Controlled Release of L-Arginine from Poly(lactide-co-glycolide) Nanoparticles for the Inhibition of Vascular Smooth Muscle Cell Proliferation

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Several local drug delivery systems are currently under investigation for the suppression of vascular smooth muscle cell (VSMC) proliferation, which is thought to be the cornerstone of restenosis. Nanoparticulate sustained release carrier system is small enough in size to be delivered through the currently available balloon delivery catheters, and is able to continuously release the agent over a prolonged period of time. The administration of L-arginine, the precursor of nitric oxide, has been shown to suppress the proliferation of VSMCs. With the purpose of inhibition of VSMC proliferation, biodegradable poly(lactide-co-glycolide) (PLGA) nanoparticles were prepared. L-arginine loaded PLGA nanoparticles were formulated as a sustained release system for the prevention of restenosis by an antiproliferative effect on vascular smooth muscle cells. The size of the nanospheres was about 250 nm and the L-arginine loading was about 10-15% (w/w). L-arginine release from the nanospheres extended over 10 days. The antiproliferative effect of L-arginine released from the nanospheres was comparable to those observed with free L-arginine (1.0, 1.5, 3.0 mg/ml). The inhibitory effect of L-arginine on VSMC growth was reversible. L-arginine release was sustained. L-arginine released from PLGA nanospheres effectively reduced VSMC proliferation. Biodegradable PLGA nanospheres could be used to encapsulate L-arginine and may serve as a carrier for local drug delivery after percutaneous coronary intervention and in turn prevent restenosis.

Key words: Restenosis; L-arginine; Poly(lactide-co-glycolide) (PLGA) nanospheres; Local drug delivery

INTRODUCTION

A pharmacological approach to reduce restenosis has led to the systemic administration of many different drug classes during or after PCI (percutaneous coronary intervention). However no pharmacologic agent has reduced the rate of restenosis in humans after PCI. Also failure of systemic administration has been attributed partially to insufficient drug concentration at the site of angioplasty to suppress neointimal proliferation, which is thought to be the cornerstone of restenosis. Because restenosis is localized, systemic toxicity can be overcome by the direct intra-mural delivery of antirestenotic agents.

Local drug delivery rather than systemic administration can be a more effective way to obtain higher tissue drug levels at the site of balloon injury and at the same time decrease the potential adverse systemic drug associated side effects.

Currently several local drug delivery systems, including different balloon catheters, hydrogel-coated balloon catheters, polymeric or coated stents, and a number of other approaches are under investigation for the suppression of neointimal proliferation. However, the main disadvantages of these local systems are the very low tissue uptake of the infused agents and the lack of sustained delivery with rapid washout of the drugs by blood flow. To avoid these disadvantages, local drug delivery systems made of biocompatible and biodegradable polymeric particles or membranes have been studied.

Therapeutic microparticles containing various agents of interest have been configured as microspheres, nanospheres, microcapsules and liposomes. These dosage forms are currently of great interest because of their usefulness in both conventional and unconventional dosage forms. Microparticles and nanoparticles can be administered in a fluidized form with a liquid carrier. This permits their usefulness in intravascular infusion preparations or injectable emulsions for
both parenteral and enteric administration, as well as vaccine dosage forms for use in subcutaneous or intramuscular injection\textsuperscript{6,7}. Sustained release microparticles and nanoparticles can be formulated based upon the composition and material characteristics of the specific polymers and coingredients used\textsuperscript{8,9}. Nanoparticulate sustained release carrier system is small enough in size to be delivered through the currently available balloon delivery catheters, and is able to continuously release the agent over a prolonged period of time\textsuperscript{9}.

Nitric oxide (NO) is known to suppress the proliferation of vascular smooth muscle cells\textsuperscript{5,6,10}. In experimental animal models, administration of NO donors or augmentation of endogenous NO synthesis (as with administration of the NO precursor, L-arginine) inhibits restenosis after balloon angioplasty\textsuperscript{6,7}. L-arginine serves as the substrate for nitric oxide synthase (NOS). NOS is an enzyme to produce citrulline and NO from L-arginine. Administration of L-arginine, the precursor of NO, has been shown to suppress the formation of vascular neointima and improve endothelial function in a noninvasive rat model of balloon injury and normocolesterolemic rabbit models of restenosis\textsuperscript{6,7,10,11}.

This study was undertaken to evaluate the inhibitory effect on the proliferation of vascular smooth muscle cell of L-arginine using nanoparticles formulated with a biodegradable poly(lactic/glycolic acid) (PLGA) copolymer as an sustained-release system for the prevention of restenosis.

**MATERIALS AND METHODS**

**Materials**

L-arginine hydrochloride (Sigma Ultra, >99% TLC) was obtained from Sigma Chemical Co., USA. The biodegradable polymer used was poly(D,L-lactide-co-glycolide) (PLGA) 50:50 (Resomer RG504H, Boehringer Ingelheim, Ingelheim, Germany). All other chemicals used were of HPLC grade (Fisher). All cell culture materials (fetal bovine serum, penicillin-streptomycin solution, DMEM F12 powder, and trypsin-EDTA solution) were from Gibco BRL (Basel, Switzerland). MTT test kit was from TAKARA.

**Nanosphere preparation**

L-arginine loaded nanospheres were prepared by a double emulsion (W/O/W) technique, according to Blanco et al\textsuperscript{10}. Briefly, 0.2 ml of an aqueous solution of L-arginine was emulsified in a 4 ml solution of PLGA in methylene chloride (25 mg/ml) by sonication for 30 s in an ice bath. Then, 8 ml of a 3% (w/v) human serum albumin (HSA) solution were added to this primary emulsion and sonicated for 30 s under the above conditions to obtain the double emulsion. The double emulsion was then diluted into 160 ml of a 0.1% HSA solution and the solvent was rapidly eliminated by extraction with 160 ml of an aqueous isopropyl alcohol solution (8% v/v) under magnetic stirring. Finally, the nanospheres were isolated by centrifugation at 20,000 g for 20 min at 10°C, washed three times with water and lyophilized.

**Physicochemical properties of L-arginine loaded PLGA nanospheres**

The morphological examination of the nanospheres was performed using transmission electron microscopy (TEM). The samples were placed on copper grids with Formvar films and stained with 2% (w/v) phosphotungstic acid for TEM viewing.

Particle size of the nanospheres was determined by photon correlation spectroscopy (PCS) (Malvern Instruments). Each determination was performed on three samples from different batches.

**Determination of L-arginine content in nanospheres**

The amount of L-arginine entrapped within PLGA nanospheres was determined by an extraction technique. Two mg of the nanospheres was dissolved in 2 ml chloroform and the L-arginine was extracted into 1 ml phosphate buffered saline (PBS) by vortexing. Then, the aqueous phase containing the L-arginine was separated from the organic phase containing PLGA by centrifugation at 5000 rpm for 15 min. Afterwards, the L-arginine was assayed by high performance liquid chromatography (HPLC) according to the Zorbax Eclipse AAA\textsuperscript{TM} Method for amino acid analysis. The HPLC conditions were: column: ZORBAX Eclipse XDB-C18 column; temperature: 40°C; mobile phase: 40 mM NaHPO\textsubscript{4} pH 7.8, acetonitrile: methanol: water (45:45:10 v/v); flow rate: 2 ml/min; wavelength: 338 nm. Encapsulation efficiency represents the percentage of L-arginine encapsulated with respect to the total amount of L-arginine used in the encapsulation process. L-arginine loading represents the percentage of mass of L-arginine in nanospheres with respect to the mass of nanospheres recovered.

**In vitro release test**

In vitro drug release from the nanospheres was performed in PBS (pH 7.4) at 37°C utilizing double-chamber diffusion cells in a shaking incubator (60 rpm)\textsuperscript{12}. A Millipore hydrophilic low-protein-binding poly(vinylidene fluoride) membrane (VWLP) (Millipore Co., Bedford, MA) with 0.1 pore size was placed between the two chambers. The donor chamber was filled with 7 ml nanosphere-buffer suspension (4 mg/ml) and the receiver chamber filled with 7 ml plain buffer. At appropriate intervals, the samples (7 ml) were collected from the receiver chamber fluid and the receiver chamber was replaced with fresh buffer. L-arginine concentration in the release medium was determined by HPLC.

**In vitro biodegradation test**
In vitro biodegradation test of the nanospheres was performed in PBS (pH 7.4) at 37°C in a shaking incubator (60 rpm). Molecular weight distributions of PLGA before and after various incubation periods were determined by gel permeation chromatography (GPC) (Model 5/5 pump, 410 Differential Refractometer). The conditions were: column; three PCLel columns (CHR2, HR4 and HR5E in series); column temperature: 40°C; mobile phase: tetrahydrofuran; flow rate: 1.0 ml/min; detection: refractive index. At the appropriate intervals, after centrifugation and lyophilization, nanospheres were dissolved in tetrahydrofuran and injected into the GPC equipment. The weight average molecular weight was calculated with reference to the polystyrene standards.

**Vascular smooth muscle cells (VSMCs) culture**

Vascular smooth muscle cells (VSMCs) were isolated from the carotid arteries of Sprague-Dawley rats by an enzymatic digestion (collagenase and elastase) method and subcultured in Dulbecco’s modified Eagles medium F12 (DMEM F12) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a humidified, 5% CO₂ atmosphere. Cells were used between the fourth and tenth passages. The identity of cell types was characterized by immunofluorescent staining with a monoclonal anti-smooth muscle actin antibody.

**VSMC proliferation assay**

The VSMCs cultured in a T75 flask were washed with PBS, and harvested in 1 ml of PBS containing 1 mM trypsin-EDTA. The cells harvested were mixed with DMEM F12 containing 10% FBS. Aliquots (100 µl) of these cell suspensions were seeded on 96 well plate at a density of 3000 cells/well. After 24 h, the medium was replaced with DMEM F12 without FBS for 24 h. Thereafter the medium was replaced with a medium containing L-arginine (free drug or drug in the nanospheres, 1.0 mg/ml, 1.5 mg/ml, 3.0 mg/ml) and the cells were incubated at 37°C in a humidified, 5% CO₂ atmosphere. At the end of the incubation, the numbers of viable VSMCs were evaluated with MTT assay. 10 µl of PreMix WST-1 (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well, followed by a 1 h incubation at 37°C. Then the optical density was immediately measured at 450 nm using a microplate reader.

To test the reversibility of the antiproliferative activity of L-arginine, the drug was removed from some of the wells at 2 days, 4 days, or 5 days and the cells were incubated further in a normal growth media.

**Data analysis and statistics**

Drug release experiments were conducted in triplicate. Data are presented as percent of control and given as means ± SD. All other data are presented as mean ± SD and were conducted in triplicate. One-way factorial analysis of variance (ANOVA) was used for statistical analysis. A two-tailed P value smaller than 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Physicochemical characterization of the L-arginine loaded nanospheres**

Optical transmission micrographs of the nanospheres revealed regular shape and particle size in the range of 200 to 300 nm. The obtained nanospheres were spherical and separated each other as observed by transmission electron micrographs (Fig. 1). The surface morphology was smooth, without any visible pinholes, cracks, or pores. The average particle size of the nanospheres measured by photon correlation spectrosopy was about 250 nm, although it varied depending on the preparation condition. L-arginine loading was examined by HPLC analysis. Table 1 showed L-arginine loading, size and polydispersity of the prepared nanospheres under the varied concentrations of L-arginine.

**In vitro L-arginine release and polymer degradation studies**

L-arginine release from the nanospheres showed a triphasic profile with an initial burst, a dormant period and a final stage with an increased release rate (Fig. 2). The in vitro release profile represented the % of L-arginine released with respect to the total amount of L-arginine encapsulated. L-arginine release from the nanospheres extended over 10 days. The initial burst during the first 2 days represented about 40% of the total L-arginine content that was poorly entrapped in the polymer matrix. Thereafter, a period of
lower release rates was observed, followed finally by a re-
crease of the release rate. The dormant period lasted about
4 days. This phase of the release profiles corresponds to the
release of the entrapped protein. Commonly the release of
drugs from PLGA nanospheres is determined by the polymer
degradation time depending on the PLGA molecular weight
and the ratio of lactide and glycolide. The present nano-
spheres were prepared of PLGA polymer with molecular
weight 55,000 and the ratio of lactide and glycolide, 5:5.
Indeed, the onset of the third release stage represents the
time when polymer degradation was accelerated and accom-
panied by substantial mass loss as shown in Fig. 3. This
result of the third release stage agreed with the degradation
rate of the nanospheres (Fig. 3).

As shown in Fig. 2, L-arginine release from PLGA nano-
spheres exhibits a triphasic pattern over a prolonged period of
time with an initial burst, a dormant period and a final stage
with an increased release rate. The pattern and kine-tics of
release may have a definite effect on the biological response.
By selection of appropriate polymer types, the release profile
can be controlled, because the final stage of increased
release is closely related to polymer degrada-tion4). Thoma-

Effect of free L-arginine on proliferation of VSMCs
The antiproliferative effect of L-arginine was investigated
by culturing vascular smooth muscle cells in the medium
containing free L-arginine up to 6 mg/ml (Fig. 4). As shown in
Fig. 4, L-arginine inhibited proliferation of VSMCs with an
IC50 value of between 3.0 mg/ml (0.014 mM) and 4.0 mg/
ml (0.019 mM).

Fig. 5 illustrated that control cells incubated in a normal
growth medium free of L-arginine continued to grow through-out
the incubation time (●). On the other hand, the cells incu-
bated in the medium containing free L-arginine were attenu-
ated in depending on concentrations of L-arginine [(A]1.5
mg/ml, (B) 3.0 mg/ml [●, ▲]). However, when the medium
was replaced with normal medium (▲) at 2 days, the cells
resumed their growth thereafter, which suggests that the anti-
proliferative effect of L-arginine is reversible. When the L-argi-
nine containing medium was not replaced throughout the
incubation period, the cell growth was suppressed for 5 days
(●). As shown in Fig. 5, the inhibit-of VSMC growth was
more pronounced with the in-crease of L-arginine concentra-
tion.

Early studies demonstrated that NO donor agents inhibit
the in vitro proliferation of VSMCs isolated from rats and
rabbits in tissue culture. Such a biological activity of NO on
VSMC proliferation is associated with two distinct and revers-

<table>
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<th>L-arginine conc.</th>
<th>L-arginine loading (%) ± SD</th>
<th>Entrapment efficiency (%) ± SD</th>
<th>Particle size (nm ± SD)</th>
<th>Polydispersity index</th>
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<tr>
<td>0.125</td>
<td>12.21 ± 0.33</td>
<td>48.84 ± 0.22</td>
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<td>0.15</td>
<td>15.65 ± 0.15</td>
<td>52.17 ± 0.43</td>
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<tr>
<td>0.2</td>
<td>6.72 ± 0.38</td>
<td>16.82 ± 0.47</td>
<td>253.1 ± 13.2</td>
<td>0.136</td>
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Table 1. Effect of the internal aqueous phase concentration of L-arginine on the size and L-arginine loading of L-arginine loaded
PLGA nanospheres

Figure 2. The cumulated L-arginine in vitro release profile form
PLGA nanospheres. The 27 mg of nanospheres were dispersed in
7 ml of PBS. Values are expressed as the mean ± SD (n=4).

Figure 3. The in vitro degradation profile of PLGA nanospheres used
(n=4).
Controlled Release of L-Arginine from Poly(lactide-co-glycolide)~

Effect of L-arginine released from PLGA nanospheres on the proliferation of VSMCs

The intimal smooth muscle cell proliferation that follows vascular injury in animal models reaches a maximum at 10-14 days after injury and ceases upon re-endothelialization. Therefore, drugs need to be supplied continuously to control smooth muscle cell proliferation until a self-limiting repair mechanism is operating. The short cellular residence time of L-arginine suggests that L-arginine needs to be formulated in a sustained-release dosage form in this system. L-arginine loaded nanospheres can provide an extended period of time to inhibit vascular smooth muscle cell hyperproliferation to the localized lesion. Furthermore, the nanospheres (NS) can be delivered more efficiently to arterial tissue because of their ability to permeate cells and connective tissue.

The L-arginine loaded nanospheres (Arg NS) were incubated with vascular smooth muscle cells in a cultured medium for 2 days, and their antiproliferative effects on vascular smooth muscle cells were evaluated. Fig. 6 shows the morphometric results in the cases of control, Arg, Arg NS and NS. There was a substantial antiproliferative effect on the VSMCs in the case of Arg NS (L-arginine loaded nanospheres, containing 1.5 mg/ml L-arginine as calculated according to the time release curve shown above) and Arg (free L-arginine 1.5 mg/ml) when compared with control (normal media) and NS (media containing empty PLGA nanospheres). On the contrary, there was no significant effect on the inhibition of VSMCs growth in the case of NS. There was 18.5% reduction in growth of VSMCs treated with Arg NS compared with control VSMCs (Arg NS 81.47 ± 2.71%; Arg 82.70 ± 4.27%; NS 98.89 ± 0.74%; control 100 ± 1.50%).

The inhibitory effect of L-arginine loaded PLGA nanospheres on the VSMCs (Arg NS) was examined at concentrations of L-arginine varying from 1.0 to 3.0 mg/ml. L-arginine released from PLGA nanospheres inhibited VSMC proliferation in a dose-dependent manner (Fig. 7). As shown in Fig. 7, the Arg NS exhibited comparable antiproliferative effects to those observed with Arg (containing free L-arginine), suggesting that the nanospheres efficiently delivered L-arginine to the cells. The antiproliferative effect of Arg NS (1.0 mg/ml, 88.36 ± 0.68%; 1.5 mg/ml, 72.32 ± 1.48%; 3.0 mg/ml 63.99 ± 3.67%; containing L-arginin as calculated according to the time release curve shown above) was similar to that of directly added free L-arginine (Arg; 1.0 mg/ml, 1.5 mg/ml, 3.0 mg/ml).

Drug-releasing polymeric stents and polymer-coated stents are under investigation in different animal models, how-
however, these systems have potential disadvantages. First, most of the current stent designs cover only 5% to 12% of the arterial surface, limiting the amount of surface area in contact with the drug. Second, and perhaps more important, is that many of the polymeric materials used for retention and continuous delivery of the agent tested in different animal models do not appear to be bio-compatible with vascular tissue, as evidenced by induction of a significant inflammatory and proliferative response. In addition, use of a prosthetic, permanent stent implant is undesirable for the purpose of a short-term drug delivery. Balloon catheters for endoluminal local drug delivery may be more widely applied after coronary interventions. However, these systems have the disadvantages of not being capable of achieving high local tissue drug concentration, result in significant systemic drug levels, and most importantly, are unable to maintain therapeutic local drug levels for an adequate period of time.

This study showed that VSMC proliferation was significantly inhibited by L-arginine. Indeed, local drug administration can provide higher tissue drug levels at the site of vascular injury and avoid systemic side effects associated with the drug. However, a major problem of local injection of drugs through a catheter is low tissue uptake of the drug and lack of sustained delivery due to rapid wash-out of the agent.

To be effective in the prevention of the restenosis process, a carrier has to meet several features. First, it is essential that the size be small enough for delivery through the current catheter designs. Second, it should have a high drug loading capacity. Third, since it cannot be removed later from the lesion, as such, it should be biodegradable. Fourth, it should gradually release the agent over an extended period of time. Fifth, it must be fully biocompatible with the vascular tissue.

Recently, biodegradable and biocompatible polymers such as PLGA have been extensively studied for encapsulation of drugs responsible for cell proliferation. The PLGA nanoparticles used in this study as carriers for continuous local drug delivery possess most of the above-mentioned characteristics. The PLGA-based systems have been administered locally through currently available catheters or into the perivascular region through a surgical procedure yielding enhanced local uptake and drug concentrations as well as inhibition of intimal proliferation.

**CONCLUSION**

Biodegradable nanospheres containing L-arginine were formulated using PLGA copolymer as a controlled drug delivery system for the suppression of VSMC proliferation. In this study, L-arginine could be encapsulated into biodegradable polymeric nanospheres and subsequently be released over a prolonged time period, and L-arginine loaded PLGA nanospheres showed an antiproliferative effect on the VSMC growth comparable to free L-arginine solution.

This controlled release of L-arginine from the nanospheres may be useful to provide high concentration of L-arginine in blood vessel wall to inhibit proliferation after PCI and to minimize vascular diseases such as restenosis, respectively. It is needed further investigation in an in vivo model to confirm the antiproliferative effect of L-arginine on VSMC growth.

In conclusion, L-arginine released from PLGA nanospheres effectively reduced VSMC proliferation. Biodegradable PLGA nanospheres could be used to encapsulate antiproliferative agents and may serve as a carrier for local drug delivery after PCI and in turn prevent restenosis.

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