Sequential delivery of TAT-HSP27 and VEGF using microsphere/hydrogel hybrid systems for therapeutic angiogenesis

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Abstract

Ischemic disease is associated with high mortality and morbidity rates, and therapeutic angiogenesis via systemic or local delivery of protein drugs is one potential approach to treat the disease. In this study, we hypothesized that combined delivery of TAT-HSP27 (HSP27 fused with transcriptional activator) and VEGF could enhance the therapeutic efficacy in an ischemic mouse model, and that sequential release could be critical in therapeutic angiogenesis. Alginate hydrogels containing TAT-HSP27 as an anti-apoptotic agent were prepared, and porous PLGA microspheres loaded with VEGF as an angiogenic agent were incorporated into the hydrogels to prepare microsphere/hydrogel hybrid delivery systems. Sequential in vitro release of TAT-HSP27 and VEGF was achieved by the hybrid systems. TAT-HSP27 was depleted from alginate gels in 7 days, while VEGF was continually released for 28 days. The release rate of VEGF was attenuated by varying the porous structures of PLGA microspheres. Sequential delivery of TAT-HSP27 and VEGF was critical to protect against muscle degeneration and fibrosis, as well as to promote new blood vessel formation in the ischemic site of a mouse model. This approach to controlling the sequential release behaviors of multiple drugs could be useful in the design of novel drug delivery systems for therapeutic angiogenesis.

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1. Introduction

Ischemic vascular disease, including myocardial infarction, cardiovascular disease, and peripheral vascular disease, is associated with high risk of death or loss of limb [1–3]. It is caused by an insufficient supply of oxygen and nutrients to the tissues, leading to cellular apoptosis and tissue degeneration. Current clinical trials to treat ischemic vascular disease mainly focus on surgical treatments [4], but there are several limitations to these approaches such as reoperations caused by the high frequency of restenosis [5,6]. Site-specific or systemic drug delivery for therapeutic angiogenesis is thus a promising approach, rather than invasive surgery, allowing one to deliver angiogenic factors including genes [7] and recombinant proteins [8], or a combination of these molecules [9] to the ischemic tissues, which helps the tissues in recovering.

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels and requires the action of various growth factors and cell-adhesion molecules in endothelial cells [10,11]. Vascular endothelial growth factor (VEGF) is known as a potent angiogenic factor. Its important role in angiogenesis and vasculogenesis [12–14] has been extensively exploited for therapeutic angiogenesis for several decades. VEGF stimulates endothelial cell proliferation and migration [15], which promotes angiogenesis in subjects with vascular insufficiency [16]. Recently, it was reported that VEGF-induced therapeutic angiogenesis was successful in treatment of patients with ischemic limb and cardiac disease [17–19].

Necrosis and apoptosis are associated with ischemia, and apoptosis represents an early response to ischemia [20]. For example, apoptosis appeared after 10 min of ischemia and reached a maximum within 30 min under ischemic condition in isolated rat hearts [21]. Thus, prompt and rapid treatments to reduce cellular apoptosis are critical to avoid severe tissue damage in ischemic regions. Heat shock proteins (HSPs) are molecular chaperones that can protect cells from various stresses [22] and are named according to their molecular weights. HSP27 has a molecular weight of 27 kDa and the cytoprotective effect of HSP27 in cardiac cells against hypoxia has been demonstrated [23]. Recently, transcriptional activator (TAT) derived from the human immunodeficiency virus has been introduced as a protein transduction domain (PTD) to HSP27 that has inherently low cell membrane permeability, and TAT-HSP27 showed a significant protective effect against ischemic injury in vitro and in vivo [24].

Despite the potential usefulness of various proteins in therapeutic applications, the short half-lives of these therapeutics within the body remain a challenge to overcome before their successful clinical application [25]. Polymeric matrices can be an excellent depot for proteins, protecting them from proteolysis and prolonging their activity within...
the body [26]. Various polymeric carriers have been widely investigated in the form of microspheres, films, and hydrogels for delivery of therapeutic proteins [27–29]. Hydrogels are a polymer network saturated with water molecules which can vary in physical properties through chemical and molecular modifications [26,30]. Hydrogels have already found many useful applications in delivery of various proteins and genes, as well as in many tissue engineering applications [31].

Alginate is a naturally occurring polymer obtained from brown algae and is now known to be a linear copolymer containing blocks of (1,4-linked β-α-mannuronate (M) and α-1-guluronate (G) residues. The blocks are composed of consecutive G residues, consecutive M residues, and alternating M and G residues. Their composition and block length are dependent on the natural source [32]. Although alginate has excellent biocompatibility and low toxicity, there is still debate regarding the biocompatibility of alginate which relates to varying levels of impurities such as heavy metals, endotoxins, proteins, and polyphenolic compounds in the alginate [33]. Alginate purified by a multi-step extraction procedure to obtain a very high purity did not induce any significant foreign body reaction when implanted into animals [34]. Simple gelation with divalent cations enables wide uses of alginate in many drug delivery and tissue engineering applications [33]. However, alginate hydrogels rapidly release hydrophilic molecules at the initial stage. For example, VEGF and bFGF are completely released from alginate gels within 7 days of incubation in vitro [35]. Combination with hydrophobic carriers can overcome this limitation. We previously reported a microsphere/hydrogel hybrid system, composed of poly(ω-lactic-co-glycolic acid) (PLGA) microspheres and alginate hydrogels, for the controlled and sustained release of various proteins [36]. The release of proteins can be regulated by the mixing ratio between microspheres and hydrogels but not by the size of the microspheres [37]. In addition, the release rate of proteins from PLGA microspheres is strongly dependent on the porous structures of the microspheres embedded in hydrogels [38].

In this study, we hypothesized that the sequential and combined delivery of TAT-HSP27 and VEGF using a microsphere/hydrogel hybrid delivery vehicle could substantially improve the outcome of an ischemic rodent disease model. We designed and prepared a hybrid system capable of rapidly releasing TAT-HSP27 within a few days in order to prevent early cellular apoptosis in the ischemic site but release VEGF in a sustained manner to stimulate new blood vessel formation. Alginate hydrogels containing TAT-HSP27 were prepared, and PLGA microspheres loaded with VEGF were incorporated into the hydrogels. The release rate of VEGF from PLGA microspheres was controlled by varying the porous structures of the microspheres using bovine serum albumin (BSA) as an osmotic agent. The therapeutic efficacies of hybrid delivery systems were evaluated using an ischemic mouse hind limb model.

2. Materials and methods

2.1. Materials

Sodium alginate (MW 200,000–300,000) and PLGA (RESOMER® RG 502H, MW 10,000, 0.16–0.24 dl/g) were purchased from FMC Biopolymers (Drummen, Norway) and Boehringer Ingelheim (Ingelheim, Germany), respectively. Bovine serum albumin (BSA) was purchased from Amresco® (Solon, OH, USA). Methylene chloride was purchased from JT Baker (Phillipsburg, NJ, USA). Poly(vinyl alcohol) (PVA, MW 30,000–70,000), calcium sulfate (CaSO4), and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Duksan Pure Chemical Co. (Ansan, Korea). Dulbecco’s phosphate buffered saline (DPBS) was purchased from Gibco (Grand Island, NY, USA). The 6-His tagged bacterial expression vector pRESET-A was purchased from Invitrogen (Carlsbad, CA, USA). Anti-human HSP27 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human vascular endothelial growth factor (rhVEGF) was purchased from PeproTech Asia (Rehovot, Israel). Immunodeficient mice (BALB/c nude mice, female, 4 weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea).

2.2. Expression and purification of TAT-HSP27

TAT-HSP27 was expressed and purified as previously described [39]. Briefly, the TAT-HSP27 fusion construct was generated by cloning TAT oligonucleotide (TAT GGC AGG AAG AAG CAG CAG CCA CCA) and the coding sequence of HSP27, which was PCR amplified from a full length human HSP27 cDNA using the oligo primers 5′-CAC GAG CCG CAG CAG GAC (sense) and 5′-CAG TGG CCG CAG CAG GGG TGG (anti-sense), in frame with the 6-His tag in the bacterial expression vector pRESET-A. The TAT-HSP27 fusion protein expressed from E. coli was purified by immobilized metal ion (Ni) affinity chromatography (IMAC), dialyzed, and then concentrated. The identity of the purified fusion protein was confirmed by Western blotting with anti-human HSP27 antibody.

2.3. Preparation and characteristics of porous PLGA microspheres containing VEGF

Porous microspheres containing VEGF were prepared by a modified water-in-oil-in-water (w/o/w) double emulsion method using an osmotic phenomenon [38]. PLGA (0.1 g) was dissolved in methylene chloride (2 ml), and VEGF (0.02 mg) was dissolved in deionized water (4 ml) containing BSA as an osmotic agent [25]. Both solutions were emulsified by a probe type sonicator (Branson Digital Sonifier®; Danbury, CT, USA) for 10 s in an ice bath. The single emulsion (w/o) was poured into 4% aqueous PVA solution (30 ml) and emulsified again to form a double emulsion (w/o/w) using a homogenizer (Ultra-Turrax® T25 basic, IKA®-Werke; Staufen, Germany) for 5 min at 6000 rpm. The resultant double emulsion was poured into a 0.4% aqueous PVA solution (300 ml) and stirred at 800 rpm for 3 h to evaporate the solvent. The microspheres were washed five times with distilled water, collected, and lyophilized (−55 °C, 0.02 mbar). Porous structures were controlled by varying the BSA concentration [BSA/PLGA = 0.4 and 0.6, w/v], and non-porous microspheres were also prepared without using BSA. The morphologies of microspheres were observed by scanning electron microscopy (S-4800 UHR FE-SEM, Hitachi; Hitachinaka, Japan). To determine the VEGF content, microspheres were dissolved in a 1 N NaOH solution and neutralized with a 1 N HCl solution. The solution was filtered through a 0.2 μm filter (Millipore; Billerica, MA, USA) and analyzed using a VEGF ELISA assay kit (R&D; Minneapolis, MN, USA). The loading efficiency was determined from the ratio of the actual protein content in microspheres to the amount initially added during microsphere preparation.

2.4. Preparation of hybrid systems containing both TAT-HSP27 and VEGF

A hybrid delivery system was prepared by ionic cross-linking of alginate solution in which PLGA microspheres were suspended, using calcium sulfate, as previously described [26]. Briefly, VEGF-loaded microspheres were suspended in 2% (w/v) alginate solution dissolved with TAT-HSP27 at a mixing ratio of 2.0 (PLGA/alginate, w/w). The mixed solutions were cross-linked by calcium sulfate slurry (21%, w/v).

2.5. In vitro protein release

Hybrid systems were cut into disks (15 mm diameter and 2 mm thick), placed in 12-well tissue culture plates, and incubated at 37 °C under 5% CO2 atmosphere. The supernatant was retrieved and replaced with fresh DPBS at predetermined time intervals. The amounts of TAT-HSP27 and VEGF were determined using ELISA assay kits (Biosource, Camarillo, CA, USA; R&D, Minneapolis, MN, USA).
2.6. Ischemic hind limb model and treatment

Four-week-old nude mice were anesthetized by an intramuscular injection of ketamine (20 mg/kg) and rompun (5 mg/kg). The point above the external iliac artery and the distal point where it bifurcates into saphenous and popliteal arteries were ligated using 6-0 silk suture (Allee, Busan, Korea), followed by excision of the femoral artery between them. One day after arterial dissection, the mice were randomly assigned to each experimental group (n = 10 for each group). All of the procedures were in compliance with Hangyang University Guidelines for the care and use of laboratory animals. Arterial-dissected mice procedures were in compliance with Hangyang University Guidelines (Ailee, Busan, Korea), followed by excision of the femoral artery above the external iliac artery and the distal point where it bifurcates in the resultant microspheres [38]. Both nonporous and porous microspheres were spherical with a narrow size distribution. Porous structures were efficiently generated by the osmotic induction method, and there was no significant increase in the particle size. The pore sizes of porous microspheres increased in parallel with the BSA content, as previously reported (Table 1) [38]. The loading efficiency of VEGF in microspheres was greater than 70% for all encapsulation processes.

3.2. In vitro sequential release of TAT-HSP27 and VEGF from the hybrid system

Hybrid systems, composed of TAT-HSP27-loaded alginate hydrogels and VEGF-incorporated PLGA microspheres with different pore sizes, were prepared, and the release behaviors of proteins were monitored in vitro for 4 weeks (Fig. 2). A strong initial burst of TAT-HSP27 was observed, and release was complete within 7 days. However, VEGF release was sustained for 4 weeks. The release rates of VEGF were 2, 4, and 6 ng/day, for BSA contents of 0, 0.4 and 0.6 (BSA/PLGA, w/w), respectively. This finding clearly indicates that the releases of TAT-HSP27 and VEGF from hybrid delivery systems can be controlled in a sequential manner. In addition, the release rate of VEGF from microspheres was dependent on the pore sizes of the microspheres, and the release behavior of TAT-HSP27 was not affected by VEGF loading in microspheres.

3.3. Representative photographs and histological analysis

We next investigated whether sequential delivery of TAT-HSP27 and VEGF could improve therapeutic angiogenesis in vivo. Hybrid delivery systems were injected into the ischemic region of mice, and tissue sections were retrieved 3 days or 28 days post-surgery. The tissue samples were embedded in an optical cutting temperature (O.C.T. compound, Tissue-Tek® 4583, Sakura Finetek; Northbrook, Illinois, USA) in aluminum foil, frozen at −70 °C, and then cut into 10-μm-thick sections at −20 °C. Hematoxylin and eosin (H & E) and Masson’s trichrome staining were performed to examine muscle degeneration and tissue fibrosis in the ischemic regions, respectively. In addition, apoptosis in the ischemic region after treatment was identified using an Apoptosis Detection Kit (ApopTag® Red In Situ, Millipore; Billerica, MA, USA), according to the manufacturer’s instructions. Arterioles and capillaries were immunofluorescently stained with anti-smooth muscle (SMA) α-actin (Abcam; Cambridge, MA, USA) and anti-von Willebrand Factor (vWF; Abcam; Cambridge, MA, USA). To identify newly-formed microvessels, the tissue sections were subjected to staining with anti-BrdU antigen (Sigma; St. Louis, MO, USA). The signals for BrdU and other molecules (SMA α-actin and vWF) were visualized with rhodamine- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA, USA). All the tissue sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and then observed by fluorescence microscopy (TE2000-E, Nikon; Melville, NY, USA).

All data are presented as the mean ± standard deviation. Statistical analysis was performed with Student’s t-test. Values of *P < 0.05 and **P < 0.01 were considered statistically significant.

3. Results

3.1. Preparation of VEGF-loaded PLGA microspheres

We prepared VEGF-loaded PLGA microspheres with or without porous structures by a double emulsion method and observed their morphologies (Fig. 1). BSA induces osmotic gradient between internal and external water phases during the emulsion process, which allows water molecules in the external water phase to penetrate into the internal water phase, leading to the formation of interconnected, open porous structures in the resultant microspheres [38]. Both nonporous and porous microspheres were spherical with a narrow size distribution. Porous structures were efficiently generated by the osmotic induction method, and there was no significant increase in the particle size. The pore sizes of porous microspheres increased in parallel with the BSA content, as previously reported (Table 1) [38]. The loading efficiency of VEGF in microspheres was greater than 70% for all encapsulation processes.

### Table 1

<table>
<thead>
<tr>
<th>BSA/PLGA (w/w)</th>
<th>Particle size (μm)</th>
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<td>0</td>
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<td>0.6</td>
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of their limbs were removed after 4 weeks (Fig. 3a). Rapid progression of limb necrosis was also observed when mice were treated with hybrid systems loaded with VEGF-containing PLGA microspheres only (no TAT-HSP27) (Fig. 3c). In contrast, mice treated with hybrid systems containing TAT-HSP27 only (no VEGF) showed a high rate of limb salvage only 1 week after treatment, but severe necrosis was observed after 4 weeks (Fig. 3b). Interestingly, mice treated with hybrid systems delivering both TAT-HSP27 and VEGF showed an enhanced rate of limb salvage and reduced rate of limb loss after 4 weeks (Fig. 3d and e), indicating that sequential delivery of both proteins is critical for therapeutic angiogenesis at the ischemic sites (Table 2).

Histological analysis of tissue sections retrieved from mice also supports these findings. Tissue sections were stained with H & E and Masson’s trichrome at days 3 and 28 post-treatment. Tissue sections stained with H & E showed massive muscle degeneration and infiltration of numerous granulocytes and neutrophils in the control group (Fig. 4a) and in the group treated with VEGF only (Fig. 4c). Masson’s trichrome staining indicates that the lack of VEGF in the early stage of ischemia causes severe muscle degeneration in the ischemic region (Fig. 4c). The initial burst release of TAT-HSP27 from the hybrid systems was effective in preventing muscle degeneration in the mouse model only for a few days, and massive muscle degeneration was observed after 28 days (Fig. 4b). Interestingly, prominent muscle protection from ischemic damage occurred in groups treated with hybrid systems delivering both TAT-HSP27 and VEGF in a sequential manner (Fig. 4d and e). In addition, muscle fibrosis in mice treated with hybrid systems was also significantly attenuated compared with that in other experimental groups (Fig. 4d and e).

3.4. Tunel assay

We next investigated cellular apoptosis in the ischemic region by labeling fragmented DNA using a Tunel assay. At day 3 post-treatment, a few apoptotic cells were detected in groups treated with TAT-HSP27 only (Fig. 5b) and hybrid systems (Fig. 5d and e), while numerous apoptotic cells were observed in ischemic mice treated with PBS only (Fig. 5a) and VEGF only (Fig. 5c). Cellular apoptosis was also suppressed after 28 days of treatment with the hybrid system containing both TAT-HSP27 and VEGF. This finding was consistent with data obtained from histological analysis.

3.5. Immunohistochemical analysis

Next we examined whether the sequential delivery of TAT-HSP27 and VEGF using a hybrid system effectively enhanced neovascularization in the mouse ischemia model. We performed immunofluorescent staining for smooth muscle alpha and von Willebrand factor to quantify the arteriole and capillary density, respectively (Figs. 6 and 7). In addition, BrdU-positive microvessels were considered to be newly-formed. The density of arterioles in ischemic mice treated with TAT-HSP27 and VEGF using a hybrid system containing porous microspheres was significantly increased (27.5 ± 6.5/mm²) compared with that of mice treated with the hybrid system containing nonporous microspheres (Fig. 8a). Moreover, quantification of capillary density revealed that the hybrid system composed of porous microspheres also greatly enhanced capillary formation (119.6 ± 42.5/mm²) (Fig. 8b). The number of BrdU-positive microvessels was significantly increased in groups treated with hybrid systems containing porous microspheres, compared with nonporous microspheres.

4. Discussion

Systemic and local delivery of therapeutic proteins including various angiogenic molecules has been useful for therapeutic angiogenesis [40]. To date, angiogenic factors such as bFGF and VEGF have been widely exploited to treat ischemic vascular disease, and these factors can be administered systemically or locally. However, a single angiogenic molecule may not be adequate for sufficient blood vessel formation since the mechanisms of angiogenesis are very diverse and complicated.
Recently, a synergistic effect of multiple angiogenic molecules on vascular regeneration has been reported. VEGF was used in combination with various growth factors capable of enhancing its permeability effect [41,42]. PDGFs and Ang-1, which function in vessel maturation and stabilization, have also been utilized to regenerate mature blood vessels [43,44]. However, studies of combined delivery of anti-apoptotic and angiogenic factors are lacking, despite the importance of early apoptosis at ischemic sites. A lack of oxygen and nutrients caused by disordered blood circulation leads to rapid and serious tissue damage. Up-regulation of angiogenic molecules and migration of circulating cellular components are nature’s responses to ischemic tissues, and the supply of angiogenic molecules may not be sufficient to protect tissues from ischemic stress until sufficient blood circulation is achieved, especially in the early stages of ischemic tissues.

We thus hypothesized that combined delivery with sequential release of anti-apoptotic and angiogenic molecules could be effective for angiogenesis in ischemic sites. We previously reported that TAT-HSP27 can significantly preserve cell viability under hypoxic stress in vitro and even in mice with myocardial infarction [45]. Here we developed a microsphere/hydrogel hybrid delivery system for sequential delivery of both TAT-HSP27 and VEGF. Hydrogels are excellent candidates for delivery of therapeutic proteins by injection. However, the release of hydrophilic drugs such as proteins from hydrogels is generally fast. Introduction of polymeric microspheres containing therapeutic proteins into hydrogels can slow the release rate of the proteins and allow control over the release pattern in a sustained manner for relatively long time periods [36,37]. We prepared hybrid delivery systems composed of alginate hydrogels loaded with TAT-HSP27 and VEGF-incorporated PLGA microspheres, which were still injectable using a syringe in the range of mixing ratios between alginate gels and PLGA microspheres tested in this study. PLGA microspheres with porous structures were also used to control the release rate of VEGF from hybrid delivery systems. PLGA microspheres with various porous structures were prepared using BSA as an osmotic agent (Table 1, Fig. 1), and the porous structure significantly influenced the release of VEGF from the delivery systems (Fig. 2). More than 85% of loaded VEGF was efficiently released from the delivery system over 4 weeks when porous PLGA microspheres were used compared with the use of nonporous microspheres. No interference in the release patterns of either protein was observed, and, interestingly, the sequential release of TAT-HSP27 and VEGF was achieved. We also tested the viability of cells after treating with either intact or released proteins in order to investigate whether released TAT-HSP27 and VEGF remained bioactive [24,37]. Both the released proteins showed excellent bioactivity compared to intact ones (Fig. S1).

We demonstrated that the sequential release of both proteins from hybrid systems was effective in new blood vessel formation at the ischemic site in a mouse model. Histological analysis and a TUNEL assay revealed that ischemic mice treated with hybrid systems containing TAT-HSP27 were protected from muscle degeneration at the early stage (Figs. 4, 5). However, this protection effect lasted for short time periods, leading to fibrosis and necrosis in the absence of VEGF (Figs. 4b, 5b). Mice treated with hybrid systems releasing VEGF only were largely...
unprotected from ischemic stress, likely due to initial burst release. Immunochemical staining indicated that blood vessel formation was enhanced by delivery of both TAT-HSP27 and VEGF in a sequential manner (Figs. 6, 7). In particular, the number of blood vessels significantly increased in mice treated with hybrid systems containing VEGF-loaded porous microspheres compared to hybrid systems containing VEGF-loaded, nonporous microspheres (Fig. 8). We also found that the number of BrdU-positive blood vessels significantly increased when mice were treated with hybrid systems containing porous microspheres compared with other experimental groups, indicating that neovascularization could be rapidly achieved through the sequential release of TAT-HSP27 and VEGF.

Simultaneous delivery of TAT-HSP27 and VEGF using hybrid systems were also carried out and compared with sequential delivery of the proteins. We prepared hybrid systems releasing TAT-HSP27 and VEGF simultaneously either within a few weeks (Fig. S2) or within a few days (Fig. S3), and injected them into the ischemic hind limb mouse model. Simultaneous delivery of TAT-HSP27 and VEGF using hybrid systems did not significantly enhance the new blood vessel formation in the mouse model, compared with hybrid systems releasing the proteins in a sequential manner (Figs. S2(e) and S3(e)), while keeping the same concentrations of TAT-HSP27 and VEGF in the hybrid systems. Substantial muscle degeneration was also observed 28 days post-treatment (Figs. S2(b) and S3(b)), compared with results obtained from simultaneous delivery of TAT-HSP27 and VEGF (Fig. 4e). This finding clearly indicates the importance of timely delivery of therapeutic proteins (e.g., TAT-HSP27 and VEGF) in a sequential manner, rather than simultaneous delivery. Simply increasing the dose of delivered proteins

![Fig. 5. TUNEL assay of ischemic tissues treated with microsphere/hydrogel hybrid systems containing (a) PBS, (b) TAT-HSP27 only, (c) VEGF only, (d) TAT-HSP27 and VEGF (nonporous microspheres), and (e) TAT-HSP27 and VEGF (porous microspheres) (scale bar, 20 μm).](image)

![Fig. 6. Immunohistochemical staining for SM α-actin in ischemic tissues treated with microsphere/hydrogel hybrid systems containing (a) TAT-HSP27 only, (b) VEGF only, (c) TAT-HSP27 and VEGF (nonporous microspheres), and (d) TAT-HSP27 and VEGF (porous microspheres). Blue, green and red colors represent DAPI, SM α-actin, and BrdU, respectively. Arrows indicate newly formed arterioles (scale bar, 40 μm).](image)
may provide the enhanced angiogenic effect. However, a use of hybrid systems releasing therapeutic proteins sequentially in a timely manner may reduce the side effect that may occur with high doses of therapeutics. It is worth pointing out that an intact immune system plays an important role in angiogenesis. However, it would have undermined independent evaluation of the treatment protocol. Thus, the study was conducted in immunodeficient mice in the absence of immune parameters to enable clear-cut assessment of the therapeutic benefits of just the therapeutic components. The recovery of immunodeficient mice after the ischemic procedure upon treatment with the hybrid systems encapsulating TAT-HSP27 and VEGF is an indicator of possibly improved therapeutic efficacies in the presence of an intact immune system.

Delivery of multiple therapeutic agents in an orchestrated manner is advantageous compared with single delivery in therapeutic angiogenesis including cardiovascular regeneration [46,47] since the mechanism of angiogenesis is complicated and not yet clearly understood. In addition to the use of multiple proteins in promoting therapeutic angiogenesis as demonstrated in this study, various combinations of genes and cells can be also loaded into hybrid delivery systems for many therapeutic purposes. Our approach of designing and tailoring delivery systems to release therapeutic molecules in a sustained and sequential manner is expected to have many applications in biomedical science and engineering, including therapeutic angiogenesis.

5. Conclusions

We demonstrated that TAT-HSP27 and VEGF could be sequentially released by hybrid delivery systems composed of PLGA microspheres and alginate hydrogels. The release of TAT-HSP27 from alginate hydrogels was completed within 7 days, aimed at reducing hypoxic stress at the ischemic site. In addition, the release pattern of VEGF from PLGA microspheres could be manipulated for 28 days using porous microspheres with various pore sizes, to enhance new blood vessel formation in the ischemic tissues. Hybrid delivery systems for the sequential release of
TAT-HSP27 and VEGF protected against muscle degeneration and fibrosis, and promoted new blood vessel formation in the ischemic site. This approach of controlling the release patterns of multiple drugs in a sequential manner could be useful for the design of novel drug delivery systems in many biomedical applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.12.020.

References
