Heat Enhances *Ex Vivo* Paclitaxel Delivery to Porcine Carotid Artery Wall

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Abstract We investigated the delivery of rhodamine B and Oregon Green[®]-labeled paclitaxel (OGLP) in *ex vivo* porcine carotid artery wall (CAW) samples after heating the reagents to 50–70°C for 15 s. When the isolated CAW samples were placed in the heated fluorophore solutions, the penetration depth of the hydrophobic rhodamine B increased significantly compared with reference solution at 37°C. The penetration depth of OGLP also tended to increase upon heating to 70°C for 15 s. We also studied the mechanism of this agent delivery enhancement by observing the inner surface structure and hydrophobicity of the CAW samples after heating. An expanded mesh structure at the inner surface of the heated CAW samples was observed upon heating above 70°C, and the mean hydrophobicity of the media layer also increased significantly. We hypothesize that heating at 60–70°C for 15 s enhances the delivery of fluorophores to CAW samples as a result of an expanded mesh structure at the inner surface of the CAW, along with a simultaneous increase in hydrophobicity.

Keywords: drug delivery, heating, porcine carotid artery wall, paclitaxel.

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1. Introduction

The long-term patency of blood vessels after angioplasty for peripheral stenosis is often poor. Current stent implantation methods, including the drug-eluting stents, are usually unsuitable for peripheral stenosis due to the risk of stent fracture caused by exercise [1]. Drug-coated balloons that are able to deliver anti-restenosis drugs to arterial walls suppress restenosis for an extended time without stent implantation, and have been used to treat peripheral stenotic lesions [2]. To sustain the efficacy of the anti-restenosis drugs from such a balloon, the initial quantity of drug delivered must be carefully considered, because the concentration in the artery wall decreases gradually after treatment. Indeed, some reports have shown that almost 75–90% of the drug originally taken up by the artery wall was cleared between several minutes and a few days after treatment initiation [3–5]. In the late 1980s, the development of thermal angioplasty

allowed satisfactory vessel dilatation without mechanical injury to artery walls by softening the collagen with heat [6, 7]. The short-term vascular patency after thermal angioplasty is excellent [8, 9]. However, the long-term outlook is suboptimal because of injury to the adventitia caused by overheating [9]. To obtain vascular patency without heat injury, we have developed a photo-thermo dynamic balloon (PTDB) that can dilate an artery wall by softening collagen [10]. Adventitia injury is prevented as a result of brief (< 15 s, 70°C) heating using a combination of laser light irradiation and continuous fluid flow within the PTDB [11]. Under this heating condition, thermal denaturation of collagen fibrils below the surface of the artery wall is expected, since the temperature of the surface increases to approximately 65°C [12, 13]. We hypothesize that such heating in the artery wall may alter the delivery of the anti-restenosis drug due to structural changes caused by thermal denaturation of collagen fibrils. In this study, we investigated the distribution of rhodamine B and Oregon Green[®]-labeled paclitaxel (OGLP) in the wall of porcine carotid artery after heating *ex vivo* at 50–70°C for 15 s.

2. Materials and Methods

2.1 Monitoring delivery of drug to arterial wall using fluorophores

The distribution of agents delivered to CAW samples was observed by measuring the fluorescence emitted by rhodamine B (molecular weight: 479.02, $\log P_{ow} = 1.95$; Wako Pure Chemical Industries, Osaka, Japan) and Oregon Green[®]-labeled paclitaxel (molecular weight: 1319;

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Molecular Probes, Eugene, OR, USA). Here, $\log P_{ow}$ is a standard parameter of hydrophobicity, and P_{ow} indicates the ratio of the concentration of the compound in two immiscible phases, *n*-octanol and water, at equilibrium. Rhodamine B, a hydrophobic agent, was used because of its strong fluorescence emission. Oregon Green®-labeled paclitaxel was used because it contains paclitaxel, a clinical agent that inhibits restenosis, and a fluorophore. While the hydrophobicity of rhodamine B is known, the corresponding value for Oregon Green®-labeled paclitaxel has not been reported. An upright reflective fluorescence microscope (BX71; Olympus, Tokyo, Japan) was used to observe the fluorescence intensity distribution in the arterial wall sample, as described below. The cross-section of the CAW sample perpendicular to the axis of blood flow was observed. With a mercury lamp, bandpass filters of 540–550 nm and 470–490 nm were used as the excitation light source for rhodamine B and OGLP, respectively, and fluorescence intensity was observed using bandpass filters of 575–625 nm and 510–560 nm, respectively. Digital fluorescence red-green-blue (RGB) intensity micrographs of the cross sectional samples (6.9 megapixel, 24-bit) were created using a complementary metal oxide–semiconductor sensor (D5000; Nikon, Tokyo, Japan). The exposure time of the camera was set at 1/6 s. The recorded RGB images were converted to 16-bit grayscale using Image J (National Institutes of Health, Bethesda, MD, USA) to obtain the fluorescence intensity for each pixel. Fluorescence calibration curves of measured fluorescence intensity versus fluorophore concentration were prepared as described in section 2.3.

2.2 Delivery of agents to CAW samples upon heating

The carotid arteries of domestic pigs (6 months of age; weighing 110–120 kg) removed within 12 h of culling were used. A CAW sample (length, approximately 2 cm) was immersed in a heated (50–70°C) fluorophore solution for 15 s to observe delivery of the agent to the CAW sample under the condition of reversible thermal denaturation [11, 14, 15]. The concentration of rhodamine B was 3.5 µg/cm³, and that of OGLP was 1.0 µg/cm³, to maintain the signal-to-noise ratio. Immediately after heating, the CAW sample was replaced in physiological (0.9%) saline for 60 s at 37°C to remove residual fluorescein on the surface. After fluorophore delivery, the CAW sample was cooled to –20°C in a cryostat (CM1510S; Leica, Wetzlar, Germany) for 30 min. The frozen CAW sample was sliced along the direction of blood flow to a thickness of 20 µm. A glass coverslip was placed on the CAW sample to prevent drying, and fluorescence was measured around 1 h after agent delivery.

Fig. 1 Schematic description of the definition of penetration depth in an arterial wall sample.

2.3 Calibration curves of measured fluorescence intensity vs. agent concentration

Calibration curves for rhodamine B and OGLP were created by plotting the fluorescence intensity as a function of concentration. From the calibration curve, the concentration of the agent in a stained CAW sample could be determined. The fluorescence intensity of the agent solutions (0.35 ng/cm³ to 0.35 µg/cm³ for rhodamine B, 8.54 ng/cm³ to 0.854 µg/cm³ for OGLP) was observed using a fluorescence microscope following the procedure described in section 2.1. The thickness of the rhodamine-B solution and OGLP solution was identical to that of the sliced CAW sample described in section 2.2. An intra-lipid solution of 2.6% was added to the rhodamine B and OGLP solutions to simulate the scattering characteristics of the sliced CAW sample [16].

2.4 Evaluation of Drug Delivery

Figure 1 shows the definition of the “penetration depth” of the delivered agent, which is defined as the radial depth from the inner surface of the lumen to the location at which the agent concentration was 10⁻⁸ M (= rhodamine B: 4.79 ng/cm³, OGLP: 8.54 ng/cm³). This concentration is the minimum threshold that inhibits the proliferation of smooth muscle cells [17]. The average concentration of the delivered agent was determined by the sum of the quantities of agent [obtained from the calibration curve described in section 2.3, corresponding to equation (1) of section 3.1] along the radial axis from the lumen to the defined penetration depth divided by the volume of CAW samples.

2.5 Observation of the inner surface of the artery wall sample after heating

The inner surface of the heated CAW sample was observed using a field-emission scanning electron microscope (S4700; Hitachi, Tokyo, Japan). The CAW sample was heated in 0.9% saline at 70°C for 15 s following the procedure described in section 2.2. The sample was then fixed with 10% formalin for 2 days and then immersed repeatedly in 50%, 75%, and 100% ethanol for 30 min

each to achieve dehydration. The dehydrated sample was placed in t-butyl alcohol and dried in a freezer (VFD-21S; Shinku, Ibaraki, Japan). Finally, the dried sample was coated with osmium (HPC-1S; Shinku) to render the sample surface conductive. Field-emission scanning microscopy was performed at an applied voltage of 5 kV at $\times 35,000$ magnification.

2.6 Observation of hydrophobicity of CAW samples after heating

The hydrophobicity of a heated CAW sample was observed using 8-anilino-1-naphthalenesulfonic acid (ANS; Life Technologies Japan, Tokyo, Japan) staining. The fluorescence intensity of the reagent was reported to increase with increase in hydrophobicity of the environment around the reagent [18, 19, 20]. The artery wall sample was heated to 60°C or 70°C in saline for 15 s and processed to create a sliced CAW sample by the procedure described in section 2.2. The sample was then stained with 5×10^{-4} M ANS for 5 min. A mercury lamp with a bandpass filter of 360–370 nm was used as the excitation light source, and a high bandpass filter of 420 nm was used to observe the fluorescence intensity of ANS. RGB color microscope images of the fluorescence intensity of ANS in stained CAW samples were obtained and analyzed following the procedures described in sections 2.1 and 2.4.

3. Results

3.1 Calibration curves of measured fluorescence intensity vs. agent concentration

We obtained calibration curves of the fluorescence intensity as a function of the concentrations of rhodamine B and OGLP using the method described in section 2.3. There is a logarithmic relationship between the fluorescence intensity and concentration of rhodamine B within the range of 0.35 ng/cm³ to 0.35 μ g/cm³. The calibration curve is shown in **Fig. 2a** and can be described by equation (1):

$$y_1 = (3.39 \times 10^{-1})\ln(x_1) + (8.56 \times 10^4) \quad (1)$$

$$(R^2 = 0.98)$$

where x_1 is the concentration of rhodamine B (ng/cm³) and y_1 is the fluorescence intensity (a.u.). In the case of Oregon Green®-labeled paclitaxel, the fluorescence intensity also has a logarithmic relationship with the agent concentration within the range of 8.54 ng/cm³ to 0.854 μ g/cm³. The calibration curve is shown in **Fig. 2b** and is given by equation (2):

Fig. 2 Calibration curves of measured fluorescence brightness vs agent concentration of (a) rhodamine B and (b) Oregon green®-labeled paclitaxel ($N = 4-5$).

$$y_2 = (6.28 \times 10^{-1})\ln(x_2) + (9.41 \times 10^4) \quad (2)$$

$$(R^2 = 0.89)$$

where x_2 is the concentration of OGLP(ng/cm³) and y_2 is the fluorescence intensity(a.u.).

3.2 Distribution of agents in heated CAW samples

A representative example of the concentration distribution in a CAW sample along the radial axis one hour after delivery of rhodamine B at 37°C and with heating to 70°C for 15 s is shown in **Fig. 3**. The concentration of rhodamine B in the CAW sample was higher after heating than that at 37°C, especially in the region between 0 μ m to 90 μ m from the surface of the lumen. The penetration depths and average concentrations of rhodamine B delivered at various temperatures are shown in **Figs. 4 and 5**, respectively. The same data were used in both **Figs. 4 and 5**, but different analyses were employed. When the CAW sample was heated above 65°C for 15 s, we found a significant difference in the penetration depth compared with the 37°C reference solution (**Fig. 4**: 37°C vs 65°C; $p = 0.02$, 37°C vs 70°C; $p = 0.0001$). As shown

Fig. 3 Representative example of the concentration distribution in the carotid artery wall along the radial axis after delivery of rhodamine B at 37°C and after heating at 70°C for 15 s.

Fig. 4 Penetration depths of rhodamine B in the arterial wall sample at different temperatures ($N = 4$).

in **Fig. 4**, the penetration depth after heating at 70°C for 15 s was 76 μm , which was approximately 7.5-fold deeper than that of the 37°C reference solution. When the CAW sample was heated above 60°C for 15 s, we found a significant difference in average concentration compared with the 37°C reference solution (**Fig. 5**: 37°C vs 60°C, $p = 0.02$; 37°C vs 65°C, $p = 0.09$; 37°C vs 70°C, $p = 0.008$). The penetration depth and average concentration of rhodamine B delivered to the CAW sample tended to increase with increasing temperature up to 70°C. **Figure 6** shows the concentration distribution in the CAW sample along the radial axis after delivery of OGLP at a reference temperature of 37°C and heating at 70°C for 15 s. The concentration of OGLP delivered to the CAW sample upon heating was substantially higher than that observed at 37°C, especially in the region between 0 μm to 10 μm from the surface of the lumen.

Fig. 5 Average concentrations of rhodamine B delivered at different temperatures ($N = 4$).

Fig. 6 Concentration distribution of representative sample along the radial axis after delivery of Oregon Green®-labeled paclitaxel at 37°C and after heating at 70°C for 15 s.

3.3 Observation of inner surface of CAW samples after heating

Field-emission scanning electron micrographs of the inner surface of heated CAW samples are shown in **Fig. 7a and b**. An expanded mesh structure of the internal elastic lamina of the heated CAW sample was visible after heating at 70°C for 15 s.

3.4 Observation of hydrophobicity of CAW samples after heating

Micrographs of CAW samples stained by ANS after heating at 37°C (reference), 60°C, and 70°C are shown in **Figure 8a, b, and c**, respectively. At 37°C, the fluorescence of ANS showed the lamina structure of the media (**Fig. 8a**). At 60°C and 70°C, the lamina structure in the media was more homogeneous compared with that observed at 37°C (**Fig. 8b, c**). With regard to the intima and

(a)

(b)

Fig. 7 Field-emission scanning electron micrographs of the inner surface after heating at (a) 37°C (reference) and (b) 70°C for 15 s.

adventitia, the fluorescence intensity of ANS did not change upon heating to 60°C or 70°C for 15 s. The dependence of mean fluorescence intensity of ANS along the radial axis of the CAW sample, as defined in section 2.6, at various temperatures is shown in **Fig. 9**. The mean fluorescence intensity of ANS in the CAW sample increased with increasing temperature.

4. Discussion

4.1 Increases in penetration depth and average concentration of delivered agents in CAW samples upon heating

As shown in **Figs. 4 and 5**, the penetration depth and average concentration of agent delivered to CAW samples showed similar increases after heating. We speculate that the increase in penetration depth of the agent upon heating may be responsible for the increase in average concentration of agent delivered. This is because there was little difference in agent concentration on the surface of the CAW sample at 70°C compared with that at 37°C (**Fig. 3**). Endothelial cells on the surface of the CAW

Fig. 8 Micrographs of a carotid artery wall stained by ANS after heating at (a) 37°C (reference) (b) 60°C and (c) 70°C for 15 s.

Fig. 9 Mean fluorescence intensity of ANS along the radial axis at various temperatures ($N = 4$).

samples were “peeled off” during heating, and the protein mesh of the internal elastic lamina was visible (Fig. 7). The internal elastic lamina is the primary barrier against blood contamination [21], and its thickness has been reported to be approximately 60 μm [22]. Therefore, heating at 70°C for 15 s may facilitate the delivery of the agent through the internal elastic lamina. With regard to OGLP, a similar enhancement of delivery was obtained, even though the molecular weight of OGLP is 2.5-fold greater than that of rhodamine B. Thus, heating at 70°C for 15 s may be effective in enhancing the delivery of anti-stenosis agents.

4.2 Mechanism of increased agent delivery upon heating

As shown in Fig. 7, an expanded mesh at the inner surface of the CAW sample was visible upon heating at 70°C for 15 s. A similar phenomenon has been reported; thermal denatured type I collagen showed 1.7-fold increase in fibril diameter and rougher surface compared with the state prior to denaturation [23]. We hypothesize that expansion of the mesh structure of protein fibrils in the internal elastic lamina upon heating at 70°C for 15 s may enhance the penetration depth and total quantity of agent delivered to the CAW samples. As shown in Fig. 9, the mean hydrophobicity of the media layer increased significantly upon heating at 60°C or 70°C for 15 s compared with that of 37°C. A similar phenomenon has been observed regarding the hydrophobicity of a protein surface after heating [18, 19]. Moreover, it has been reported that the hydrophobicity of an agent plays an important role in transportation of agents into artery walls, since hydrophobic agents bind to hydrophobic sites in tissues [24, 25]. We hypothesize that heating may increase hydrophobic binding in CAW samples, which enhances drug delivery.

5. Limitations

Our study had two main limitations. First, we employed rhodamine B and OGLP as experimental agents, instead of paclitaxel, itself. The hydrophobicity of rhodamine B ($\text{Log}P_{ow} = 1.95$) is lower than that of paclitaxel (2.83), and the hydrophobicity of OGLP is not known. In addition, we were not able to obtain reliable data of penetration depth and average concentration of OGLP since few data was obtained using OGLP because of the hydrophobicity. Second, we investigated agent delivery using isolated porcine carotid arteries *ex vivo*. The components of porcine CAWs are different than those of human arteriosclerotic arteries. The number of endothelial cells, the tension in arterial walls, and blood flow may all be different *in vivo*.

6. Conclusion

We investigated the distribution of rhodamine B and OGLP in porcine CAWs after heating these agents at 50–70°C for 15 s *ex vivo*. The penetration depth of rhodamine B increased significantly upon heating to >65°C for 15 s, compared with the same procedure performed at 37°C. In the case of OGLP, the penetration depth also increased upon heating at 70°C for 15 s. We also studied the mechanism of enhancement of agent delivery by observing the structure of the inner surface and hydrophobicity of CAW samples after heating. An expanded mesh structure at the inner surface of heated CAW samples became visible upon heating at 70°C for 15 s. The mean hydrophobicity of the media layer increased significantly after heating, compared with that at 37°C. We hypothesize that heating may enhance the delivery of agents to CAW samples because of the expanded mesh structure of the inner surface as well as the increased hydrophobicity.

Conflict of interest

The authors declare no conflicts of interest associated with this study.

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