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ORIGINAL ARTICLE

Sustained delivery of vascular endothelial growth factor using a dextran/poly(lactic-co-glycolic acid)-combined microsphere system for therapeutic neovascularization

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Abstract

We hypothesize that the controlled delivery of vascular endothelial growth factor (VEGF) using a novel protein sustained-release system based on the combination of protein-loaded dextran microparticles and PLGA microspheres could be useful to achieve mature vessel formation in a rat hind-limb ischemic model. VEGF-loaded dextran microparticles were fabricated and then encapsulated into poly(lactic-co-glycolic acid) (PLGA) microspheres to prepare VEGF–dextran–PLGA microspheres. The release behavior and bioactivity in promoting endothelial cell proliferation of VEGF from PLGA microspheres were monitored in vitro. VEGF–dextran–PLGA microsphere-loaded fibrin gel was injected into an ischemic rat model, and neovascularization at the ischemic site was evaluated. The release of VEGF from PLGA microspheres was in a sustained manner for more than 1 month in vitro with low level of initial burst release. The released VEGF enhanced the proliferation of endothelial cells in vitro, and significantly promoted the capillaries and smooth muscle α-actin positive vessels formation in vivo. The retained bioactivity of VEGF released from VEGF–dextran–PLGA microspheres potentiated the angiogenic efficacy of VEGF. This sustained-release system may be a promising vehicle for delivery of multiple angiogenic factors for therapeutic neovascularization.

Keywords Vascular endothelial growth factor · Microsphere · Sustained release · Ischemia · Neovascularization

Introduction

Critical limb ischemia represents a complication of peripheral arterial stenosis and is characterized by rest pain, non-healing ulcers, and gangrene of the diseased leg. It is a major cause of decreased mobility, quality of life, and functional capacity, as well as an increased risk of amputation or death. Some patients with critical limb ischemia are not candidates for endovascular or surgical procedures and have no other options besides an amputation. Therapeutic neovascularization has attempted to offer an alternative treatment for this subgroup of patients. It refers to deliver angiogenic growth factors and associated genes or cells in a targeted and controlled manner so as to stimulate physiological neovascularization [1–3].

Vascular endothelial growth factor (VEGF) is well known to play a crucial role in migration, proliferation and survival of vascular endothelial cells (ECs). It can induce angiogenesis, resulting in the proliferation and expansion of the capillary network that is necessary to maintain or restore local oxygen and nutrition supply to cells and decrease cellular ischemia [4–8]. Therefore, localized drug delivery systems using biocompatible and biodegradable polymeric systems can release VEGF in a controlled manner at the desired site, and have been tested extensively as a potential treatment of ischemic disease. For example, hydrogels, polyglycol ethylene (PEG), poly(lactic-co-glycolic acid) PLGA and dextran have been extensively utilized to date [9–14].

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Unlike small molecular or peptide drugs, proteins possess tertiary structures which are easily denatured or aggregated during the microencapsulation processes involving water–oil or water–air interfacial tensions [15, 16]. Denatured or aggregated proteins may not only lose therapeutic activity, but also evoke immunogenicity or toxicity [17–19]. To overcome these limitations, a novel protein sustained-release system based on the combination of protein-loaded dextran microparticles and PLGA microspheres was developed [20–23]. These protein–dextran–PLGA microspheres were produced by loading protein into dextran microparticles, and then encapsulating them into PLGA microspheres using solid-in-oil-in-water (S/O/W) method. It was found that the first step protected the protein from denaturing during the manufacturing and releasing process, and the S/O/W method showed stable sustained release of protein for a prolonged time without aggregation. The protein–dextran–PLGA microspheres using the S/O/W method had the higher encapsulating efficiency, preserved protein integrity and bioactivity, and lower level of initial burst release than protein-loaded PLGA microsphere using W/O/W method [20–28].

In this study, PLGA microspheres containing VEGF-loaded dextran microparticles were prepared. The morphology and size distribution of microspheres were characterized by scanning electron microscopy (SEM). The encapsulation efficiency, in vitro release profile and bioactivity in promoting ECs proliferation of the released VEGF were investigated. We also tested if VEGF–dextran–PLGA microspheres could enhance neovascularization efficiently in vivo by intramuscular injection into the ischemic limb of rats. The therapeutic efficacy was evaluated.

Materials and methods

Preparation of VEGF–dextran–PLGA microspheres

A mixture of recombinant rat VEGF 165 (0.5%, w/w) (Pepro Tech, Rocky Hill, NJ, USA), dextran (5%, w/w) (Sigma-Aldrich, Louis, MO, USA), and PEG (5%, w/w) (Sigma-Aldrich, Louis, MO, USA) in a ratio of 1:5:50 (w/w/w) was prepared, followed by stirring at the speed of 1500 rpm for 60 s at 0 °C, and then reduced temperature in a freezer at − 80 °C overnight and subjected to lyophilization below 0.1 mbar for 24 h. The lyophilized powders were washed with dichloromethane, and then centrifuged at 12,000 rpm for 5 min to remove the supernatant containing the PEG in the continuous phase, and these two procedures were repeated three times. The final VEGF-loaded dextran microparticles collected were evaporated at 1.33 Pa at 25 °C for 24 h using a vacuum dryer to remove the dichloromethane residues.

The VEGF-loaded dextran microparticles were suspended in a dichloromethane solution of PLGA (15%, w/w) (Sigma-Aldrich, USA), and emulsified into an aqueous continuous phase (fivefolds large in volume) containing polyvinyl alcohol (1%, w/w) (Sigma-Aldrich, Louis, MO, USA) and NaCl (5%, w/w) (Sigma-Aldrich, Louis, MO, USA), followed by stirring at 2000 rpm for 60 s to form the initial VEGF–dextran–PLGA microspheres. This emulsion was immediately transferred into a large volume of another aqueous continuous phase (200-folds larger in volume and containing 10%, w/w NaCl) for solidification. The sample was stirred gently at 150 rpm and 4 °C for 4 h during which the dichloromethane in the PLGA microspheres was extracted by water and their PLGA matrix was hardened. Finally, the solidified VEGF–dextran–PLGA microspheres were rinsed with deionized water to remove surface-adsorbed NaCl, and then lyophilized.

Morphological characterization of VEGF–dextran–PLGA microspheres

Scanning electron microscopy (SEM) images of VEGF–dextran–PLGA microspheres were obtained using a FEI Sirion 200 SEM (FEI, Hillsboro, OR, USA). Prior to image scanning, the samples were coated with gold vapor under an argon atmosphere and at 5–10 keV.

Encapsulation efficiency of VEGF–dextran–PLGA microspheres

VEGF–dextran–PLGA microspheres, 10 mg, were dissolved in 10 mL of dichloromethane. The resultant mixture was centrifuged at 12,000 rpm for 5 min to remove the PLGA-containing supernatant. These procedures were repeated three times, and the insoluble pellets containing VEGF-loaded dextran particles were remained. Harvested pellets were dissolved in PBS (pH 7.4) and concentration of VEGF was measured by a VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA). VEGF encapsulation efficiency of the microspheres was calculated using equation:

$$\text{Encapsulation efficiency } (\%) = W/\text{Wt} \times 100$$

In the equation, W is the actual total weight of VEGF encapsulated into PLGA microspheres and Wt is the theoretical amount of VEGF encapsulated into the microspheres. The standard deviation for drug loading was calculated based on the experiments which were repeated for six times.

$$\text{Loading efficiency } (\%) = W/M \times 100$$

In the equation, W is the actual total weight of VEGF encapsulated into PLGA microspheres and M is the weight of PLGA microspheres. The standard deviation for drug

loading was calculated based on the experiments which were repeated for six times.

In vitro release profile of PLGA microspheres

10 mg of the VEGF–dextran–PLGA microspheres was suspended in 1 mL release medium [20 mM of pH 7.4 PBS and 0.02% (w/v) sodium azide] and shaken at 37 °C. The release medium was collected completely and fresh medium was added every 2 days. The concentration of VEGF in each collected medium was determined using a VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA). The amount of VEGF released during each time interval was determined by multiplying VEGF concentration with the medium volume. For cumulative release profiles, the accumulated release amount was plotted against time for each sampling interval.

In vitro ECs proliferation with released VEGF

Human umbilical vein ECs (HUVECs) were seeded onto 24-well tissue culture plates (5×10^3/well) and cultured in endothelial growth media (EGM-2) (Cloneticsᵀᴹ, Lonza; Basel, Switzerland) for 12 h at 37 °C under 5% CO2 atmosphere. The cells were then treated with the media containing no VEGF (control group, $n = 6$), intact VEGF (VEGF group, VEGF = 2 ng/well, $n = 6$) or VEGF released from VEGF–dextran–PLGA microspheres as determined by VEGF ELISA (VEGF–PLGA group, released VEGF = 2 ng/well, $n = 6$), and the number of cells were counted using a 3-(4,5-dimethylthiazol-2-yl)-2,5-driphenyltetrazolium bromide (MTT) assay. The growth rate of HUVECs was calculated from cell counts comparing the cell number at day five to the cell number at 12 h post-seeding.

Ischemic hind-limb model and treatment

Sprague–Dawley rats (male, 6 weeks old, average weight 200 g) were anesthetized by an intraperitoneal injection of ketamine (60 mg/kg). The proximal point above the external iliac artery and the distal point where it bifurcates into the saphenous and popliteal arteries were ligated using a 6–0 grade silk suture, followed by excision of the femoral artery between them. One day after arterial dissection, the rats were randomly divided into four groups ($n = 12$ for each group). The sealer protein component (Fibrinogen 50–75 mg/mL, Factor XIII 10–50 U/mL) (Bioseal, Guangzhou, China) was reconstituted with a fibrinolysis inhibitor solution (Aprotinin 3000 KIU/mL) and spiked with PLGA microspheres. The thrombin component (400 IU/mL) was reconstituted with CaCl2 (40 μmol/mL). Then fibrin gel was made by mixing 1 ml of the sealer protein and VEGF–dextran–PLGA microspheres with 1 ml of the thrombin component. In VEGF–PLGA group, the rats were treated with fibrin gel containing VEGF–dextran–PLGA microspheres by intramuscular injection into the tibialis anterior muscle (injection volume = 200 μl, VEGF = 70 μg/rat). In VEGF group, the rats were treated with fibrin gel containing VEGF (VEGF = 70 μg/rat). In PLGA group, the rats were treated with fibrin gel containing dextran–PLGA microsphere without VEGF. In group PBS, the rats were treated with PBS. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were preapproved by the Institutional Animal Care and Use Committee of Nanchang University.

Histological and immunohistochemical analysis

The tibialis anterior muscle samples were harvested on days 5 and 28. The tissue samples were embedded in OCT compound, snap-frozen in liquid nitrogen, and then cut into 6-μm-thick sections. The sections were histochemically stained for alkaline phosphatase to detect capillary endothelial cells. The arterioles in the regions were immunohistochemically stained with a rabbit polyclonal antibody against smooth muscle α-actin (SMA) (Abcam; Cambridge, MA, USA) and the sections were also immunohistochemically stained with the following antibodies: rabbit polyclonal antibodies against CD45 and VEGFR-2, a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA), and rabbit monoclonal antibodies against CD34 and c-kit (Abcam; Cambridge, MA, USA). Protocols for immunohistochemistry were according to the Elite ABC Kit (Vector Labs, Burlingame, CA, USA). The stained arterioles and capillaries of the tissue sections were quantified by image analysis using Image-Pro Plus Software.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical analyses were performed using Student's *t* test. Values of $^*p < 0.05$ were considered statistically significant.

Results

Morphology of VEGF–dextran–PLGA microspheres

Morphology of the VEGF–dextran–PLGA microspheres was characterized using SEM. As shown in Fig. 1, VEGF–dextran–PLGA microspheres possessed spherical shapes, smooth surface and diameters ranging from 40 to 100 μm (Fig. 1).

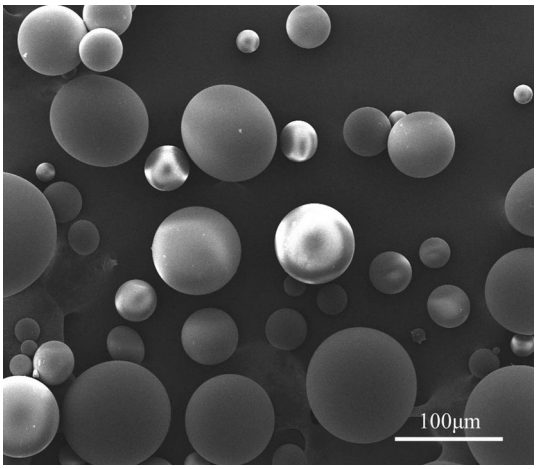

Fig. 1 Scanning electron micrograph of VEGF–dextran–PLGA microspheres

VEGF encapsulation efficiency of VEGF–dextran–PLGA microspheres

VEGF encapsulation efficiency was measured using a VEGF ELISA kit. Encapsulation efficiency for the VEGF–dextran–PLGA microspheres was 85% ± 4.1 (w/w) and VEGF loading efficiency was about 1.72%.

In vitro VEGF release from VEGF–dextran–PLGA microspheres

The release behavior of VEGF from the VEGF–dextran–PLGA microspheres was monitored in vitro. As shown in Fig. 2, the release of VEGF sustained more than 1 month, and the cumulative release of VEGF was 89.7%. The amount of burst release was about 21.4% during the first 2 days.

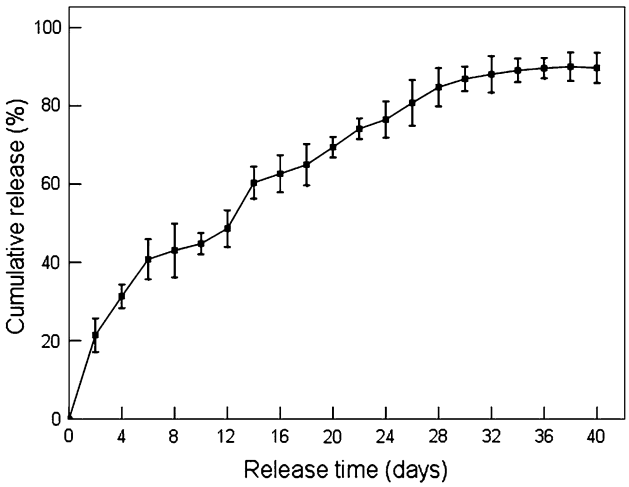

Fig. 2 In vitro release profiles of VEGF from VEGF–dextran–PLGA microspheres

HUVECs proliferation in the presence of VEGF released from VEGF–dextran–PLGA microspheres

To test the bioactivity of VEGF released from VEGF–dextran–PLGA microspheres, in vitro HUVECs proliferation in the presence of released VEGF was investigated. As shown in Fig. 3, the growth rate of cells significantly increased when the cells were cultured in media containing VEGF (0.83 ± 0.09 day^{-1} for VEGF–PLGA group and 0.90 ± 0.07 day^{-1} for VEGF group).

Neovascularization by VEGF–dextran–PLGA microspheres in ischemic limb

As shown in Fig. 4, at postoperative day 5, histological analysis of the ischemic hind-limb skeletal muscle tissues with alkaline phosphatase staining revealed that the capillary densities (number/muscle fiber) in VEGF–PLGA group and VEGF group were significantly higher than those in PLGA group and PBS group ($p < 0.01$).

As shown in Fig. 5, at postoperative day 28, immunohistochemical analysis using an antibody against α-SMA to detect the density of SMA-positive vessels showed that the density of SMA-positive vessels was significantly higher in VEGF–PLGA group than in VEGF group, dextran–PLGA group and PBS group ($p < 0.01$).

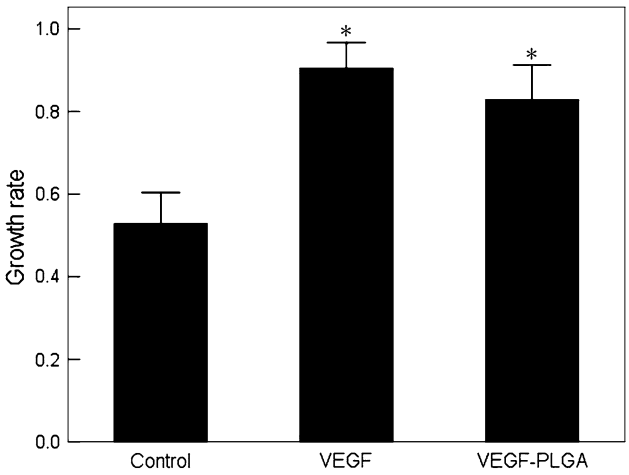

Fig. 3 Effect of VEGF released from VEGF–dextran–PLGA microspheres on human umbilical vein endothelial cell (HUVECs) proliferation in vitro. HUVECs were cultured in endothelial growth media without VEGF (control group), with intact VEGF (VEGF group), or with released VEGF from VEGF–dextran–PLGA microspheres (VEGF–PLGA group, VEGF = 2 ng/well, $n = 6$), and growth rates of the cells were calculated from changes in the number of cells. *$p < 0.05$ versus control group

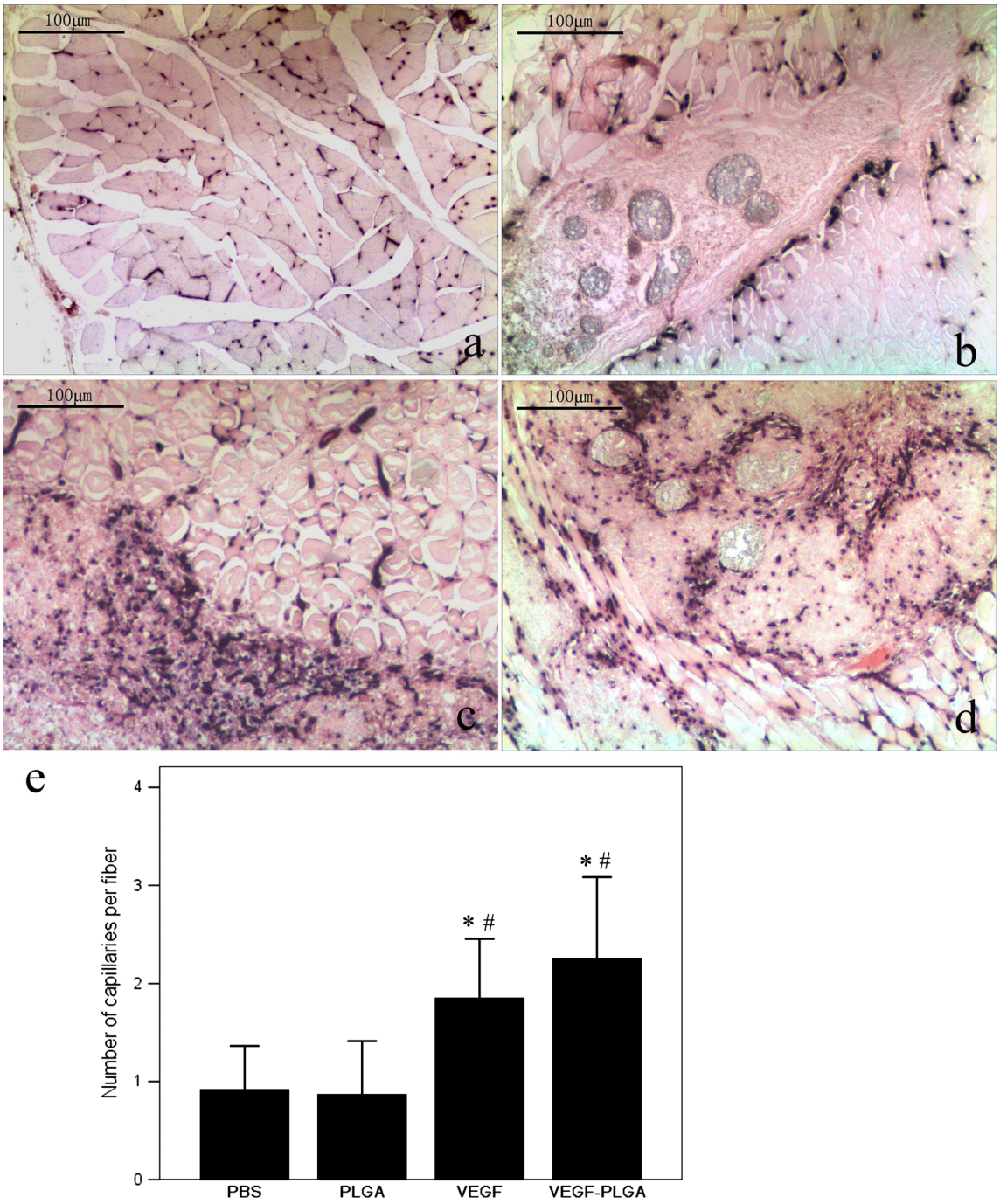

Fig. 4 Capillary density in ischemic muscle obtained from the rats at postoperative day 5. Representative microscopic photographs of capillaries identified by staining of alkaline phosphatase. **a** PBS group; **b** PLGA group; **c** VEGF group; **d** VEGF-PLGA group. Quantification of capillary density as number of capillaries per fiber on the tissue sections. $^{*}p < 0.05$ vs. PBS group, $^{\#}p < 0.05$ vs. PLGA group

These results showed that the injection of VEGF–dextran–PLGA microsphere-loaded fibrin gel into ischemic limbs of rats induced more extensive neovascularization than any of the other groups. These suggested that VEGF–dextran–PLGA microspheres led to sustained release of bioactive VEGF in the ischemic tissues that potentiated the angiogenic efficacy of VEGF administration.

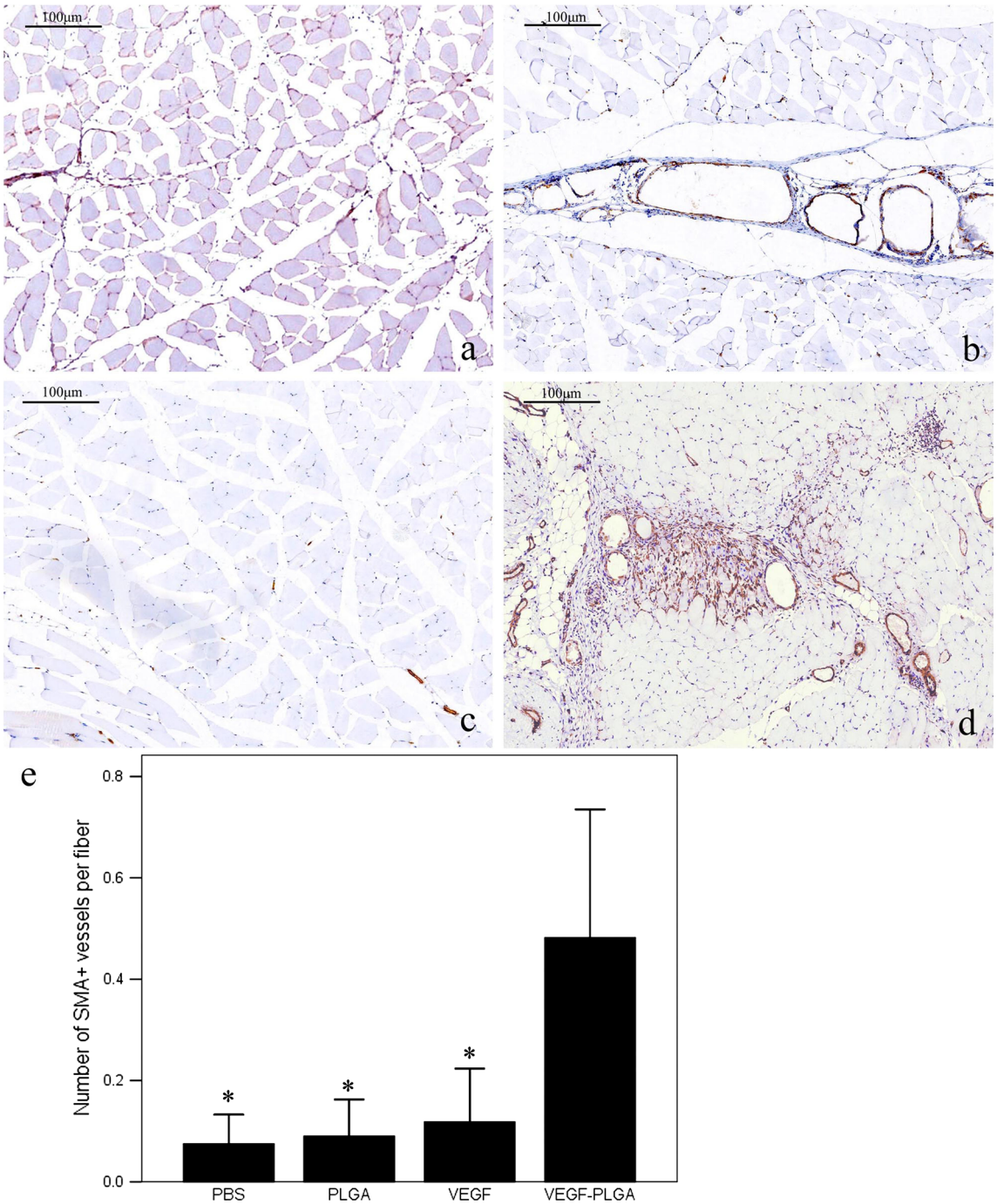

Fig. 5 α-SMA-positive vessel density in ischemic muscle obtained from the rats at postoperative day 28. Representative microscopic photographs of vessels immunostained with an anti-smooth muscle α-actin antibody. **a** PBS group; **b** PLGA group; **c** VEGF group; **d** VEGF–PLGA group. Quantification of α-SMA-positive vessel density as number of SMA^+ vessels per fiber on the tissue sections. $^*p < 0.05$ vs. VEGF-PLGA group

Characterization of infiltrating cells in ischemic limb treated with VEGF–dextran–PLGA microspheres

Histological sections were stained with hematoxylin and eosin at postoperative day 28 and muscles received VEGF–dextran–PLGA microsphere-loaded fibrin gel which showed remarkably increased cellularity compared to any other groups (Fig. 6). To understand the source of these infiltrating cells, immunohistochemistry with different antibodies was performed. As shown in Fig. 7, few cells

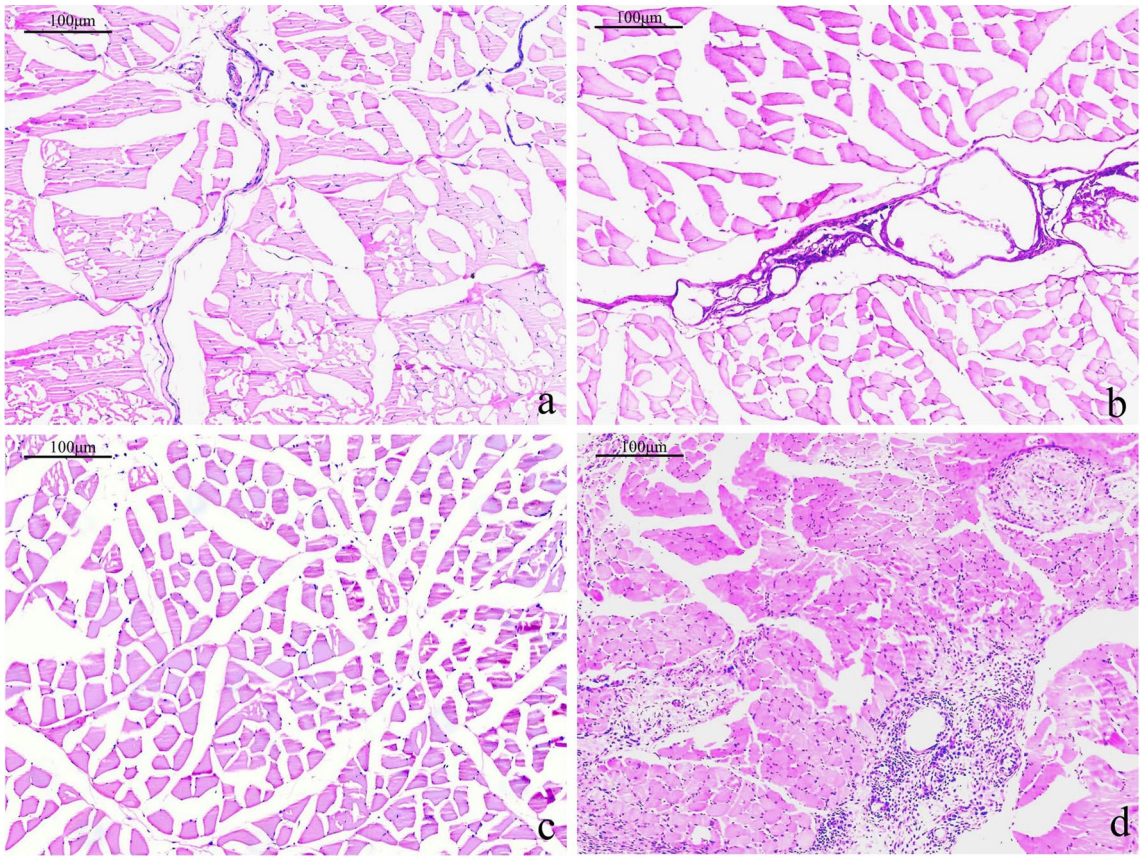

Fig. 6 Representative microscopic photographs of ischemic muscle tissue stained with hematoxylin and eosin at postoperative day 28. **a** PBS group; **b** PLGA group; **c** VEGF group; **d** VEGF–PLGA group

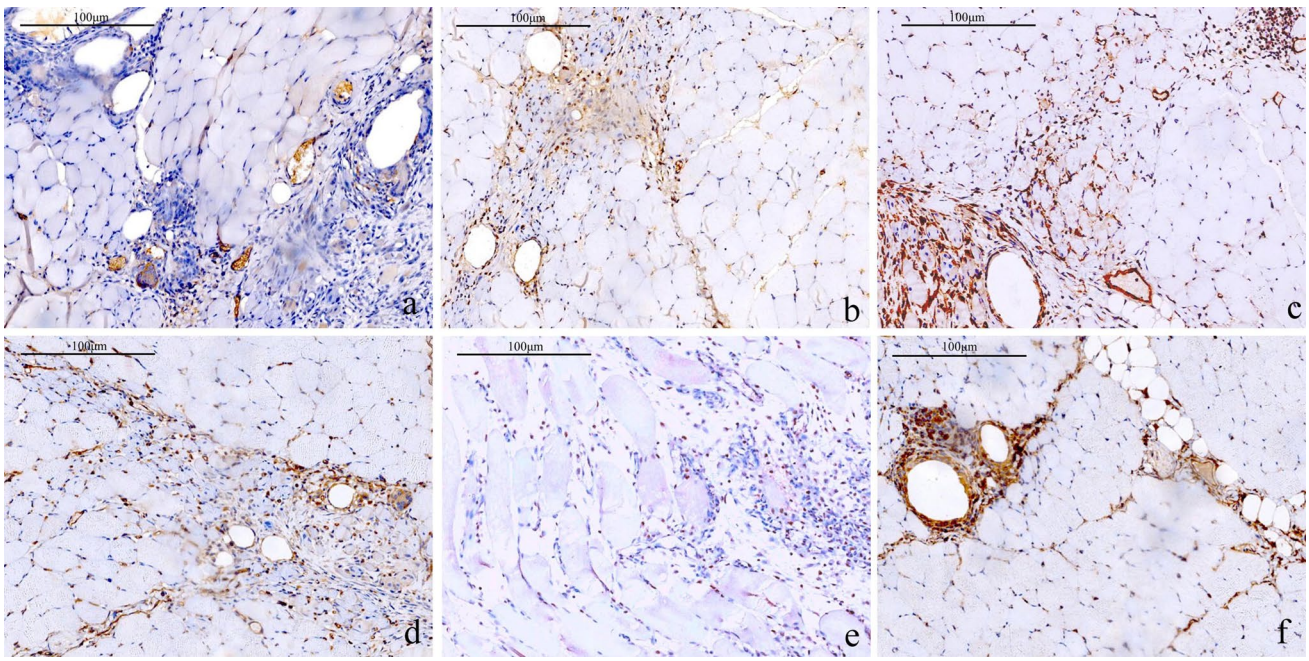

Fig. 7 Representative microscopic photographs of ischemic muscle tissue immunostained with antibodies against CD45 **a**, proliferating cell nuclear antigen **b**, smooth muscle α-actin **c**, CD34 **d**, c-kit **e** and vascular endothelial growth factor receptor 2 **f** in VEGF-PLGA group

were positive for pan-leukocyte CD45 marker. It seemed that cellular infiltration was not an inflammatory response. Some of these recruiting cells were positive for expression of PCNA, a marker associated with cellular proliferation. Some cells were positive for smooth muscle cell-specific SMA marker. Some cells were also positive for CD34, C-kit and VEGFR-2.

Discussion

As a potent mitogen, VEGF induces angiogenesis by stimulating ECs proliferation, migration, and new vessel formation. It represents the major molecular target for the therapeutic angiogenesis [4–8, 29–31]. But bolus injection of therapeutic angiogenic factors does not provide efficient blood vessel formation due to the short half-lives of these factors in vivo. Polymeric systems including hydrogels,PEG, dextran, PLGA and chitosan have been extensively utilized as localized drug delivery systems. These systems have the potential in protecting proteins or peptide drugs from enzymatic degradation, and can allow drugs' activity in the body to be maintained for relatively long time periods [9–14].

PLGA-based drug delivery system is approved by the Food and Drug Administration, and has been used extensively in drug delivery because of its inherent advantages of biodegradability and biocompatibility. But unlike small molecular or peptide drugs, proteins possess tertiary structures which are easily denatured or aggregated during the microencapsulation processes involving water–oil or water–air interfacial tensions [15, 16]. Denatured or aggregated proteins may not only lose therapeutic activity, but also evoke immunogenicity or toxicity [17–19]. To overcome these limitations, a new protein delivery system based on the combination of protein-loaded dextran microparticles and PLGA microspheres was found [20, 21].

In this study, based on this sustained-release protein delivery system, PLGA microspheres containing VEGF-loaded dextran microparticles using S/O/W method were produced. It was showed that these sustained-release microspheres had a relatively high encapsulating efficiency and achieved releasing VEGF in a sustained manner for more than 1 month with a low level of initial burst release and a zero-order release profile. Theoretically, these microspheres could allow tissues at the injection region to be exposed to VEGF at a constant concentration for a long period. And in practice, it was found that the VEGF released from VEGF–dextran–PLGA microspheres obviously enhanced the proliferation of HUVECs in vitro and potentiated the angiogenic efficacy of VEGF administration in a rat limb ischemic model.

The retained bioactivity of released VEGF suggested that VEGF in microspheres had been well protected during all the preparation processes. This is probably because protein-loaded dextran microparticles might avoid creation of oil–water and water–gas interfaces which may result in the loss of factors bioactivity, and a low-temperature aqueous–aqueous-phase separation method was used to load protein into dextran microparticles, wherein water-soluble proteins were partitioned in the polysaccharide dispersed phase preferentially and solidified by lyophilization. Once pre-loaded in the microparticles, the protein conformation is immobilized in the hydrophilic matrix of the polysaccharide, and can, therefore, be safely microencapsulated in PLGA microspheres through a so-called "S/O/W" process [21, 25, 32].

In this study, it was found that the number of capillaries per muscle fiber in samples treated with VEGF–dextran–PLGA microspheres was two times of that in control group and PLGA group. And the number of SMA-positive vessels per muscle fiber in samples treated with VEGF–dextran–PLGA microspheres was also five times of that in any other groups. The injection of VEGF–dextran–PLGA microsphere-loaded fibrin gel into ischemic limbs of rats induced more extensive neovascularization than either fibrin gel with or without VEGF.

Besides being a potent mitogen for endothelial cells, VEGF also has a direct effect on smooth muscle cells to promote vessel maturation [33–35]. Experiments performed by implantation of myoblasts constitutively expressing VEGF also revealed the formation of smooth muscle-coated vessels similar to arterioles directly adjacent to the implantation site [36]. Finally, analogous findings had also been reported by studying the effect of VEGF overexpression in a transgenic mouse model [34]. Premature cessation of the VEGF stimulus could lead to regression of most acquired vessels. VEGF expression shorter than about 4 weeks was insufficient to stabilize normal newly induced vessels, leading to their regression after stimulus cessation [34]. The persistence of neovasculature was gained after 28 days but not 14 days of continuous VEGF expression in mice hearts [35]. All these findings explained that the released VEGF from VEGF–dextran–PLGA microspheres sustained more than 1 month in the injection site, which could promote the formation of mature vessels.

In VEGF–dextran–PLGA group, muscles showed remarkably increased cellularity compared to any other groups. By immunohistochemistry, some of the infiltrating cells were positive for expression of PCNA, SMA, CD34, C-Kit, and VEGFR-2, but negative for expression of pan-leukocyte CD45 marker. These antigens start to be expressed by pluripotent bone marrow-derived stem/progenitor cell population comprising the early hematopoietic/endothelial cell precursors (EPCs). Our results suggest that prolonged expression of VEGF might be one of the local triggers for stem/progenitor cell recruitment,

accumulation and proliferation which contributed for local neovascularization through postnatal vasculogenesis and arteriogenesis mechanisms.

VEGF could promote adult vasculogenesis by enhancing EPC recruitment from VEGF receptors flt1 (VEGFR1) or flk1 (VEGFR2) [37–40]. Blocking VEGFR2 signaling resulted in poor mobilization of EPCs [41]. VEGF164 gene transfer augmented proliferative activity, adhesion, and incorporation of EPCs to EC monolayers in vitro and in vivo engraftment to ischemic limbs [42].

VEGF is sufficient for organ homing of circulating mononuclear cells which served as arteriogenic cells distinct from EPCs [39, 43]. This perivascular positioning of incoming monocytes can exert a paracrine accessory role [5–8, 39, 43]. Extravasated monocytes were shown to participate in the process of arteriogenesis, remodeling of existing small vessels into larger vessels via promotion of in-wall proliferation of endothelial cells, in the context of the compensatory response to vessel occlusion [39]. They could secret a number of cytokines and growth factors including fibroblast growth factors, transforming growth factor β, platelet-derived growth factor, as well as numerous matrix-degrading enzymes to promote arteriogenesis [5, 8, 39, 43].

Altogether these observations suggested that bone marrow and blood-derived mononuclear cells and stem/progenitor cells might sense the long presence of VEGF and migrate to the local tissue, where they promoted neovascularization by mechanisms of postnatal vasculogenesis and arteriogenesis.

In conclusion, we produced VEGF-loaded dextran microparticles and encapsulated them into microspheres to prepare VEGF–dextran–PLGA microspheres. VEGF released from PLGA microspheres was in a sustained manner for more than 1 month in vitro with low level of initial burst release, obviously enhanced the proliferation of HUEVCs in vitro, and significantly promoted the capillaries and SMA-positive vessels formation in vivo. The retained bioactivity of VEGF released from VEGF–dextran–PLGA microspheres potentiated the angiogenic efficacy of VEGF. This sustained-release system may be a promising vehicle for delivery of multiple angiogenic factors for therapeutic neovascularization.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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