Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model

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Abstract

The use of pro-angiogenic growth factors in ischemia models has been associated with limited success in the clinical setting, in part owing to the short lived effect of the injected cytokine. The use of a microparticle system could allow localized and sustained cytokine release and consequently a prolonged biological effect with induction of tissue revascularization. To assess the potential of $VEGF_{165}$ administered as continuous release in ischemic disease, we compared the effect of delivery of poly(lactic-co-glycolic acid) (PLGA) microparticles (MP) loaded with $VEGF_{165}$ with free VEGF or control empty microparticles in a rat model of ischemia-reperfusion. $VEGF_{165}$ loaded microparticles could be detected in the myocardium of the infarcted animals for more than a month after transplant and provided sustained delivery of active protein in vitro and in vivo. One month after treatment, an increase in angiogenesis (small caliber caveolin-1 positive vessels) and arteriogenesis (α-SMA positive vessels) was observed in animals treated with VEGF microparticles ($p<0.05$), but not in the empty-microparticles or free VEGF groups. Correlating with this data, a positive remodeling of the heart was also detected in the VEGF-microparticle group with a significantly greater LV wall thickness ($p<0.01$). In conclusion, PLGA microparticle is a feasible and promising cytokine delivery system for treatment of myocardial ischemia. This strategy could be scaled up and explored in pre-clinical and clinical studies.

Key words: angiogenesis; VEGF; PLGA microparticles; Controlled Release; myocardial infarction.

1. Introduction

Cardiovascular diseases remain the first cause of morbidity and mortality in the developed countries accounting for almost 30% of all deaths [1]. Despite recent evidence indicating that the heart is endowed with a regerative potential based on the presence of cardiac progenitors/stem cells, this is insufficient overall to prevent the development of cardiac failure after myocardial infarct in the majority of patients [2, 3]. While heart transplant remains the only curative option for patients with end-stage heart failure, new approaches such as gene (reviewed in [4, 5]) and stem cell therapy (reviewed in [6, 7]) or even the direct administration of pro-angiogenic growth factors have been explored in recent years [8, 9]. In the case of cell therapy, the current view suggests that stem cells contribute to cardiac repair through a paracrine effect associated with the release of growth factors rather than by directly contributing to tissue regeneration [10-14].

If the role of paracrine mechanisms is taken as a starting point, the administration of growth factors to promote tissue revascularization represents an attractive option that has been explored in animal models of limb ischemia or myocardial infarction. Pro-angiogenic cytokines such as VEGF (Vascular Endothelial Growth Factor), FGF (Fibroblast Growth Factor) or HGF (Hepatic Growth Factor) [15-18] have been administered either as natural recombinant human proteins or by gene transfer. However, although preclinical animal models and initial clinical trials suggested a beneficial effect [19-21], double-blinded clinical trials with large cohorts of patients failed to show efficacy [22-25]. These disappointing results were attributed, at least partially, to the short lived effect and high instability of the protein when injected as a bolus. On the other hand, gene delivery through naked plasmids or integrative viral vectors was associated with low efficacy, or even with risk of genome integration, which hinders their use in the clinical setting [23, 25, 26].

An alternative approach is the development of biocompatible delivery systems, allowing for sustained and controlled release of growth factors that could prevent some of the problems described, and which would at the same time facilitate stable prolonged treatment in the damaged tissue (reviewed in [27, 28]). To explore this possibility, we prepared poly(lactide-co-glycolide) (PLGA) microparticles (MP) containing the angiogenic cytokine $VEGF_{165}$ by water/oil/water (W/O/W) multiple emulsion solvent evaporation using the Total Recirculation One-Machine System (TROMS), a suitable technique for encapsulating proteins [29]. Next, we analyzed the vasculogenic effect of small-sized biodegradable and biocompatible VEGF-loaded microparticles in a rat model of myocardial infarction induced by ischemia reperfusion.

2. Materials and Methods

2.1 Materials

Recombinant human $VEGF_{165}$ (rhVEGF, Sf21-derived) was purchased from R&D Systems (Minneapolis, MN, USA). Quantikine VEGF ELISA kit was obtained from R&D Systems and used according to manufacturer's instructions. PLGA with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA), bovine serum albumin (BSA) and sodium azide were provided by Sigma–Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Rhodamine B isothiocyanate was from Sigma-Aldrich (Barcelona, Spain). A human iliac artery endothelial cell line (HIAE-101, ATCC, USA) was used in the bioactivity studies. Rabbit polyclonal anti-human VEGF-A (clone A-20, sc-152) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

ECL™ anti-Rat IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Anti-caveolin-1α antibody was purchased from BD Biosciences Pharmingen (Heidelberg, Germany) and Alexa Fluor 594 goat conjugated anti mouse IgG was from Invitrogen (Eugene, OR, USA). Anti-alpha smooth muscle actin-Cy3 was provided by Sigma (St. Louis, MO, USA). Rabbit anti-caveolin-1 antibody was supplied by Cell Signaling Technology (Danvers, MA, USA) and donkey anti-rabbit FITC was from Jackson ImmunoResearch (West Grove, PA, USA).

2.2 Preparation of PLGA microparticles containing VEGF

rhVEGF$_{165}$-loaded microparticles were prepared by the solvent extraction/evaporation method using TROMS [29]. Briefly, 50 mg of PLGA were dissolved in 2 ml of a dichloromethane/acetone mixture. This polymer solution was injected into the inner aqueous phase (W_1) containing 35 µg of VEGF, 5 mg of HSA and 5 µl of PEG 400 dissolved in 200 µl of phosphate-buffered saline (PBS). Next, the previously formed inner emulsion (W_1/O) was recirculated through the system for 3 min under a turbulent flow regime. After this step, the first emulsion was injected into the outer aqueous phase (W_2), composed of 30 ml of a 0.5% PVA solution, resulting in a multiple emulsion ($W_1/O/W_2$), which was homogenized by circulation through the system for 4 min. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Microparticles were washed three times with ultrapure water by consecutive centrifugation at 4°C (20000 *g*, 10 min). Finally, the particles were resuspended in 1 ml of ultrapure water, frozen at -80°C, lyophilized (Genesis 12EL, Virtis) and stored at 4°C. For fluorescence-labeled microparticle formulation, rhodamine B isothiocyanate (0.5 mg/mL) was added to inner aqueous phase and microparticles were prepared as described.

2.3 Characterization of microparticles

2.3.1 Particle size analysis

Particle size and particle size distribution were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, UK). A suitable amount of freeze-dried microparticles was resuspended in deionized water before measurement. The average particle size was expressed as the volume mean diameter in micrometers.

2.3.2 Determination of VEGF Encapsulation

Encapsulation efficiency was determined via extraction with dimethyl sulfoxide (DMSO). Freeze-dried loaded microparticles (2 mg, n = 3) were dissolved with 250 µl of DMSO as previously performed [30]. The amount of VEGF entrapped in the particles was measured using the Quantikine VEGF ELISA kit following the manufacturer's protocol.

VEGF content into microparticles was also quantified using Western blot analysis. After VEGF extraction from microparticles with DMSO, SDS-PAGE was performed onto 12% polyacrylamide gels and after electrophoresis the proteins were transferred onto nitrocellulose membranes. After 1 h blocking with 5% nonfat dried milk in TBS plus 0.05% Tween 20, nitrocellulose sheets were incubated overnight at 4 °C with primary rabbit antibodies against VEGF-A (A-20): sc-152 (diluted 1:2000). The binding of primary antibodies was performed by incubating membranes with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (diluted 1:2000). Immunoreactive bands were, after several washes, visualized using LumiLight Plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). Quantitative analysis of MP-extracted VEGF bands was performed by densitometry using Quantity One software (Bio-Rad Laboratories Inc., Munich, Germany). Sample values were quantified using a standard curve.

2.3.3 In vitro release of VEGF from PLGA microparticles

For determination of the cytokine release profile, 2 mg of VEGF-loaded microparticles (n = 3) were dispersed in 100 µl of PBS, pH 7.4, containing 0.1% BSA and microbiologically preserved with 0.02%$_{w/w}$ sodium azide. Incubation took place in rotating vials maintained at 37°C for 28 days. At defined time intervals (1 hour, 6 hours, 1, 2, 4, 7, 14, 21 and 28 days), sample tubes were centrifuged (25000 *g*, 15 min) and the supernatant was removed and frozen at −80°C. The removed solution was replaced with an equal volume of fresh medium. Supernatant protein content was determined by ELISA and western-blot assays.

2.3.4 Bioactivity of released VEGF

The bioactivity of the VEGF released from the microparticles was evaluated *in vitro* by determining the proliferative capacity of an endothelial cell line (HIAEC) after VEGF treatment. HIAECs were cultured in F12K media supplemented with 30 µg/mL endothelial cell growth supplement (ECGS), 10% fetal bovine serum, 1% heparin and 1% penicillin/streptomycin. In order to determine the endothelial cell proliferation capacity after VEGF stimulation, the HIAECs were plated into 24-well culture plates at a density of 1.25×10^3 cells/well and microparticles were placed in an upper chamber by using transwells (0.4 µm pore size, tissue culture treated polycarbonate membrane - Corning, USA). Cells were incubated for 3 or 7 days, with supernatant from non-loaded or VEGF-loaded microparticles, free VEGF (at 10 or 25 ng/mL), or medium alone as control. The number of viable cells in each experimental group was determined by ATP quantitation, which signals the presence of metabolically active cells by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA). Moreover, in order to confirm the VEGF bioactivity, the capability of the released VEGF to activate its receptor (KDR) in the cultured HIAECs was evaluated by measuring KDR-tyrosine phosphorylation using an ELISA assay (DuoSet® IC for human phospho-VEGF R2/KDR, R&D Systems, Minneapolis, MN, USA).

2.4 In vivo experiments

2.4.1 Myocardial Infarction Model

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC. A total of 30 female Sprague-Dawley rats (250-300 g) were obtained from Harlan-IBERICA (Barcelona, Spain). Animal experiments were carried out using a rat model of cardiac acute-reperfusion ischemia. Rats were initially anesthetized with 4% isoflurane in an induction chamber. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously, fentanyl 0.15 mg/kg and heparin 0.1 mg/kg both administered by intraperitoneal route. The rats were then intubated and ventilated at 90 cycles/min (1.5-2% isoflurane was maintained for continuous anesthesia). A left thoracotomy through the fourth intercostal space was performed, and the left anterior descending (LAD) coronary artery was occluded 2-3 mm distal from its origin for 1 hour and then re-opened. The chest was then closed in layers and rats allowed to recover. Those rats with an ejection fraction bellow 55% (as determined by echocardiography) 2 days after myocardial infarction were included in the study.

2.4.2 Intramyocardial administration of microparticles

Four days after LAD coronary artery occlusion, 2 mg of VEGF-loaded microparticles or free VEGF (100 ng), or non-loaded microparticles (NL-MP) were injected with a 29-gauge needle into 4 regions in the border zone surrounding the infarct. Prior to injection, freeze-dried

microparticles were dispersed in a sterile buffered solution consisting of 0.1% (w/v) carboxymethylcellulose, 0.8% (w/v) polysorbate 80 and 0.8% (w/v) mannitol in PBS, pH 7.4. All groups received the same volume of buffered solution (100 µl) and 6 animals were included in each group. The chest was closed and rats were allowed to recover.

2.4.3 PLGA-microparticles visualization in the myocardium

In order to evaluate the persistence of PLGA microparticles in the heart tissue, a group of infarcted animals was sacrificed 8 and 30 days after fluorescent-labelled microparticle administration (drug-free). Rhodamine B was used as a fluorescent marker to localize the injected microparticles by fluorescent microscopy in the heart tissue.

2.5 Morphometric Study

Four weeks post-injection, animals were sacrificed and their hearts were collected for subsequent morphometric and histological analysis. After being harvested, the hearts were weighed and perfused-fixed in 4% paraformaldehyde at 4°C, and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4°C and embedded in paraffin. Sections (5 µm) were cut from each segment and stained with Sirius Red as previously described [31] to evaluate infarct wall thickness. These morphometric parameters were measured in images made with a 5× objective of Sirius Red-stained sections viewed with a Zeiss Axio Imager M1 microscope (Carl Zeiss AG, Oberkochen, Germany) and captured using an Axio Cam ICc3 video camera and Axiovision software (4.6.3.0 version). Fibrosis was measured in high power photographs within the infarct border as the percentage of collagen area (red) vs. total tissue area, using AnalySIS® software (Soft Imaging System GmbH, Münster, Germany).

2.6 Histological Study

For the analysis of capillary density (capillaries/mm^2), 9 sections per heart were stained with an anti-caveolin-1α antibody (diluted 1:50) and 2 peri-infarct and 2 intra-infarct images per section were analyzed. Secondary antibody was Alexa Fluor 594 goat conjugated anti mouse IgG (diluted 1:100). The arteriolar density and arteriolar area were quantified in the same way after staining with anti-alpha smooth muscle actin-Cy3 (α-SMA, diluted 1:500) in the following sections. For vessel counting, images were acquired using the Axio Cam MR3 video camera at 20× connected to the Zeiss Axio Imager M1 microscope equipped with epifluorescence optics. Digital images were analyzed using MatLab® software platform (Mathworks Inc., Natick, MA, USA). Also, double immunostaining with anti-alpha smooth muscle actin-Cy3 and rabbit anti-caveolin-1 (diluted 1:125) was performed. Secondary antibody was donkey anti-rabbit FITC (diluted 1:200) and nucleus were stained with TOPRO-3 (diluted 1:50 in PBS-glycerol). For confocal microscopy, a LSM 510 META (Carl Zeiss, Minneapolis, USA) microscope was utilized.

2.7 Statistical analysis

Results are expressed as mean ± SEM. Statistics were calculated with SPSS computer software for Windows (version 15.0, SPSS Inc, Chicago, Ill). Non-parametric statistical analyses were used when values were not normally distributed. The differences among the groups were first evaluated using the Kruskall–Wallis test, followed by Mann–Whitney U-test comparing individual groups where necessary. The differences among the groups were assessed by ANOVA with a Tukey post hoc correction when the measured values were normally distributed. Shapiro-Wilk test was used to justify the use of a parametric test. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1 Characterization of PLGA-microparticles, $VEGF_{165}$ release and bioactivity

VEGF-loaded microparticles (VEGF-MP) were prepared with a diameter of 5.1±1.3 µm (Fig.1A). The total amount of loaded VEGF was 0.58 µg per mg of polymer, which corresponds to an encapsulation efficiency of 83.8±6.6% determined by ELISA and confirmed by Western Blot analysis (not shown). The yield of the microencapsulation process was over 80%.

The *in vitro* release kinetics was performed in PBS (pH 7.4) at 37 ºC for 28 days. VEGF released within the first 6 hours (*burst* effect) was 10±1%, followed by a phase of sustained release of the cytokine with almost 75% of VEGF being released within 28 days (Fig.1B).

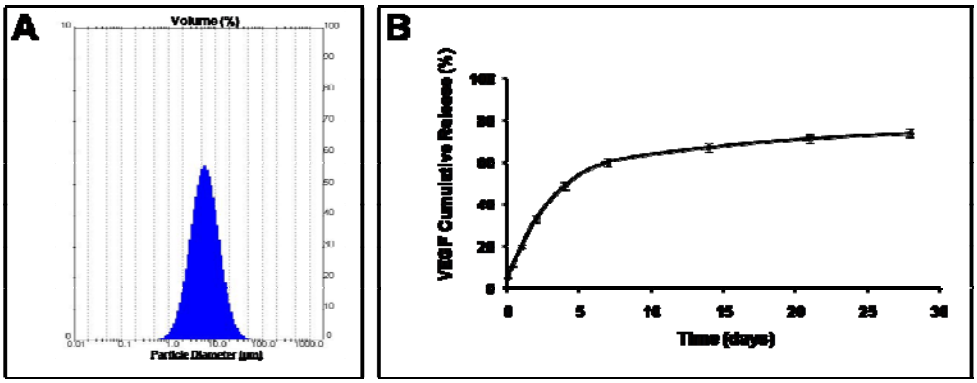

Figure 1: Microparticle characterization and Release Profile. A. Representative particle size distribution for PLGA microparticles prepared by TROMS employed in the *in vivo* studies. **B.** *In vitro* release of VEGF from PLGA microparticles.

The bioactivity of the encapsulated $VEGF_{165}$ released from the microparticles was examined by determining its capacity to induce proliferation of endothelial cells (Human Iliac Artery Endothelial Cells HIAEC). VEGF-MP (10 or 25 ng/mL) induced a 2-3 fold increase in proliferation of HIAEC in comparison with control (no cytokine) or non-loaded MP (NL-MP) after 3 and 7 days in culture ($p<0.01$). This increase was similar to that observed when HIAEC cells were cultured with daily addition of free VEGF at doses of 10 or 25ng/mL (Fig.2A). In addition, bioactivity of the VEGF-MP was further determined by quantifying the tyrosine phosphorylation of the VEGF receptor KDR. Similar levels of stimulation in the HIAEC cells treated either with the free-VEGF or the VEGF-MP was detected, confirming the bioactivity of the released protein (Fig.2B).

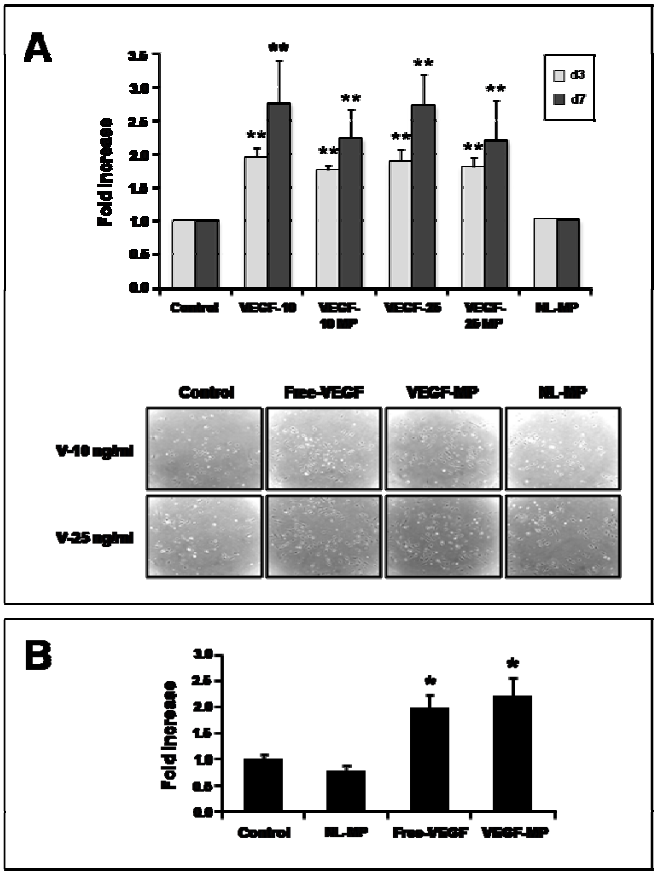

Figure 2: Bioactivity of VEGF-MP. **A.** Proliferation of HIAEC cells was induced by free VEGF at 10ng/mL (VEGF-10) or 25ng/mL (VEGF-25) or VEGF-MP at the same concentration and compared to culture medium alone (control) for 3 and 7 days. Non-loaded microparticles (NL-MP) did not induce cell proliferation. Representative pictures of HIAECs density 7 days after treatment are shown. **B.** KDR activation in HIAECs induced by VEGF stimulation (free or encapsulated at 10ng/mL) and NL-MP (y axis represents fold increase *versus* non treated group)**.** **P*<0.05 and ***P*<0.01.

3.2 ***Microparticle visualization in the heart tissue***

In order to confirm the capacity of the microparticles to remain in the myocardium for a prolonged period of time -a requirement for sustained cytokine treatment-, the fate of the particles after *in vivo* administration was assessed. Rhodamine-labeled microparticles were injected into the peri-infarcted area of the myocardium and animals were sacrificed at 1 and 4 weeks. The fluorescent-labeled microparticles were visible by fluorescence microscopy for up to a month post-implantation (Fig.3A-E). Furthermore, partial degradation of the particles was observed after one month, indicating the biodegradable nature of the co-polymer (Fig.3D,E).

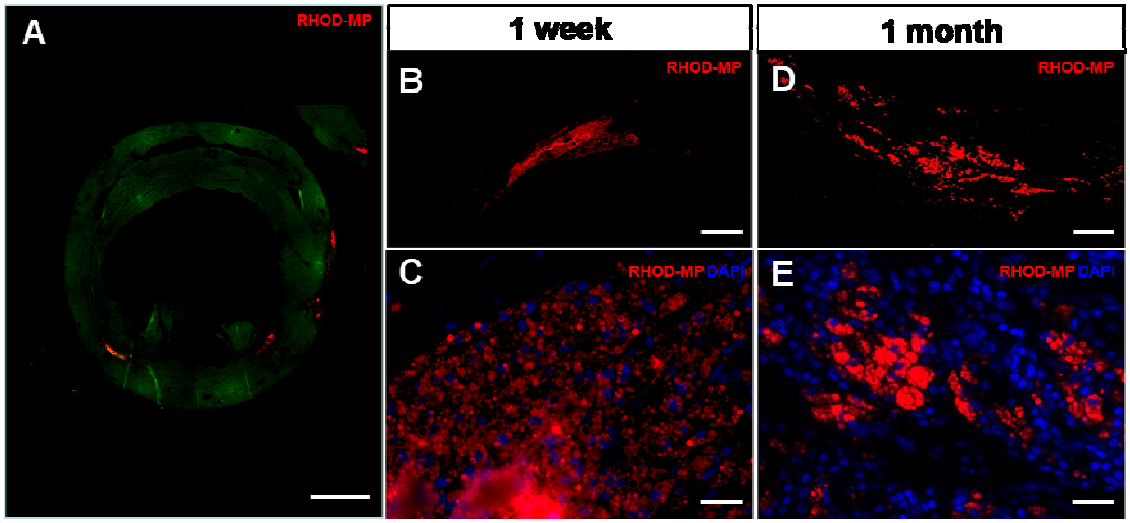

Figure 3: (A) PLGA-microparticles visualization in the heart tissue. A. Microscopy fluorescence visualization of a heart cross-section after fluorescent-labelled microparticle administration**. B-E**. Rhodamine-labeled-microparticles distribution 1 week **(B,C)** and 1 month **(D,E)** after injection. Nuclear staining was performed with DAPI (blue). Scale bars: 500 µm **(A)**, 100 µm **(B,D)** and 20 µm **(C,E)**.

3.3 Vasculogenic and tissue remodeling effect of treatment with VEGF-MP

Administration of VEGF-MP in infarcted rats was associated with a statistically significant increase in the number of capillaries in the infarct and peri-infarct areas of the injured hearts in comparison with the control group (non-loaded microparticles: NL-MP) or with animals treated with free VEGF (NL-MP: 579.5±33.8; VEGF-MP: 704.9±31.75, $P<0.05$; Free-VEGF: 571.6±37.3, P=NS, capillaries/mm^2) (Fig.4A). Treatment with VEGF-MP not only induced a significant increase in small caliber vessels, but also in the number of arterioles (α-SMA coated vessels). This effect was only detected in the hearts treated with the VEGF-MP and not in the hearts injected with the free cytokine, which showed a similar vessel density to animals treated with non-loaded MP (NL-MP: 70±6.7; VEGF-MP: 95±8.9, $P<0.05$; Free-VEGF: 55±4.5, P=NS, arterioles/mm^2) (Fig.4B). In line with these results, the area occupied by α-SMA-positive-vessels (µm^2) was significantly increased in animals treated with VEGF-MP (NL-MP: 3347±183; VEGF-MP: 6590±764, $P<0.001$; Free-VEGF: 2170±328, P=NS). No hemangioma formation or leaking vessels were detected in the VEGF-MP group in the analysis of hematoxylin-eosin stained sections, confocal 3D analysis of caveolin-1$^+$ stained vessels -where non-leaking vessels were detected- (Fig.5A,B), and confocal analysis of caveolin-1$^+$/α-SMA double-stained vessels, which showed a tightly association between the endothelial and mural cell layers (Fig.5C-E).

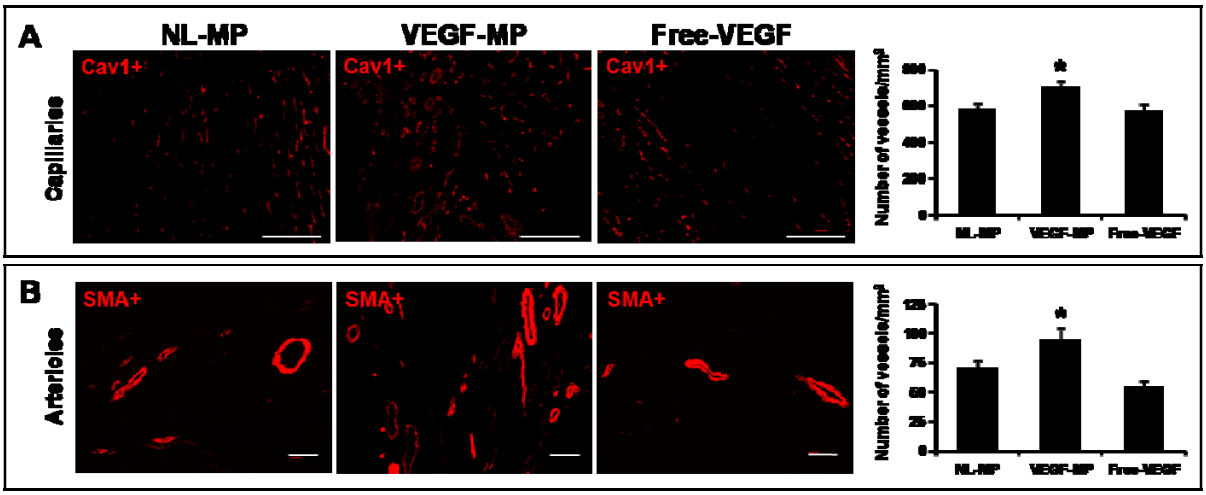

Figure 4: *In vivo* effects of VEGF-MP. Capillary **(A)** and arteriole **(B)** densities were determined by quantification of the small caliber (<15µm) caveolin-1-positive capillaries/mm^2 and α-SMA-positive vessels/mm^2 in the infarcted and peri-infarcted areas, 1 month after administration of non-loaded MP (NL-MP) (control group), VEGF-MP or free-VEGF. Representative images for caveolin-1α and α-SMA immunofluorescence stainings are shown. A significant increase in capillary and arteriole densities was determined in the hearts injected with VEGF-MP in comparison with the control group (**P*<0.05). No significant increase was detected in the free-VEGF group. Data are presented as mean ± SEM. Scale bars: 50 µm.

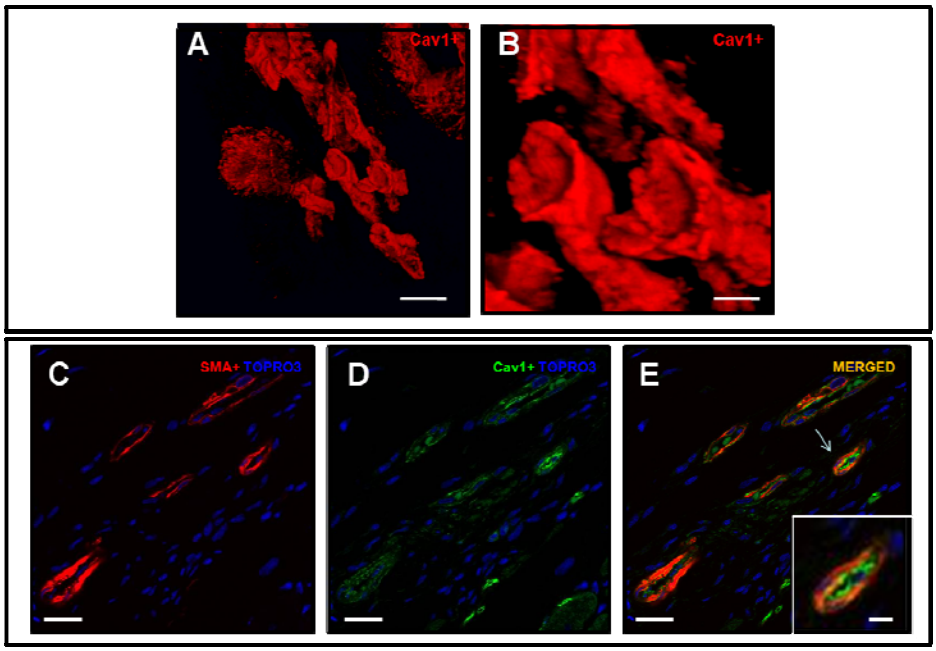

Figure 5: Vessel structure. A,B. Tri-dimensional views of caveolin-1-positive vessels in the VEGF-MP group. Note that the vessels display a regular endothelial structure. **C-E.** Representative pictures of αSMA-Cy3 (red) and Caveolin-1 (green) double immunostained vessels in VEGF-MP-treated heart sections, showing tightly contact between the smooth muscle and the endothelial cell layers. Nuclear staining was performed with TOPRO-3 (blue). Scale bars: 100 µm (**A**), 400 µm (**B**), 20 µm (**C-E**), 5 µm (**E**, insert).

The increased revascularization of the tissue translated into a beneficial effect in the remodeling processes, with a significantly greater thickness of the left ventricle wall in the VEGF-MP treated animals in comparison with the control group (NL-MP: 1.07±0.02 mm; VEGF-MP: 1.30±0.05 mm (*P*<0.01); Free-VEGF: 1.07±0.10 mm (*P*=NS)) (Fig.6), suggesting a potential benefit related to the administration of VEGF through a sustained release system in comparison with injection of the free cytokine.

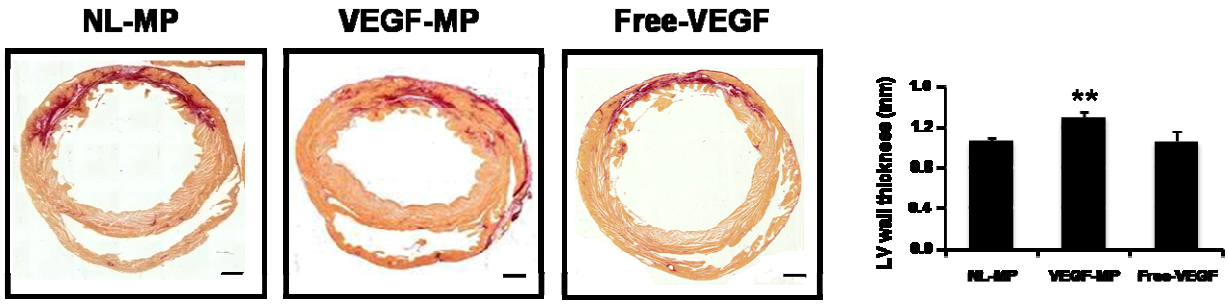

Figure 6: Heart remodeling. Representative Sirius red stained heart sections show greater thickness of the LV wall of the VEGF-MP group in comparison with the NL-MP (control group) (***P*<0.01). No significant increase was detected in the free-VEGF treated group. Scale bars: 1 mm.

4. Discussion

In patients with myocardial ischemia, therapeutic angiogenesis by direct delivery of the VEGF protein or using gene therapy approaches has not yielded the expected results in clinical practice [32]. Some important limitations, such as the short half-life of the protein, as well as the relatively short effect of naked plasmids or adenovirus transfection, seemed to be responsible, at least partially, for the lack of success. Alternative strategies that allow a local, sustained delivery of VEGF or other angiogenic cytokines are worth exploring in order to induce a therapeutic effect. The development of PLGA microparticles loaded with VEGF shown in our study fulfills many of the requirements for a potentially successful therapy like the possibility of being readily administered and their permanence in the myocardium for at least 30 days, being capable of a sustained delivery of active cytokine during that period of time.

Therefore, treatment with VEGF-MP was associated with the expected biological effect: increased angiogenesis and arteriogenesis in an acute ischemia-reperfusion model. Other delivery systems based on the use of hydrogels, liposomes, nanoparticle-fibrin complex, collagen-bound proteins or polymer scaffolds with VEGF or other cytokines have also been explored, showing an angiogenic effect after sustained cytokine treatment [33-37]. However, in general the control of release rate of growth factors from hydrogels is difficult and a strong initial burst release is generally observed which has been associated with severe side effects, such as hypotension [33]. Interestingly, targeted delivery has recently been achieved by anti-P-selectin-conjugated liposomes, which induced an increase in tissue vascularization and an improvement in the cardiac function [34]. Unfortunately, the need for very early delivery (a down-regulation of the receptor in the infarcted area occurs 24h after the ischemia) [38] represents a challenge for its therapeutic use in patients.

VEGF is known to be a potent key regulator of blood vessel formation during both angiogenesis and vasculogenesis (reviewed in [39]). Endothelial cells are activated by VEGF, which results in increased vessel permeability, cell migration and proliferation. Indeed, local high levels of VEGF may result in deleterious effects including the formation of irregularly shaped sac-like vessels associated with massive and highly disruptive edema [40] or even formation of endothelial cell-derived intramural vascular tumors [41]. The sustained controlled release provided by the PLGA particles seems to circumvent these potential problems, as none were found in our study. The positive effects of VEGF-MP on the induction of angiogenesis were not observed in animals treated with NL-MP or even with free-VEGF, demonstrating the greater effect of long-term-release VEGF. As it has been previously demonstrated, a short VEGF stimulus, if it is not sustained, is associated with the regression of the newly formed vessels when discontinued [40].

Treatment with VEGF-MP induced not only an increase in capillaries, but also in vessels of greater caliber, suggesting that prolonged release of VEGF indirectly promotes arteriogenesis by stabilizing the vessels through pericyte and/or smooth muscle recruitment and proliferation (reviewed in [42]). It has been suggested that VEGF can induce the proliferation of local pericytes by stimulating endothelial cells to express PDGF (platelet-derived growth factor) [43], which exerts a chemotactic and differentiation effect in pericytes. Furthermore, the presence of the VEGF receptor in pericytes has also been shown [44], implying that VEGF can act directly on the pericytes by stimulating migration and proliferation. Interestingly, sustained VEGF-MP treatment induced an increase in the number of arteriolar vessels, favoring tissue irrigation [45] and consequently, rescuing the tissue areas at risk. It is possible that preexisting capillaries could undergo enlargement and/or fusion and recruit a smooth muscle cell coating as a consequence of the sustained VEGF-stimulation [45-47]. On the other hand, the presence of VEGF receptors in cardiomyocytes and their association with a

protective effect has been reported [48], which could be responsible, together with the revascularization effect, for the rescue of the cardiac tissue, which translated in a positive remodeling of the heart.
Timing and combination of cytokine administration is an important issue. VEGF-MP were administered alone and shortly after the ischemic event. From the results of this study and others where cytokine was given immediately after myocardial infarction, it seems that very early injection of the cytokine might favor its therapeutic benefit by rescuing hibernating cardiac tissue. On the other hand, the combination of other cytokines that contribute to vessel maturation such as PDGF, TGFβ, FGF, or angiopoietins or even combination of cytokines and (stem) cells could induce a stronger beneficial effect [49-51]. Thus, for example, it has been shown, that subcutaneous implantation of a polymer scaffold that released $VEGF_{165}$ and PDGF-BB induced rapid formation of a mature vascular network [52].

Conclusions

In summary, we have demonstrated that a single cytokine, VEGF, could exert not only an angiogenic but also an arteriogenic effect when delivered *in vivo* in a sustained manner, which translates into positive remodeling of the heart. Moreover, the use of microparticles allows a dose-controlled release of the protein that can be easily and safely translated to patients.

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