microdroplets. The suspension was continuously mixed by magnetic stirring to prevent MS clumping, which would possibly lead to non-homogeneous MS distribution within the MC. The dispersion was continuously dropped in a 1.2% $CaCl_2$ bath, whereby they immediately turned into gel microbeads. The beads obtained were collected, washed in saline and sequentially incubated with 0.12% (10 min) and 0.05% (6 min) aqueous solutions of low molecular weight PLO (15–30 kD, Sigma, Milan, Italy) stepwise. Finally, the MC were dispersed into a diluted ALG solution to restore the outer coat biocompatibility. MC degelling was obtained by dispersion into 55 mM sodium citrate (Sigma, Milan, Italy) for 4 min. The final preparation was incubated for about 12 hrs in HAM F12 medium supplemented with nicotinamide, IBMX, antibiotics and 0.5% fraction V bovine serum albumin, at 37°C in 95:5 air/CO_2.

In Vitro Release Study

The *in vitro* SOD and KET release was determined by suspending about 10 mg of loaded MS in 10 ml of 0.1 M pH 7.4 phosphate buffer at 37°C. At predetermined intervals, samples were centrifuged (2,000 rpm, 1 min, room temperature), and 1 ml of the supernatant was removed to perform Micro-BCA protein assay and UV analysis and replaced with an equal volume of fresh medium. Analyses were performed in triplicate. Likewise, SOD and KET release from multicompartmental ALG/PLO/ALG MC was investigated by dispersing an amount of freshly prepared MC approximately containing 10 mg of loaded MS in 15 mL of 0.1 M pH 7.4 phosphate buffer at 37°C. The procedure was the same as reported above. Analyses were conducted in triplicate, and the error was calculated as S.D..

Additional studies were conducted to rule out possible PLO leakage from the capsule membrane. This study was performed by changing ALG concentration and time of coating during the second coating step. A control consisting of phosphate buffer solution was run as well. A PLO solution was also incubated in the same conditions to exclude possible PLO adsorption to the walls of the container used for the study. Samples were subjected to Micro-BCA analysis in triplicate.

Transplant Procedure

All preparations were incubated in HAM F12 medium at 37°C in 95:5 air/CO_2, for at least 12 h prior to transplantation in order to evaluate possible microorganism contamination. The experiment was performed according to a protocol previously approved by the Ethic Committee of the University of Perugia (p.n. 19382 PRE 805/2003) and adhered to the "Principles of Laboratory Animal Care" (NIH publication #85–23, revised in 1985).

CD1 mice (average weight 25 g, Charles River Laboratories, Wilmington, MA) were divided into three groups ($n=9$): one control group implanted with conventional MC, one group with multicompartmental MC containing SOD and one group with multicompartmental MC containing KET, to be terminated at day 10, 20, and 30.

Under general anesthesia, induced by intraperitoneal administration of 100 mg/Kg ketamine (Parke-Davis/Pfizer, Karlsruhe, Germany) and 15 mg/Kg xylazine, (Bayer, Leverkusen, Germany), the MC were collected by a sterile transfer pipette and delivered into the peritoneal cavity of the recipient CD1 mice through a small abdominal incision. Each mouse received 1.5 mL of MC suspension.

Evaluation of Recovered MC Cell Overgrowth

At each time point (day 10, 20, 30), three mice from each group were sacrificed. The MC were collected by intraperitoneal lavage with a phosphate buffer saline (PBS) solution. Conventional and SOD- and KET-containing multicompartmental MC were qualitatively examined to assess morphology and cell pericapsular overgrowth.

Morphological Analysis

The recovered MC were examined by optical microscopy (Leica, Italy) in order to evaluate changes in morphology of both the collected MC and the entrapped MS, and the entity of cell overgrowth.

Flow Cytometry

The capsules recovered by intraperitoneal lavage with PBS solution at each time point were washed twice with PBS and suspended in 0.05% trypsin, 0.02% EDTA PBS solution and incubated in 5% CO_2 at 37°C for 20 min. The supernatant was recovered and incubated in the same conditions in a 25 cm^2 chamber for 3 days. Samples for flow cytometry were obtained by detaching the formed cell monolayers with 2 mM EDTA solution.

For Fluorescence-Activated Cell Sorting (FACS) analysis, the detached cells were treated with rat anti-CD16/32 (2.4G2) for 30 min at 4°C for blockade of Fc receptors before assaying on an EPICS flow cytometer using EXPO 32 ADC software (Beckman Coulter). The following Abs were applied: FITC-conjugated rat anti-F4/80 (BM8, Biolegend) or PE-conjugated rat anti-CD11b (M1/70, Biolegend), FITC-conjugated rat anti-CD8 (GK1.5, Biolegend) in combination with PE-conjugated rat anti-CD25 (7D4, Miltenyi) and PE-conjugated rat anti-CD69 (H1.2F3 BD-PharMingen). Each staining was compared against the relevant isotype controls to verify specificity.

RESULTS

SOD and KET MS Characterisation

SOD- and KET-loaded PLGA and PLA MS were obtained by using methods previously developed (16,37,38). An efficient protein loading inside the MS was achieved by employing RG504H polymer that afforded 90% efficiency and 9% drug content. The mean volume diameter was not modified by the enzyme encapsulation and was around 15 μm. Size distribution profiles outlined the presence of multiple populations at about 5, 12, 23 up to 50 μm (Fig. 2). A span of 2.46 reflected the wide MS population distribution observed. Such results are predictable when dealing with a double emulsion preparation method, which seldom produces unimodal particles due to the not homogeneous size of water droplets within the organic phase upon stirring. However, the

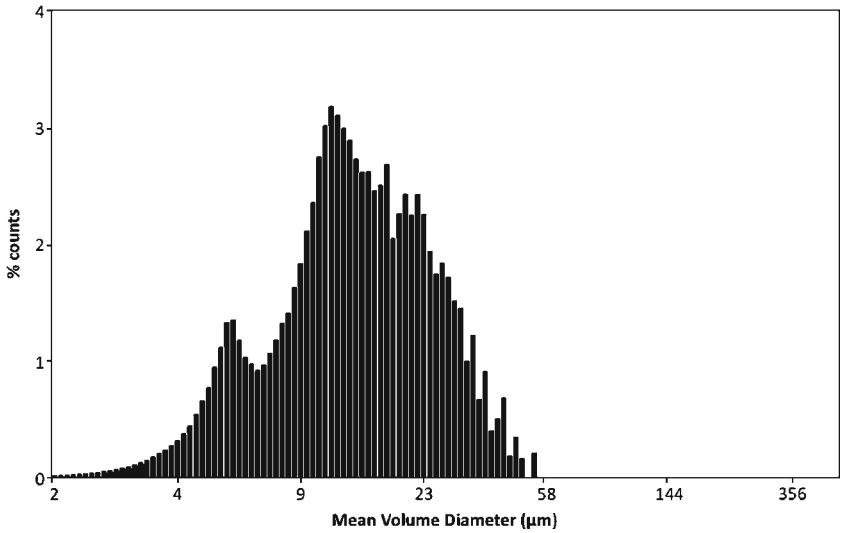

Fig. 2. Size distribution of SOD-loaded PLGA MS obtained by W/O/W double emulsion-solvent extraction/evaporation method.

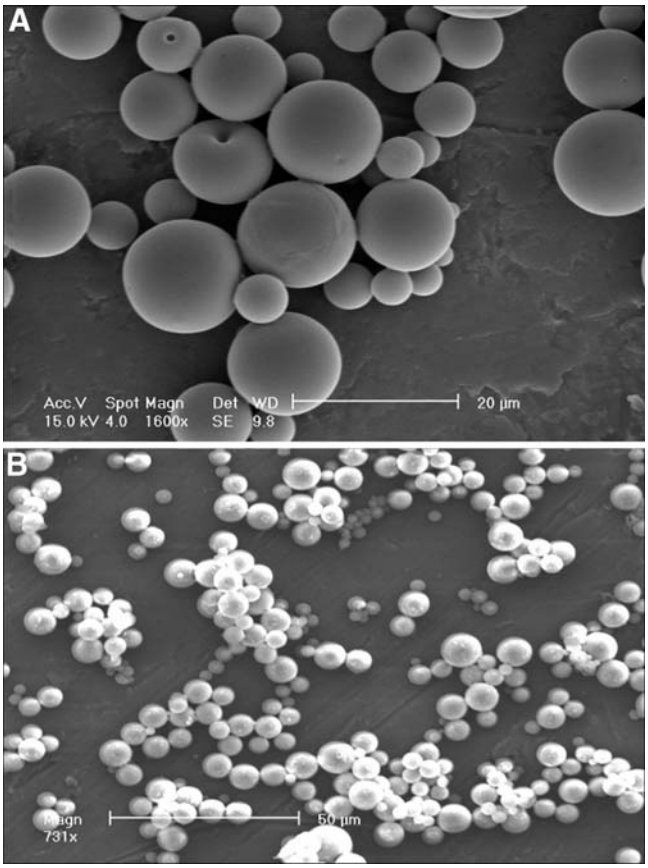

Fig. 3. SEM microphotographs of A) SOD-loaded PLGA MS and B) KET-loaded PLA MS.

populations obtained were found suitable to be encapsulated within multicompartmental MC. On the other hand, KET-loaded PLA MS showed the same characteristics of the batches produced in previous works with average particle size around 5 µm and a narrow size distribution, being 99% of the particles <20 µm (37,38) (data not shown). Drug content and encapsulation efficiency were 17% (w/w) and 75%, respectively.

Optical microscopy pictures showed a certain homogeneity and characteristic biphasic inner structure of SOD-loaded MS (data not shown). SEM microphotographs highlighted a rather smooth surface and fairly regular shape for both MS formulations (Fig. 3).

The enzyme activity measured by the pyrogallol autoxidation assay was entirely retained during the preparation process due to the presence of PEG400, already found essential in ensuring activity preservation upon enzyme encapsulation (37). The stabilizer was able to preserve the protein from coming into contact with the organic solvent, being this the main reason of denaturation and activity loss when double emulsion evaporation methods are being employed. One unit of active enzyme corresponded to 50 µg/mL of SOD.

Multicompartmental MC Characterization

Optical microscopy investigation showed MC of rather regular and uniform shape and size (Fig. 4). Both blank (Panel 4A) and multicompartmental (Panel 4B, 4C) had the same features, proving that the addition of MS did not affect and alter MC characteristics, which are essential to preserve biocompatibility (25). All MC showed similar size around 450 µm. The MS distribution within the MC was homogeneous, and no adhesion to the double-coated ALG

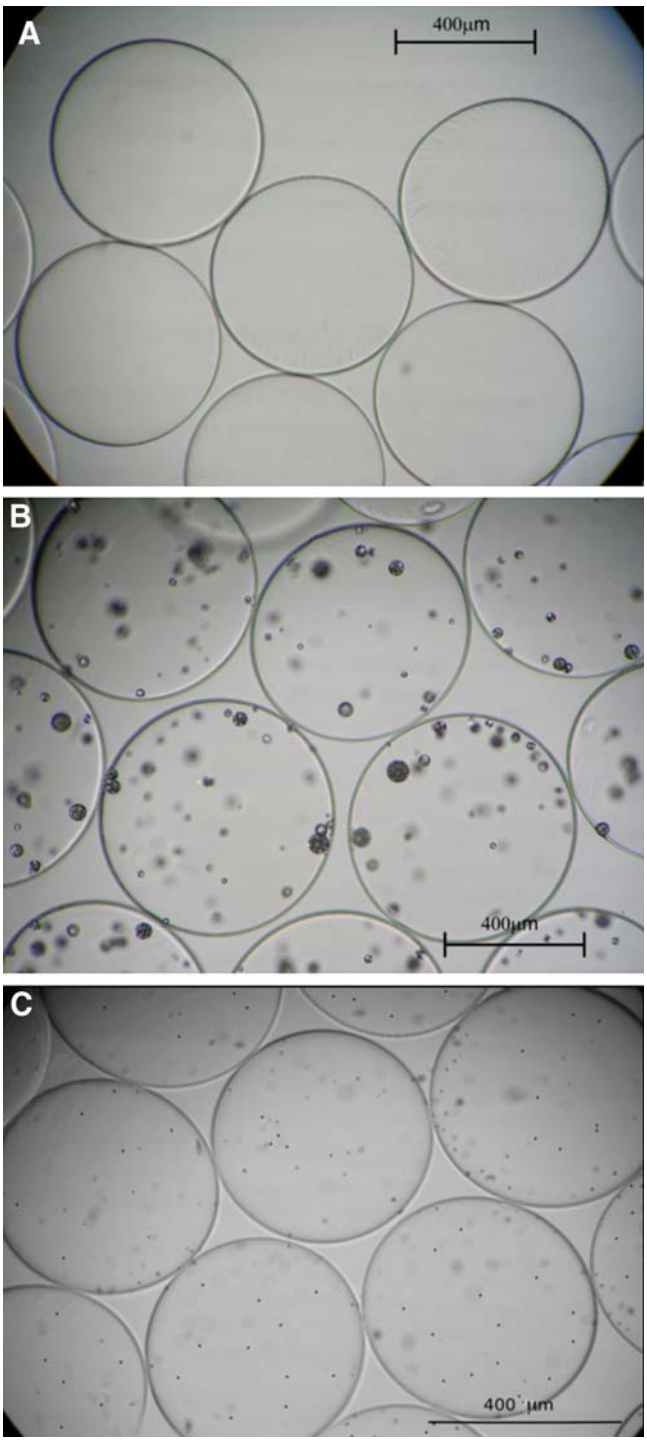

Fig. 4. Pictures of the obtained conventional and multicompartmental ALG/PLO/ALG MC: A) conventional, B) SOD-containing multicompartmental MC and C) KET-containing multicompartmental MC.

membrane was observed. The latter is important, as the medium viscosity (c.a. 200 cps, 4°C) of the ALG solution employed can affect MS dispersion, producing irregular distribution of particles and particle clusters within the capsule.

Investigation of SOD release from the MS and from the multicompartmental MC is shown in Fig. 5. SOD had a sustained release from PLGA MS with just about 25% initial burst, and the release reached 90% after 64 days in an almost linear manner (r^2=0.993) after day 1 (Fig. 5). This highly sustained behavior is the result of a biphasic trend with an initial diffusion-driven mechanism and a second phase in which erosion becomes prevalent. On the other hand, SOD release from multicompartmental MC barely approached 40% at 30 days with a slow progression over time and practically stopped afterwards (Fig. 5).

KET release from the PLA MS and from the multicompartmental system matched that already reported, with 90% of the drug released after 7 days, while it dropped down to 70% from inside the MC over the same timeframe (16,38) (data not shown).

Evaluation of possible PLO release from the membrane showed clearly that not significant amounts of PLO (2–3% w/w) were found in the release medium up to 10–12 days (Fig. 6). No differences were observed from the control even when varying ALG concentration of the coating solution and time of the ALG coating process. In addition, no PLO adsorption on the container was observed (data not shown). These findings suggest that the PLO-ALG interaction is strong enough to avoid polycation release into the surrounding environment, thus preventing the *in vivo* response to PLO.

In Vivo Effect of SOD and KET Controlled Release on MC Grafts and Cell Expression

Optical microphotographs of the MC collected at day 10, 20 and 30 clearly show a difference between conventional and the multicompartmental MC containing KET and SOD. In fact, already at day 10, conventional MC were completely coated by cells showing the characteristic "rice cake" peculiar to a post-transplant acute inflammatory response (Fig. 7). Usually, this phenomenon occurs within

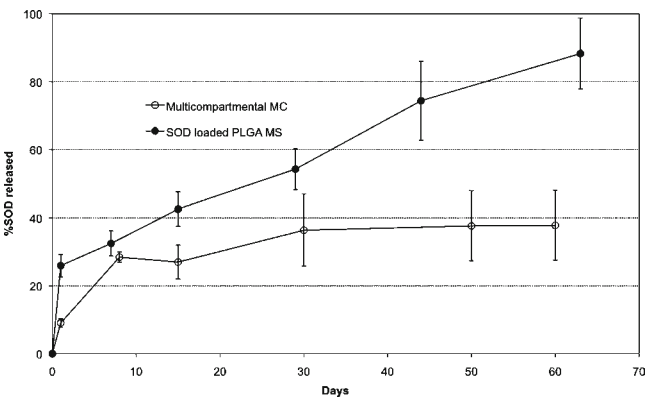

Fig. 5. SOD *in vitro* release profiles from loaded PLGA MS and multicompartmental MC.

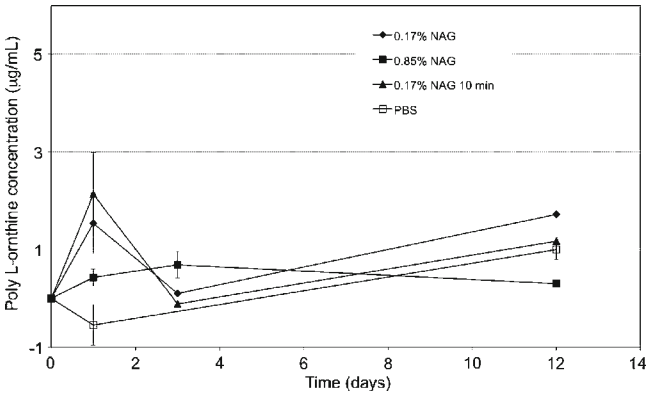

Fig. 6. *In vitro* release profile of PLO from blank ALG/PLO/ALG MC. ALG concentration and coating time were changed.

a week after transplantation and in most cases is reversible when other concomitant factors do not worsen the host response. In this case, an acute irreversible response was not expected, as mice were implanted with a purified ALG that showed a level of endotoxins below 100 EU/g of polymer and foreign protein concentration below the 0.5% threshold (44). Moreover, metals content was within the established range, with the exception of calcium, which resulted 17-fold higher (data not shown). However, the high calcium content should not represent an issue, as calcium is used as a gelling agent and has not been previously found to affect biocompatibility (45).

In spite of all parameters being within accepted range, this ALG batch led to a strong response upon grafting (Fig. 7). As a consequence, pericapsular overgrowth was observed in the controls as early as day 10 (Fig. 7A) and slightly on the multicompartmental MC containing KET, which became completely covered by cells after day 20 (Fig. 7B). The multicompartmental MC containing SOD did not show significant cell growth over the entire post-transplant follow-up (Fig. 7C). Moreover, at day 30, the partial disappearance of the SOD-loaded MS inside the MC is observed. This is correlated with polymer degradation that is reported to effect more than 60% of mass loss within 60 days (38). A slight cell growth was observed at day 20 on the MC with SOD, but, beyond being very limited, it reversed at day 30, resulting in MC almost completely free from macrophages. In all the other cases the process was not reversible.

Flow cytometry characterization of cell overgrowth outlined the presence of activated macrophages and a certain host immune response to ALG (Figs. 8, and 9). The profile of cell phenotypes recovered from the collected MC is summarized in Table I. In detail, the cell population for the control group was comprised at day 10 by a 50% of F4/80+ peritoneal macrophages, which decreased to about 40% at day 30 (Table I, Fig. 8A). 30% of the macrophages expressed the typical macrophage marker CD11b+, which remained unchanged up to day 30 (Table I, Fig 8A). On the other hand, the KET group presented 92% CD11b+ cells since day 10, which at day 30 decreased down to 51%, while the F4/80+ cells were 61% (Table I, Fig. 8B). Regardless of the relative fractions of cell populations determined by cytometry, as

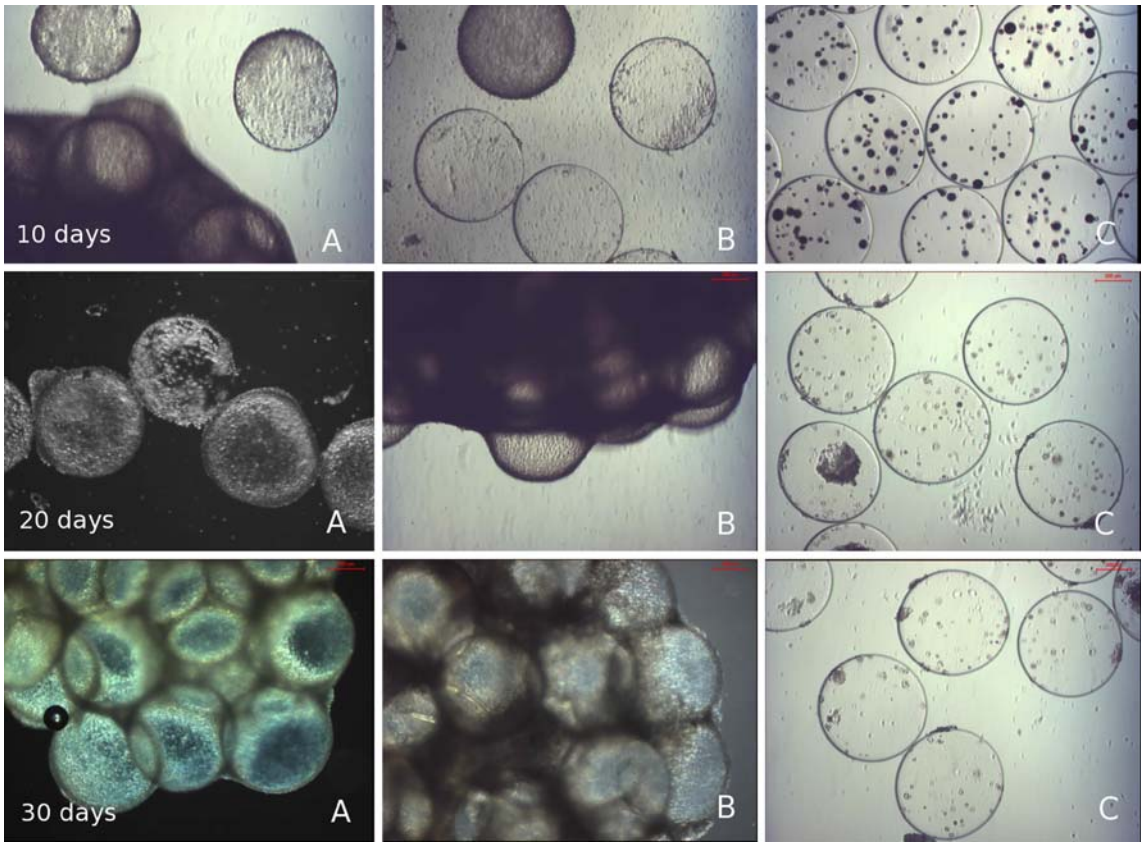

Fig. 7. Microscopy observations of ALG/PLO/ALG MC overgrowth at each time point for A) Blank MC, B) MC embedded of KET-loaded PLA MS and C) MC embedded of SOD-loaded PLGA MS.

stated previously and shown by optical microscopy, the total mass of cells recovered from the SOD group was always very low at all time points. Therefore, day 20 and 30 were used for cytometry due to the insufficient mass of cells recovered at day 10. In this regard, as by optical observations, at day 20 slightly higher mass of cells was observed on the MC with SOD as demonstrated by the presence of 61% of F4/80+ cells, among which 48% expressed also for CD11b+ (Table I, Fig. 8C). Such phenomenon reversed at day 30 displaying a bare 8% of F4/80+ cells, no CD11b+ phenotype and an almost negligible mass of cells recovered from the collected MC.

Furthermore, the analysis of T-cell expression at day 30 showed the presence of about 19% CD8+ in the control group, 6% in the KET group and barely 4% in the SOD group (Table I, Fig. 9). Moreover, in the control group, 50% of CD8+ expressed an activated phenotype, as measured by CD25 and CD69 analysis (data not shown).

DISCUSSION

The cell encapsulation strategy possesses a number of advantages over other approaches to T1DM therapy. Among others, the possibility to achieve a complete control on blood glucose level over time by tight regulation of insulin production is considered the main potential of such strategy. Nevertheless, this procedure is not free from risk of adverse recipient response to MC graft. A number of concomitant causes can provoke post-transplant inflammatory and immune reactions that need to be prevented to ensure treatment success. Therefore, radical scavenger and anti-inflammatory agent controlled release may warrant such a goal. As already pointed out, this study represents a proof-of-concept on the application of such systems to control unexpected post-grafting adverse reactions. For this purpose, all the other concurrent factors have been controlled and accurately optimized. First of all, MS morphological and dimensional features, which indeed affect the way particles distribute inside the MC, can drastically influence biocompatibility by altering membrane surface properties and MC uniformity. In this regard, MS clusters within MC were avoided, thus warranting reproducible membrane homogeneity, absence of deformations, roughness and protrusions either in blank or multicompartmental MC. In addition, the MS release behavior, according to the optimal characteristics obtained, should warrant islet graft protection over the immediate post-transplant throughout long-term periods. In fact, the release profiles suggest a KET effect over the initial acute response due to post-transplant shock (16), while SOD should grant for defense against the chronic oxidative stress occurring over time.

The fast KET release, unusual for high MW PLA MS, has been ascribed to drug and water polymer plasticization, which lead to an increase of the drug diffusion coefficient (46,47), while ALG hydrogel viscosity as well as KET-PLO

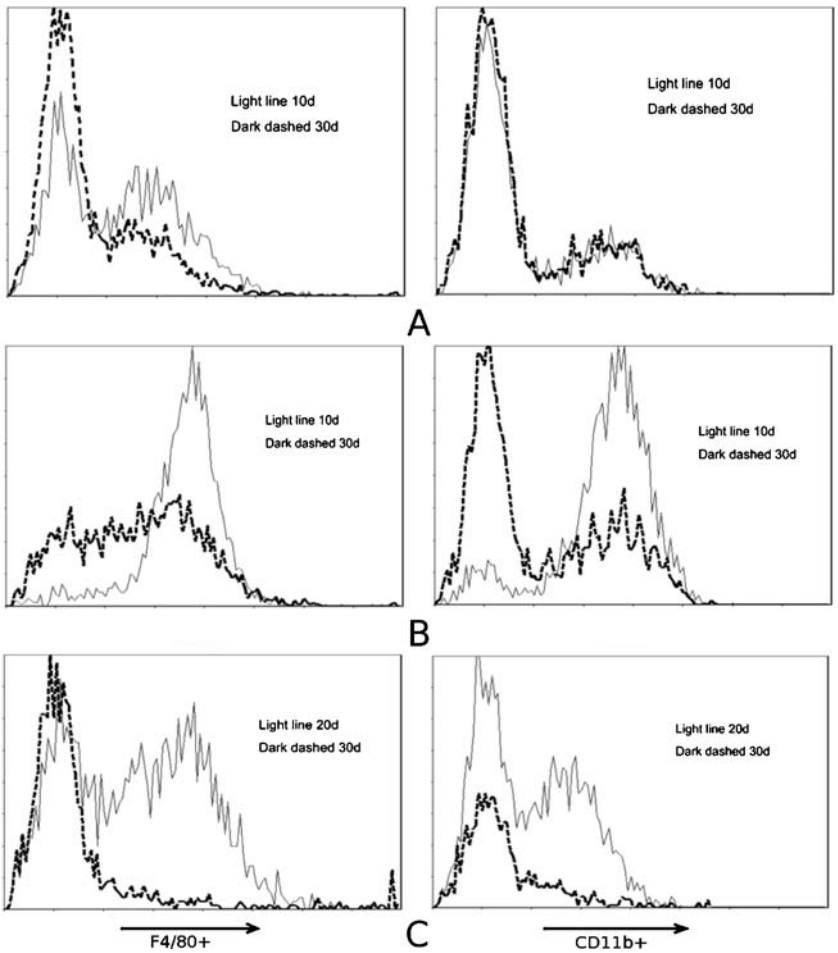

Fig. 8. Comparison of cells expressing for F4/80 and CD11b over time collected from the recovered ALG/PLO/ALG MC at each time point. A) Blank MC, B) MC embedded of KET-loaded PLA MS and C) MC embedded of SOD-loaded PLGA MS.

interaction might explain the slower KET diffusion from the MC (16,38).

An additional issue that was ruled out is the possible leakage of PLO from the membrane, which indeed can induce a strong immune response. Such molecule serves to reduce the large molecular weight cut-off of the ALG gel that otherwise may not warrant immunoprotection (48). The presence of PLO is then being masked by the ALG outer coat, which is necessary to restore MC biocompatibility.

Therefore, in light of such observations, the high unexpected response recorded by MC grafting was ascribed to the ALG batch employed. In fact, blank MC themselves were able to trigger a strong reaction, even though MC features were optimized according to what previously discussed.

ALG contaminants were considered the reason for the observed adverse *in vivo* response, albeit, as mentioned above, the employed purified ALG batch complied with all requirements in terms of endotoxin, protein and metal contents. This erratic behavior of the host response to purified graft materials poses serious concerns to the clinical use of ALG and other naturally occurring polymers for human cell transplant. Therefore, the availability of a system able to prevent or control such issue would have a remarkable impact on cell transplant procedures. In this regard, as a result of the strong host response observed, the control group and the KET group at day 20 developed the typical 'rice cake' structure. This is a cluster formation of microcapsules, which adhere to each other and to the surrounding organs as a result of cell pericapsular overgrowth. Therefore, it is clear that in such conditions, KET limits the response over the immediate post-transplant period but not in the long term. A possible improvement of the KET effect could be obtained by increasing KET concentration and/or extending drug release over a longer period of time.

On the other hand, SOD totally preserved MC from being engulfed by macrophages by modulating cell expression and immune response (49). In fact, at day 30, both the control and the KET group, beyond showing a consistently larger mass of recruited macrophages as compared to the SOD group, provided a significantly higher amount of T-cells as well. This proved that the enzyme sustained-release from biodegradable MS can indeed prevent cell expression and host immune response over a wide timeframe. These findings open some new perspectives about the use of these controlled release systems to improve cell transplant success. In fact, the evidences here provided claim for the employment of the

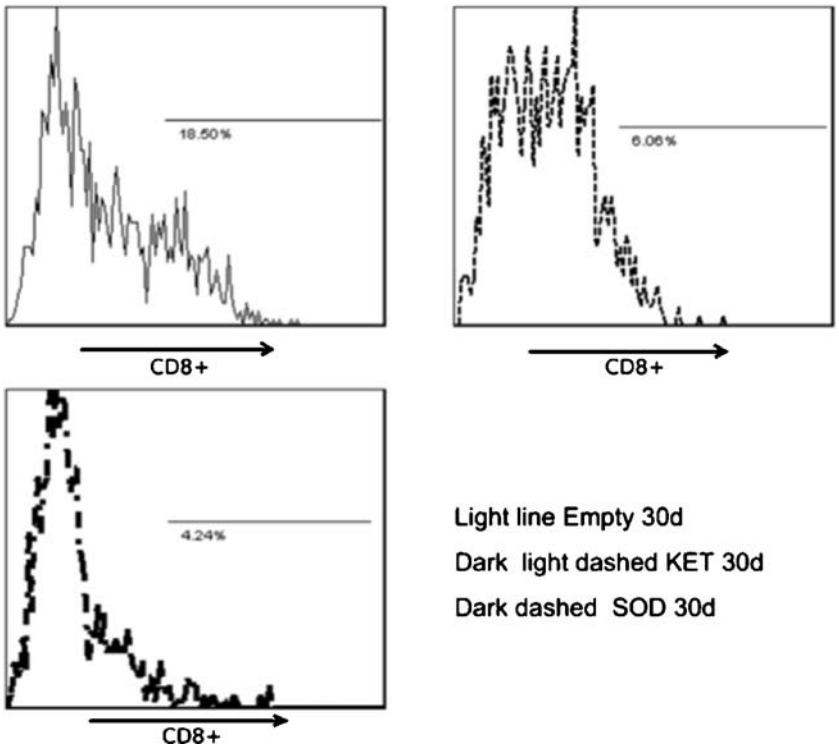

Fig. 9. Comparison of cell expression for CD8 at day 30 for the control, KET and SOD groups. The percentages reported in the panels express the fraction of CD8+ phenotype of the cell population being considered.

SOD and KET releasing MS as potential new tools to improve success rate of encapsulated cell transplantation, especially considering the unpredictability of recipient reaction to grafts.

CONCLUSIONS

The use of both anti-inflammatory drugs and antioxidants sustained release from polymeric MS can be of great importance in cell transplantation, not only on behalf of cell survival and post-grafting reaction control, but to prevent possible unpredictable host response to the material employed as well. In fact, it must be stressed that, even though using materials of tested clinical-grade, host reactions can never be completely avoided. Therefore, the use of such systems is proposed as a novel tool to preserve the efficiency of grafts in the immediate and longer post-transplant follow-up and to warrant success over cell transplantation and survival. Moreover, a combination of KET- and SOD-releasing MS can be theorised, especially in the presence of Langerhans islets. In this regard, studies are ongoing to demonstrate the *in vivo* efficacy of such systems when neonatal porcine islets are encapsulated within multicompartmental MC.

Table I. Cell Expression on the Recovered MC. The Percentages Relate to the Fractions of Cell Population Expressing the Relative Marker Collected at Each Timepoint. The CD11b+ (between Brackets) are Subpopulation of the F4/80+ Cells

	Control		KET		SOD	
Time points (days)	[a]F4/80+ (CD11b+)	CD8+	[a]F4/80+ (CD11b+)	CD8+	[a]F4/80+ CD11b+	CD8+
10	50% (30%)	–	100% (92%)	–	n.a.[b]	–
20	[c]–	–	–[c]	–	61% (48%)	–
30	40% (30%)	19%	61% (51%)	6%	8% (n.d.)[d]	4%

[a] Total activated cells = F4/80+. CD11b+ is a fraction of the F4/80+ phenotype. The number of the recovered cells was qualitatively estimated being in the order: Control group > KET group >> SOD group at all time points.
[b] Not applicable. The mass of cells collected at day 10 for the SOD group was not sufficient to allow flow cytometry analysis. As alternative, data at day 20 were used.
[c] Data for day 20 for the control and KET groups were not used as a slight contamination was observed.
[d] Not detected

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