Development of a Sustainable Release System for a Ranibizumab Biosimilar Using Poly(lactic-co-glycolic acid) Biodegradable Polymer-Based Microparticles as a Platform

Yusuke Tanetsugu, a Tatsuaki Tagami, a Takayuki Terukina, a Takaya Ogawa, b Masato Ohta, b and Tetsuya Ozeki a

a Drug Delivery and Nano Pharmaceutics, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan; and b Chemistry, Manufacturing & Control Research Laboratories, Meiji Seika Pharma Co., Ltd.; 760 Morooka-cho, Kohoku-ku, Yokohama 222–8567, Japan.

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Regular Article

Ranibizumab is a humanized monoclonal antibody fragment against vascular endothelial growth factor (VEGF)-A and is widely used to treat age-related macular degeneration (AMD) caused by angiogenesis. Ranibizumab has a short half-life in the eye due to its low molecular weight and susceptibility to proteolysis. Monthly intravitreal injection of a large amount of ranibizumab formulation is a burden for both patients and medical staff. We therefore sought to develop a sustainable release system for treating the eye with ranibizumab using a drug carrier. A ranibizumab biosimilar (RB) was incorporated into microparticles of poly(lactic-co-glycolic acid) (PLGA) biodegradable polymer. Ranibizumab was sustainably released from PLGA microparticles (80+% after 3 weeks). Assay of tube formation by endothelial cells indicated that RB released from PLGA microparticles inhibited VEGF-induced tube formation and this tendency was confirmed by a cell proliferation assay. These results indicate that RB-loaded PLGA microparticles are useful for sustainable RB release and suggest the utility of intraocular sustainable release systems for delivering RB site-specifically to AMD patients.

Key words ranibizumab; poly(lactic-co-glycolic acid) (PLGA); controlled release; anti-vascular endothelial growth factor (VEGF) therapy; biosimilar; age-related macular degeneration (AMD)

Age-related macular degeneration (AMD) results in reduced eyesight and is the most common cause of blindness in the elderly in developed countries. As life expectancy increases, the number of AMD patients is expected to increase and AMD will become a public health problem and an economic burden in the future. Consequently, various therapeutic approaches for treating AMD are in development. The wet form of AMD, also called the exudative form, is typified by choroidal neovascularization, which is angiogenesis under and/or through the retinal pigment epithelium. Vascular endothelial growth factor (VEGF) is a key molecule that plays an important role in angiogenesis and its inhibition is therefore being targeted as a therapy for AMD. Anti-VEGF agents such as aptamer (pegaptanib, Macugen®), antibody (bevacizumab, Avastin®), and soluble decoy receptor fusion protein (aflibercept, VEGF Trap-eye®) are currently on the market. Ranibizumab, a Fab fragment of humanized monoclonal antibody against VEGF, can bind all isoforms of VEGF-A. The efficacy of ranibizumab has been investigated in several trials such as the MARINA study (ranibizumab alone at various doses), the ANCHOR study (ranibizumab vs. photodynamic therapy with verteporfin), and the PIER study (optimization of dose interval). Ranibizumab is a first line drug for AMD and, following several trials, has been widely approved for treating macular edema of retinal vein occlusion and for diabetic macular edema.

Although there are established treatment regimens using ranibizumab, limitations are becoming apparent. The HARBOR study, in which the dosage (2.0 vs. 0.5 mg) and timing (monthly or pro re nata) of ranibizumab administration were varied, showed that a higher dose of ranibizumab did not provide an increased therapeutic effect for AMD patients. The half-life of ranibizumab is relatively short (2.88 d, rabbit) compared with other anti-VEGF agents such as bevacizumab (4.32 d, rabbit) and aflibercept because of its low molecular weight (48 kDa) and thus its ready ability to penetrate tissues and be distributed widely in the body. In addition, ranibizumab is easily degraded by non-specific proteases present in the eyes. The eyes of patients with angiogenesis likely contain many compounds, including proteases, introduced from the bloodstream and causing the eyes to become increasingly inflexed. Repeated intravitreal injection of ranibizumab at shorter intervals should improve therapeutic efficacy but is undesirable by both patients and medical staff due to the cost and invasiveness of this approach. In addition, systemic serious side effects such as cardiovascular and cerebrovascular events after therapy with ranibizumab have been reported. It is clearly important to provide a sustained supply of ranibizumab specifically and only to the site exhibiting pathological angiogenesis.

Here we propose a sustained delivery system for ranibizumab using poly(lactic-co-glycolic acid) (PLGA)-based microparticles. PLGA is a safe and biodegradable copolymer that is partially hydrolyzed into water and carbon dioxide in the body and partially degraded to lactic acid which is subsequently utilized in the tricarboxylic acid (TCA) cycle. PLGA has been used to treat prostate cancer using, for example, leuprolineloaded PLGA microparticles (Leuplin®, owned by Takeda Pharmaceutical Industries Ltd., Tokyo, Japan) and other applications of PLGA for treating other diseases have been expanding. Peptides, and proteins such as ranibizumab, can likely...
be protected from proteases in tissues by entrapment into PLGA microparticles. Several articles very recently reported the use of ranibizumab-loaded PLGA-microparticles prepared by coaxial electrospray and the complexation of ranibizumab with chitosan improved drug release. However, there is currently little information regarding the encapsulation, sustainable release properties, and other characteristics of ranibizumab-loaded PLGA microparticles. In addition, earlier studies used authentic ranibizumab, rather than the ranibizumab biosimilar (RB) used in the present study. Biosimilars are very similar to the original biological drug and are attracting interest because the manufacturing process used in their production can and must be regulated to control the bioequivalence and therapeutic effect of the drug. Investigation of the handling of biosimilar-loaded PLGA microparticles as drug carriers may provide useful information regarding the characteristics of biosimilar-loaded drug nanocarriers. We incorporated RB into PLGA microparticles and the RB-loaded PLGA microparticles were characterized.

MATERIALS AND METHODS

Reagents RB was obtained from Meiji Seika Pharma (Tokyo, Japan). PLGA (PLA:PGA=75:25, molecular weight (MW)=15000), VEGF-A165, polyvinyl alcohol (PVA) and dichloromethane were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of RB-Loaded PLGA Microparticles RB-loaded PLGA microparticles were prepared using the water-in-oil-in-water (W/O/W) double emulsion technique to entrap RB in PLGA microparticles, as previously described. Briefly, RB solution (500 μL, 0.1%) was mixed with PVA solution (125 μL, 1.25%) to prepare the “W1” solution. Next, 250 mg of PLGA was dissolved with 7 mL dichloromethane to prepare the “O” solution. Then, 625 μL of W2 solution was dropped into the O solution with constant homogenization (3 min, 10000 rpm) using a Polytron homogenizer (PT3100; Kinematica AG, Luzern, Switzerland) to prepare W1/O and the resulting W1/O sample was placed on ice for 15 min. The W1/O sample was then dropped into the PVA solution (400 mL, 0.5%, “W2” solution) with constant homogenization (3 min, 6000 rpm) using a Polytron homogenizer (PT3100; Kinematica AG, Luzern, Switzerland) to prepare W1/O/W2. The resulting W1/O/W2 sample was stirred with a propellant overnight to remove dichloromethane. The samples were centrifuged (10000 rpm, 5 min, 4°C) in a Himac CF15RX II centrifuge (Hitachi Koki Co., Tokyo, Japan), the supernatants were removed, then the samples were washed with water and centrifuged again. After removing the supernatant, the samples were freeze-dried for 24 h to provide the RB-loaded PLGA microparticles. The PLGA samples were preserved below −80°C until use. The appearance of the RB-loaded PLGA microparticles was observed using a scanning electron microscope (SEM, S-4300, Hitachi, Tokyo, Japan).

Encapsulation Efficiency of RB in PLGA Microparticles

The encapsulation efficiency of RB in PLGA microparticles was evaluated by dispersing 5 mg of RB-loaded PLGA microparticles in 100 μL of phosphate buffered saline (PBS) and incubating at 37°C. After 1.5 months of incubation, the samples were completely degraded and presumably 100% of the RB had been released from the PLGA microparticles. The encapsulation efficiency of RB in the PLGA microparticles was calculated by: Encapsulation efficiency (%) = Protein concentration following 100% release/Protein concentration theoretically encapsulated. The protein concentration was determined using a DC Protein Assay kit (BioRad, Hercules, CA, U.S.A.) and the absorbance of the sample solution was measured using a plate reader (Wallac 1420 ARVO, PerkinElmer, Inc., Waltham, MA, U.S.A.; wavelength, 600 nm).

Release of RB from PLGA Microparticles RB-loaded PLGA microparticles (5 mg) were dispersed in 100 μL of PBS in a microtube and the microtube was incubated at 37°C. At the appropriate time point, the microtube was removed and centrifuged (10000 rpm, 4 min). The supernatant contained the released RB and was collected. The protein concentration in the supernatant and the absorbance of the sample solution were determined as described above. The released amount of RB was calculated as follows: Released RB (%) = Protein concentration at time t of incubation/Protein concentration following 100% release.

Cell Culture Human umbilical venous endothelial cells (HUVECs, HUVEC-umbilical vein, pooled cells) were purchased from Lonza (Basel, Switzerland). The cells were maintained in complete EGM-2 medium (prepared using an EGM-2 bullet kit, Lonza) at 37°C with 5% CO2 in an incubator. The cells were passaged when semi-confluent. Cells within six passages were used for the experiment.

Cell Proliferation Assay HUVECs dispersed in EGM-2 medium without VEGF as an added factor were seeded into a 96-well plate at a density of 5000 cells/well and the cells were pre-cultured for 24 h. The VEGF and RB formulations, shown in Table 1, were premixed with each other for 1 h to allow “antigen-antibody” reaction and then added to the wells. Untreated RB solution was used as control (“untreated”) and the RB solutions collected after the release of RB from PLGA microparticles were used as the test compound (“released”). Following addition of the formulations to the cells, the cells were further incubated for 24 h.

Cell proliferation was determined by the WST assay using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) as described previously. Briefly, 110 μL of medium mixture (containing 100 μL of medium and 10 μL of CCK-8 solution) was added to the cells and then incubated at 37°C for 4 h. The absorbance at 450 nm was measured using a Wallac 4000 ARVO multi-label counter (PerkinElmer, Inc.).

Tube Formation Assay An in vitro tube formation assay was conducted as previously described. Briefly, 50 μL of Matrigel (Corning Inc., Corning, NY, U.S.A.) was added to each well of a 96 well plate and then incubated at 37°C for 1 h to allow solidification and pre-coating of the gel. Then, 50 μL of cell suspension (10000 cells/well) was added to the wells. The premixed formulations of VEGF and RB shown in Table 2 were added to the wells. Untreated RB solution was used as control and the RB solutions collected after the release of RB from PLGA microparticles were used as the test compound, as described above. After 4 h incubation, tube formation by

| VEGF-A/RB (molar ratio) | Untreated | 1/0 | 1/1 | 1/2 | 1/4 |
|------------------------|-----------|-----|-----|-----|-----|
| VEGF-A (ng/well)       | 0         | 10  | 10  | 10  | 10  |
| RB (ng/well)           | 0         | 0   | 12.5| 25  | 50  |

Table 1. Premixing of RB and VEGF for the Cell Proliferation Assay
the HUVECs was observed under a light microscope (TS-100; Nikon Co., Kyoto, Japan). Five areas were chosen randomly and the number of completely connected tubes were counted.

Statistics One-way ANOVA with Bonferroni’s Multiple Comparison Test was conducted using GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.). \( p < 0.05 \) was regarded as statistically significant.

RESULTS AND DISCUSSION

The RB-loaded PLGA microparticles were characterized. The microencapsulation of drugs into PLGA is well-established. As reported previously, drug release from PLGA microparticles can be controlled by using various PLGA polymers differing in drug concentration, molecular weight, and the polymerization ratio between lactic acid and glycolic acid. A typical PLGA polymer (MW: 15000, PLA/PGA=75:25) was selected for the current study and images of RB-loaded PLGA microparticles are shown in Fig. 1. The PLGA microparticles were spherical and approximately 20 \( \mu \)m in diameter. The encapsulation efficiency was 89.0±4.2\% \((n=3)\). A study of the release profile of RB from PLGA microparticles was conducted to understand the sustained release properties of RB from PLGA microparticles (Fig. 2). The current dosing regimen requires the monthly intraocular injection of ranibizumab and thus a RB-loaded PLGA microparticle formulation providing sustained release for approximately one month was designed. Approximately 80\% of the RB was released within 3 weeks and the remainder of the RB was likely gradually released from the PLGA microparticles. Complete degradation of the PLGA microparticles was visibly evident after 1.5 months of incubation in PBS at 37°C. The mechanism of drug release from microparticles can be classified into one of four categories: by diffusion through water-filled pores, diffusion through the polymer, osmotic pumping, and erosion.\(^{23}\) The initial burst release is related to the heterogeneity of particle diameters and this heterogeneity is affected by the preparation process (such as the selection of organic solvents and polymers).\(^{25}\) The half-life of ranibizumab in eye tissue is several days and accordingly, the current regimen is monthly intravitreal injection.\(^{14}\) The RB release profile presented in Fig. 2 suggests that the interval between doses could be extended due to the sustained release of RB. The selection of another type of PLGA polymer might further extend the period of sustained release of RB.

Next, the ability of RB to specifically bind to VEGF and inhibit its function was assessed before and after the preparation of PLGA microparticles. Protein can be deactivated during PLGA microparticle preparation and during drug release from PLGA microparticles,\(^{26,27}\) due to the organic solvents used to prepare PLGA microparticles and the acidification caused by hydrolysis of PLGA microparticles affecting the higher order structure of the protein.\(^{19}\) Loss of function of tumor necrosis factor-alpha following the preparation of PLGA microparticles was also reported.\(^{28}\) However, to our knowledge, the influence of organic solvent on protein activity is protein-dependent and additives and salts can affect protein stability.\(^{28–31}\) We had no direct method to determine structural changes in RB after the preparation of PLGA microparticles and therefore we compared and evaluated its pharmacological effect against cells before and after incorporation into a microparticle preparation. Experiments to determine the effects of encapsulating RB on the proliferation of and tube formation by HUVECs were assayed. First, differences in anti-proliferation caused by RB before the preparation of PLGA microparticles ("untreated" control) and following the preparation of PLGA microparticles ("released" sample) were compared (Fig. 3). The proliferation of HUVECs was induced by the addition of VEGF, as expected. In contrast, premixing VEGF and RB allowed RB to bind to and inhibit the function of VEGF, and no cell proliferation was observed. These results are consistent with other reports that ranibizumab inhibits HUVEC proliferation.\(^{32}\) There was no significant difference in the activity of RB before and after encapsulation in PLGA microparticles, showing that the PLGA preparation process has minimal effect on RB activity.

An \textit{in vitro} tube formation model involving the combination of HUVEC and Matrigel was used to assess the influence of RB released from PLGA microparticles or untreated RB on angiogenesis. The results of tube formation by HUVEC are shown in Fig. 4 as images and in Fig. 5 as quantitative results. The addition of VEGF alone enhanced tube formation compared with control (Untreated cells), whereas premixing with an equal amount of RB (1:1) remarkably inhibited tube formation, suggesting that RB has high specificity against VEGF.

Table 2. Premixing of RB and VEGF for the Tube Formation Assay

| VEGF-A/RB (molar ratio) | (Untreated) | 1/0 | 1/1 | 1/2 | 1/4 |
|------------------------|------------|-----|-----|-----|-----|
| VEGF-A (ng/well)       | 0          | 5   | 5   | 5   | 5   |
| RB (ng/well)           | 0          | 0   | 6.25| 12.5| 25  |

Fig. 1. Image of RB-Loaded PLGA Microparticles Observed by SEM

Fig. 2. Release Profile of RB from PLGA Microparticles in PBS Buffer at 37°C

The data represent the mean±standard deviation (S.D.) \((n=3)\).
Fig. 3. Anti-proliferation Activity of RB Released from PLGA Microparticles

The experimental conditions are described in Table 1 and in Materials and Methods. Solid bar, untreated RB; Open bar, released RB. The data represent the mean±S.D. (n=3). There was no significance among the groups.

Fig. 5. The Inhibitory Effect of RB Released from PLGA Microparticles on Tube Formation by HUVECs

Solid bar, untreated RB; Open bar, released RB. The experimental conditions are described in Table 2 and in Materials and Methods. The data represent the mean±S.D. (n=3). *p<0.05; **p<0.01, statistically significant compared with 1/0 group.

Fig. 4. Typical Images of HUVEC-Based Tube Formation in Vitro

The experimental conditions are described in Table 2 and in Materials and Methods. Scale bar indicates 500 µm.
Addition of the RB–VEGF mixture greatly inhibited tube formation by HUVECs to the same level as the control and was dependent on the amount of RB. In addition, there was no significant difference between RB released from PLGA microparticles and untreated RB. These results confirmed the minimal influence of the PLGA preparation process on RB activity. The tube formation assay results showed that RB has remarkable ability to inhibit tube formation. RB-loaded PLGA microparticles can inhibit the function of VEGF and may therefore be an effective formulation not only for treating AMD, but also VEGF-related diseases such as cancers and rheumatism.

PLGA microparticles provide a prototype platform for sustained RB delivery to the eye after intravitreal injection. High amounts of PLGA microparticles can cloud vision\textsuperscript{33,34} and thus implant-type PLGA carriers are more practical\textsuperscript{33,35}. For example, rod shape\textsuperscript{36} and disc shape\textsuperscript{37} PLGA implants exhibiting long-term release of a low molecular weight drug have been reported. The development of PLGA implants containing RB will be studied in our laboratory in the future.

CONCLUSION

In conclusion, RB has remarkable anti-angiogenic ability and is used in AMD therapy. RB was incorporated into biodegradable PLGA polymer microparticles to produce a controlled and sustainable release system. RB showed sustainable release from PLGA microparticles over several weeks, suggesting that a PLGA-based sustainable release system for RB delivery to a targeted site is feasible, thereby reducing the number of intravitreal injections required and reducing the burden on patients and medical staff. This study demonstrated a system for the controlled release of antibody fragments. Although further studies are necessary to provide a system for practical use, the present findings will be useful for future studies on the controlled delivery of drugs to AMD patients.

Conflict of Interest Tetsuya Ozeki received financial support from Meiji Seika Pharma Co., Ltd.

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