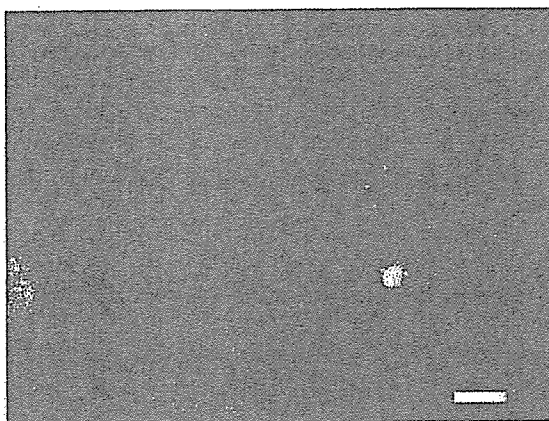


Summary: An amphiphilic poly(*N*-propargylamide) with galactose and lauryloyl groups was synthesized by copolymerization of the corresponding *N*-propargylamide monomers using a Rh catalyst. The obtained copolymer formed a one-handed helical conformation and molecular aggregates in water. The observations by fluorescence microscopy in a cell culture experiment in the presence of dye-labeled copolymer indicated that the copolymer was incorporated into the cells.



Localization of rhodamine B-labeled copolymer 8 in human aortic endothelial cells (fluorescence image).

Amphiphilic Poly(*N*-propargylamide) with Galactose and Lauryloyl Groups: Synthesis and Properties

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Introduction

Synthesis of polymers having sugar residues, so-called glycopolymers, has been widely investigated to seek biological applications because of their versatile functions.^[1] It has been demonstrated that these glycopolymers can bind specifically to carbohydrate-recognition proteins, toxins, viruses, and cells, and, thus, these polymers can be utilized as cell culture substrates with specific cell recognition sites, as well as in targeting drug delivery systems.^[2] The clustered saccharide ligands conjugated to the polymeric main chains are involved in these specific recognition processes. Most of the previously prepared glycopolymers have been based on a flexible polymer backbone, such as polystyrene and polyacrylamide.^[3,4]

This flexible nature of the glycopolymers causes the disordered orientation of the sugar residues in the glycopolymers. Regular orientation of the sugar residues is necessary for efficient interaction between the glycopolymers and receptor molecules. In this sense, the spatially regulated orientation of the sugar residues should be realized by attaching the sugar residues to a polymer backbone with a rigid conformation. The sugar residues attached to the rigid polymer backbone may give rise to the ordered orientation that can improve the molecular recognition of sugar residues by specific cell receptors. This is because the spatial regulation of the sugar residues is significant in molecular recognition as well as the chemical structure of the sugar molecules.^[5]

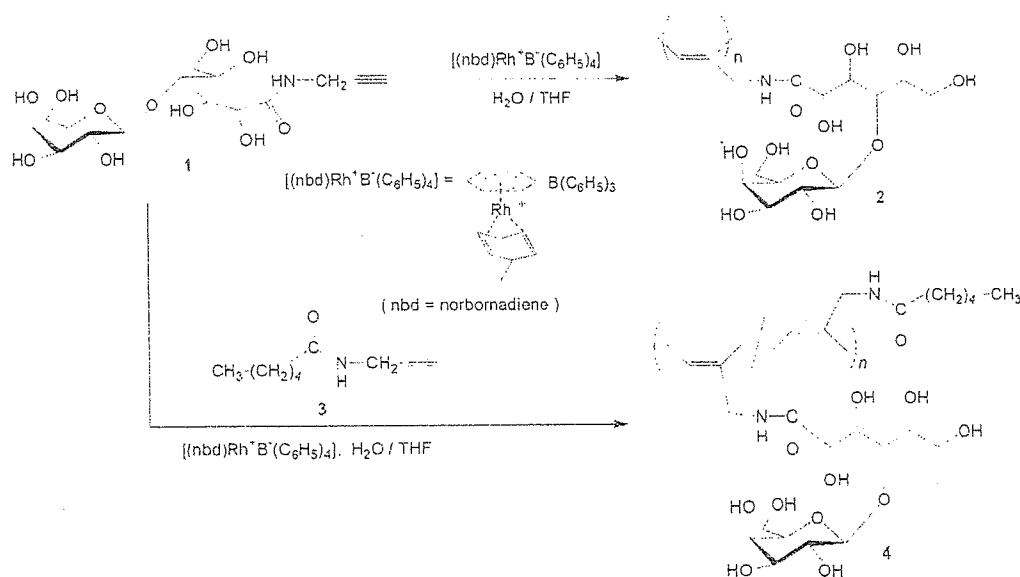
From the above viewpoints, rigid glycopolymers with π -conjugated polymer backbones would be a novel type of bio-inspired polymers, which could exhibit multiple valence states and interact specifically and firmly with targets such as cell surface receptors and biomacromolecules. In some previous works, rigid glycopolymers having various π -conjugated main chains, such as polythiophene,¹⁶¹ poly(*p*-phenylene ethynylene),¹⁷¹ polyisocyanide,¹⁸¹ polyaniline,¹⁹¹ poly(*p*-phenylene vinylene),¹¹⁰¹ and poly(phenylacetylene), have already been synthesized.¹¹¹¹ In the series of these studies, we reported the synthesis of poly(*N*-propargylamide) (**2**) with sugar residues. It contained a *cis*-polyacetylene main chain and was obtained by the rhodium-catalyzed polymerization of a *N*-propargylamide monomer (**1**) that had a galactose residue (Scheme 1).¹¹²¹ Since polymerizations of the *N*-propargylamide monomers having various substituted groups using Rh catalyst have been widely reported to produce the corresponding poly(*N*-propargylamide) derivatives with *cis*-isomers,¹¹³¹ we also investigated the copolymerization of **1** with *N*-propargylamide derivative **3** having a hexanoyl group to produce the amphiphilic glycopolymer **4**, as shown in Scheme 1. We tested the solubility of copolymer **4** in various solvents to confirm whether the copolymer exhibits an amphiphilic property. Although the homopolymer **2** is insoluble in common organic solvents, the copolymer **4** can be dissolved in some polar organic solvents, such as dimethyl sulfoxide (DMSO) and *N,N*-dimethylformamide (DMF), as well as in aqueous medium. However, the copolymer still exhibits a hydrophilic nature rather than an amphiphilic nature. We assumed that insufficient amphiphilicity of copolymer **4** could be attributed to poor hydrophobic property of the hydrophobic part.

In this study, we chose a more hydrophobic monomer: *N*-propargylamide monomer **5** having a longer alkyl chain, i.e., the lauryloyl group, as the hydrophobic part of the amphiphilic copolymer. The monomer **5** was copolymerized with **1** in the presence of Rh catalyst to give the corresponding amphiphilic copolymer **6** (Scheme 2). The resulting copolymer **6** can be expected to have the ability to conduct molecular aggregation in water, which is driven by intermolecular and intramolecular association of the hydrophobic lauryloyl groups.

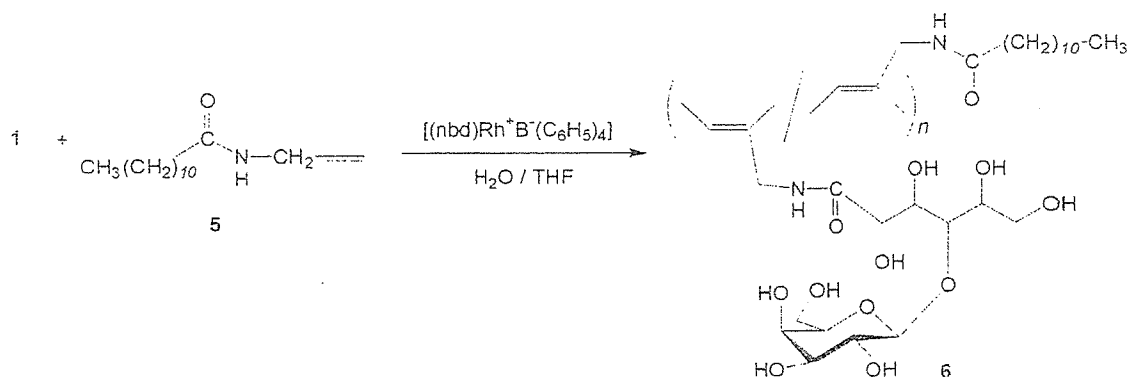
We believe that such molecular aggregates of the amphiphilic copolymer should play a significant role in the field of targeted drug delivery. The idea is supported by the following characteristics of the molecular aggregates:

- 1) Drug carriers with nanometer dimensions can be obtained by molecular aggregation of amphiphilic copolymers.
- 2) Nano-sized aggregates can remain in the bloodstream for an extended period because of the size-dependent uptake in the reticuloendothelial system.
- 3) Drug molecules with hydrophobic natures can be loaded into hydrophobic milieu formed by the association of hydrophobic long alkyl chains; otherwise the drug molecules could be directly attached to the constituent monomer molecules of the copolymer.
- 4) Sugar residues of the copolymer can function as recognition sites for target cells and tissues as well as providing the hydrophilic character of the amphiphilic copolymer.

Therefore, we studied the *in vitro* cell uptake of the molecular aggregates of the amphiphilic copolymer. For this purpose, the fluorescent marker, rhodamine B, was introduced into the amphiphilic copolymer. In this article, we report the synthesis of the amphiphilic



Scheme 1. Polymerization of **1** and copolymerization of **1** with **3**.



Scheme 2. Copolymerization of 1 with 5.

poly(*N*-propargylamide) 6 by Rh-catalyzed copolymerization, evaluation of its molecular aggregation in water [gel permeation chromatography (GPC) characterization, scanning electron microscopy (SEM) observation, and dynamic light scattering (DLS) measurement] and secondary conformation [circular dichroism (CD) spectra], and cell uptake of the nanoaggregate of the rhodamine-labeled amphiphilic copolymer (fluorescence microscopy).

Experimental Part

Materials

Monomer 1 and catalyst $(\text{nbd})\text{Rh}^+\text{B}^-(\text{C}_6\text{H}_5)_4$ were prepared according to the literature.^{112,141} Tetrahydrofuran (THF) used as polymerization solvent was purified by distillation. Other reagents and solvents were used as received without further purification.

Synthesis of Monomer 5

Monomer 5 was synthesized by a method similar to that used for 3.¹¹⁵¹ Under argon, *N*-propargylamine hydrochloride (0.915 g, 10.0 mmol) was dissolved by slight warming in anhydrous acetonitrile (12.0 mL), and triethylamine (3.35 mL, 24.0 mmol) was added to the solution at room temperature. Then a solution of lauryloyl chloride (2.36 mL, 10.0 mmol) in anhydrous acetonitrile (12.0 mL) was added dropwise to the solution. After the mixture was stirred for 2 h, the reaction solution was concentrated by evaporation. The residue was dissolved in ethyl acetate and the solution was washed successively three times with 2 mol·L⁻¹ hydrochloric acid and with saturated NaHCO₃ aqueous solution. The organic layer was dried over anhydrous Na₂SO₄, filtered, and evaporated. The residue was subjected to column chromatography on silica gel (hexane:ethyl acetate = 4:1, v/v) to isolate 5 (1.38 g, 5.82 mmol) in 58.2% yield.

¹H NMR (CDCl₃): δ = 0.88 (t, J = 7.2 Hz, CH₃, 3H), 1.28 [m, CH₂(CH₂)₈, 16H], 1.62 (m, CH₂CH₂C=O, 2H), 2.19 (t, J = 7.8 Hz, CH₂C=O, 2H), 2.23 (t, J = 2.4 Hz, H-C≡C, 1H), 4.05–4.06 (m, CH₂N, 2H), 5.57 (s, NH, 1H).

Synthesis of Monomer 7

Under argon, triethylamine (0.836 mL, 6.00 mmol) was added to a solution of *N*-propargylamine hydrochloride (0.549 g, 6.00 mmol) and rhodamine B (1.92 g, 4.0 mmol) in anhydrous methanol (10.0 mL) at 0 °C. To the solution was added 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (1.15 g, 6.00 mmol) as a condensing agent at 0 °C and the mixture was stirred for 17 h at room temperature. The precipitated material was isolated by filtration and dried under the reduced pressure to give 7 (0.472 g, 0.915 mmol) in 23.0% yield.

¹H NMR (CDCl₃): δ = 1.16 (m, CH₃, 12H), 1.77 (t, J = 2.4 Hz, H-C≡C, 1H), 3.33 (m, NCH₂CH₂, 8H), 3.95 (d, J = 1.8 Hz, CH₂C≡, 2H), 6.26–7.93 (m, aromatics, 10H).

Copolymerization of 1 with 5

A typical copolymerization procedure was as follows (entry 1, Table 1). Under argon, a solution of 5 (0.0356 g, 0.150 mmol) in THF (0.90 mL) and a solution of catalyst (0.00630 g, 0.0125 mmol) in THF (0.90 mL) were added to a solution of 1 (0.0397 g, 0.100 mmol) in water (0.20 mL) in this order at 30 °C. After the mixture was stirred at 30 °C for 140 min, the reaction mixture was concentrated by evaporation and dried under reduced pressure. The residue was dissolved in a small amount of DMSO and the solution was poured into a large amount of methanol to precipitate the polymeric product. The precipitate was isolated by filtration and was dried under reduced pressure to give 6 (0.0588 g) in 78.1% yield.

¹H NMR (DMSO-*d*₆): δ = 0.84 (CH₃), 1.21 [CH₂(CH₂)₈], 1.45 (CH₂CH₂C=O), 2.15 (CH₂C=O), 3.0–3.9 [–CH(O–*D*-gal)–CH(OH)–CH₂OH, =CCH₂–, H2–H6 of *D*-gal], 4.04 [C(=O)CH(OH)CH(OH)–], 4.29 [C(=O)CH(OH)– and H1(β) of *D*-gal], 4.55, 4.79, 5.22 (OH), 6.11 (–CH=C–), 7.93 (NH).

Copolymerization of 1, 5, and 7

Under argon, a solution of 5 (0.0285 g, 0.120 mmol) and 7 (0.0929 g, 0.180 mmol) in THF (0.90 mL) and a solution of catalyst (0.0166 g, 0.0330 mmol) in THF (0.90 mL) were

Table 1. Results for copolymerization of **1** with **5** with Rh catalyst in THF–water (9:1) solvent.

Entry	Feed ratio ^{a)}	Time	Yield ^{b)}	Unit ratio ^{c)}	\bar{M}_n ^{d)}	\bar{M}_w/\bar{M}_n ^{d)}	$[\alpha]_D^{20}$ ^{e)}
	1:5	min	%	1:5			degrees
1	1.0:1.5	140	78.1	1.0:0.92	9 100	1.30	–
2	1.0:1.0	195	62.5	1.0:0.88	6 200	1.63	–
3	1.0:0.67	240	57.0	1.0:0.35	5 200	1.60	–96.8
4	1.0:0.50	260	68.9	1.0:0.32	9 600	1.64	–142.3
5	1.0:0.33	200	69.9	1.0:0.26	7 100	1.50	–173.6
6	1.0:0.20	210	78.1	1.0:0.17	9 700	1.30	–185.6

^{a)} [Catalyst]/[**1**–**5**] = 0.05, reaction temperature: 30 °C.

^{b)} Fraction insoluble in methanol.

^{c)} Determined from ¹H NMR spectra.

^{d)} Determined by GPC with water as eluent using pullulan standards, sample concentration = 0.1 mg · mL⁻¹.

^{e)} Measured by polarimetry in water, *c* = 1.0 g · dL⁻¹ at 20 °C.

added to a solution of **1** (0.143 g, 0.360 mmol) in water (0.20 mL) in this order at 30 °C. After the mixture was stirred at 30 °C for 18 h, the reaction mixture was concentrated by evaporation and dried under reduced pressure. The residue was dissolved in a small amount of DMSO and the solution was poured into a large amount of methanol to precipitate the polymeric product. The precipitate was isolated by filtration and dried under reduced pressure to give **8** (0.164 g) in 62.0% yield.

¹H NMR (DMSO-*d*₆ + D₂O): δ = 0.93 (CH₃CH₂CH₂), 1.16 (CH₃CH₂N), 1.25 [CH₂(CH₂)₈], 1.50 (CH₂CH₂C=O), 3.0–4.5 (sugar protons and =C–CH₂), 6.16 (HC=), 7.0–8.0 (aromatics).

Cell Culture Experiment

Human aortic endothelial cells (HAECs) were purchased as cryopreserved samples of third passage (Lot: 3F1346) from Cambrex (Wakersville, MD, USA). The HAECs were subcultured once and stored in liquid nitrogen until cell culture experiment. The HAECs used in the experiment were fourth passage. Each well of a 12-well plate of polystyrene (Iwaki) was filled with 1 mL of a supplemented culture medium (EGM-2; Lot: 08103123, Cambrex) and equilibrated at 37 °C in a humidified incubator under 5% CO₂ for 30 min before cell seeding. After the frozen cells were thawed at 37 °C, 10 μ L of the cell suspension was seeded in each well. The initial cell density was 2.2×10^3 cells · cm⁻². Cell viability assessed by the trypan blue exclusion test was 83% for the cell suspension. The cell seeded plates were placed in a humidified incubator at 37 °C under 5% CO₂. The HAECs were cultured for 48 h. Cell culture mediums were replaced with fresh medium 24 h after cell seeding. At 48 h after cell seeding, cell culture mediums were each replaced with an aqueous suspension of copolymer **8**. Then the HAECs were cultured in the polymer suspension for 1, 6, and 24 h in a humidified incubator at 37 °C under 5% CO₂ to study cellular uptake of nanoaggregates of copolymer **8**. For fluorescence

microscopy observation, the cells were fixed by immersion in 10% formaldehyde neutral buffer solution (Nacalai Tesque) at room temperature (22 °C) for 15 min and were washed three times with phosphate-buffered saline (PBS; Gibco). Fluorescence images of the cells were taken with a fluorescence microscope (IX71; Olympus) equipped with a CCD camera (DP70; Olympus). Fluorescence intensity of the incorporated copolymer **8** was measured by integrating the fluorescence intensity observed at each pixel of the fluorescence images using image analysis software (Fluoview ver. 5.0; Olympus).

Measurements

NMR spectra were recorded on a JEOL ECA 600 spectrometer. Optical rotations were measured with a Jasco P-1030 digital polarimeter. GPC analyses were performed by using a TOSOH 8012 with refractive index detection under the following conditions: Shodex Asahipak GF-310HQ column with water as eluent at a flow rate of 0.5 mL · min⁻¹. The calibration curve was obtained using pullulan standards. CD and UV-vis spectra were measured in a quartz cell (thickness 1 cm) at room temperature using a Jasco J-820 spectropolarimeter and Shimadzu UV160A spectrophotometer, respectively. The SEM images were obtained using a Hitachi S-4100 electron microscope. The DLS measurement was performed on a Zetasizer 3000 (Malvern Instruments). Fluorescence spectra were obtained on a fluorescence spectrometer (Shimadzu) using a quartz cuvette (1-mm path length).

Results and Discussion

Copolymerization of **1** with **5**

The polymerization of monosubstituted acetylene derivatives has been widely investigated using Rh complex catalysts, which enables stereoselective synthesis of the corresponding polyacetylenes of the *cis*-isomers.^{116f} As

already reported in our previous publication. **1** was polymerized using $(nbd)Rh^+B^-(C_6H_5)_4$ as the catalyst at around 25 to 50 °C in a mixed solvent of THF and water (9:1, v/v).^{11,21} In this study, we performed the copolymerization of **1** with **5** under similar conditions. The copolymerization with various feed ratios of **1** to **5** was carried out using the Rh catalyst (5.0 mol-% for **1** + **5**) at 30 °C in a THF-water mixed solvent under argon. After polymerization, the resulting mixture was concentrated and then dissolved in DMSO. The solution was poured into a large amount of methanol to precipitate the polymeric product. The precipitate was isolated by filtration and dried under reduced pressure to give the copolymer **6** (Table 1). The copolymer was soluble in water and DMSO, and its \bar{M}_n value was estimated by GPC analysis with water as eluent using pullulan standards. Figure 1a shows the ¹H NMR spectrum of the copolymer (entry 1, Table 1) measured in DMSO-*d*₆. The signals due to the sugar and alkyl protons

are observed at around δ 3.0–4.3 (signals e–h) and δ 0.84, 1.21, 1.45, 2.15 (signals a–d), respectively. In addition to these signals, signal i ascribed to the main-chain proton of $-CH=C-$ appears centered at δ 6.11. The chemical shift of this signal realistically corresponds to the *cis*-isomer. Furthermore, there is no signal due to the *trans*-isomer at lower magnetic field from the *cis*-signal. The NMR results support structure **6** as that of the copolymer, which is mainly composed of *cis*-isomer. The unit ratio of the copolymer is calculated by the integrated ratio of signal a and signal i.

Table 1 shows the copolymerization results obtained by the various feed ratios of **1** to **5**. The yields and the \bar{M}_n values are 57.0–78.1% and 5 200–9 700, respectively. The unit ratios of **5** in the copolymers increase with increasing molar ratios of **5** in the feeds. In all cases, however, the ratios of **5** in the copolymers are lower than those in the feeds. This is probably because the copolymers with higher

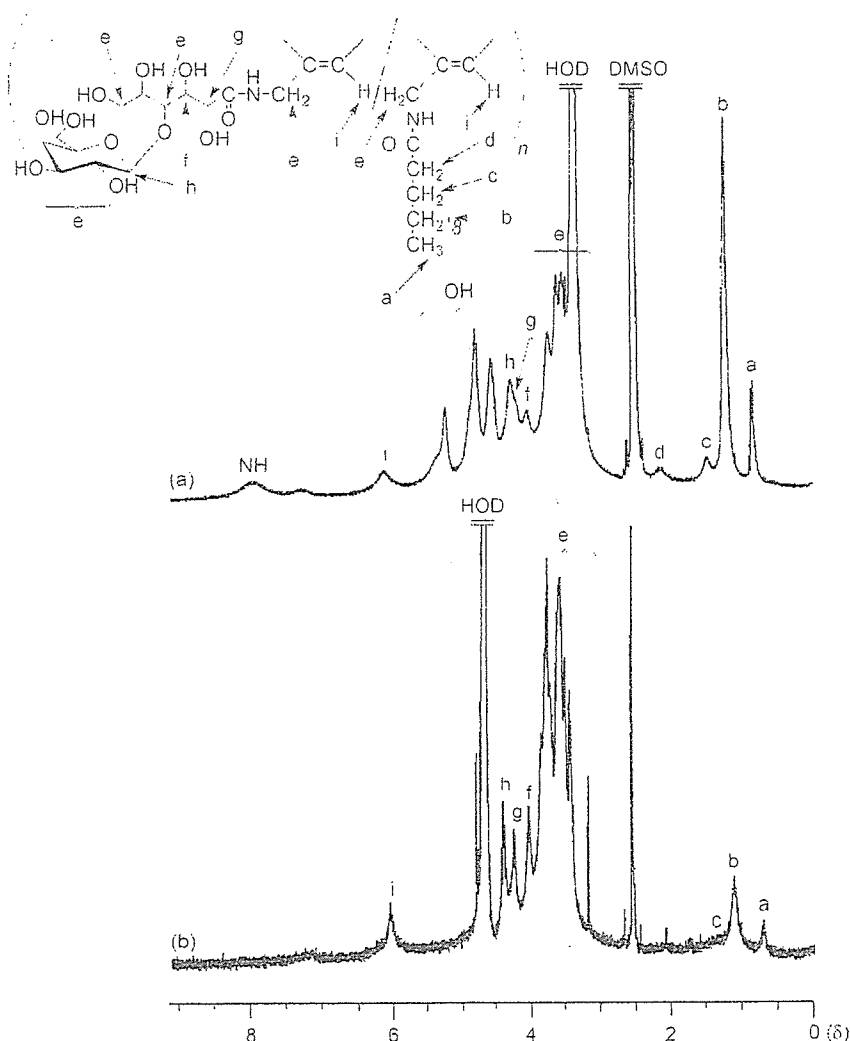


Figure 1. ¹H NMR spectra of copolymer **6** (entry 1, Table 1) in DMSO-*d*₆ (a) and D₂O (b).

contents of the unit 5 are lost as methanol-soluble fractions during the isolation procedure. The optical rotations of the copolymers with the higher contents of the sugar units were larger than those with the lower contents.

Formation of Molecular Aggregates in Water

When the copolymerization was followed by thin-layer chromatography (TLC) on silica gel (methanol: chloroform = 2:1 for 1; hexane: ethyl acetate = 1:1 for 5), 1 appeared to be consumed at the early stage of the reaction; subsequently, the consumption of 3 at a later stage was confirmed. This indicated that 6 had the block copolymeric sequence between the unit 1 and the unit 5 rather than in random style. In fact, the intensities of the alkyl signals a–d in the ^1H NMR spectrum of 6 in D_2O (Figure 1b) are obviously lower than those of the same copolymer measured in $\text{DMSO}-d_6$ (Figure 1a). The NMR results suggest formation of micelle-like aggregates having the outer hydrophilic sugar residues and the inner hydrophobic lauryloyl groups in water, and this was attributed to the block copolymeric sequence. The formation of molecular aggregates of 6 in water was also confirmed by the GPC measurements on aqueous solutions of 6 (entry 5, Table 1, the unit ratio of 1 to 5 = 1.0:0.26) ranging in concentration from 0.05 to 9.0 $\text{mg} \cdot \text{mL}^{-1}$. Figure 2 shows the relations of the \bar{M}_n values to the sample concentrations in the GPC experiments. The \bar{M}_n values increase from ca. 6300 to ca. 11000 for concentrations higher than 0.70 $\text{mg} \cdot \text{mL}^{-1}$. These data suggest the formation of molecular aggregates for the higher concentrations of 6 in water. The molecular aggregates of 6 were directly observed by SEM. The SEM image of the spin-coated sample from the aqueous solution of 6 (entry 4, Table 1, the unit ratio of 1 to 5 = 1.0:0.32) on aluminium plate (Figure 3) shows the particle-type molecular aggregates with average diameters of 20–40 nm. The

particle sizes were also confirmed by DLS measurement. The mean particle diameter of the sample shown as entry 4 in Table 1 was 85.2 ± 14.1 nm. The difference in the aggregate sizes obtained by SEM and DLS can be attributed to the difference in the sample condition: dry for SEM and wet for DLS.

Secondary Conformation of 6

We already reported in our previous report that the CD spectrum of the homopolymer 2 in water showed the positive Cotton effect at 330 nm corresponding to the main-chain UV-vis absorption.^{11,21} This indicated the possibility for formation of a one-handed helical conformation in the main chain of 2. In this study, the CD analysis was also performed to reveal the secondary conformation of copolymer 6. Figure 4 shows the CD spectra of 6 (entry 5, Table 1, unit ratio of 1 to 5 = 1.0:0.26) in comparison with those of copolymer 4 (unit ratio of 1 to 3 = 1.0:0.39) measured in DMSO and water at room temperature. The CD spectrum of 6 in DMSO (Figure 4a) shows the positive Cotton effect at 360 nm, corresponding to the main-chain UV-vis absorption. The positive Cotton effect also appeared at this region in the CD spectrum in water (Figure 4b), although its intensity was lower than that in DMSO. It has been reported that the helical structure of poly(*N*-propargylamide)s is stabilized by the intramolecular hydrogen bonds between the pendant amide groups.^{11,71} In polar solvents such as DMSO and water, therefore, the hydrogen bonds are readily broken to effect destabilization of the helical structure. The helical conformation of 6 in polar solvents is probably stabilized by the bulky substituents in the side chains of sugar and lauryloyl groups. These bulky groups shield the hydrogen bonds from the solvents, which consequently stabilizes the helical structure. This reasoning is also supported by comparison of the CD spectra of copolymer 4 (gray lines in

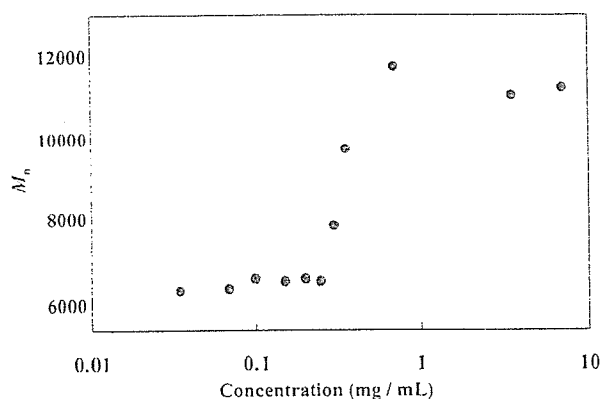


Figure 2. \bar{M}_n values versus sample concentrations in GPC measurements with water as eluent (entry 5, Table 1).

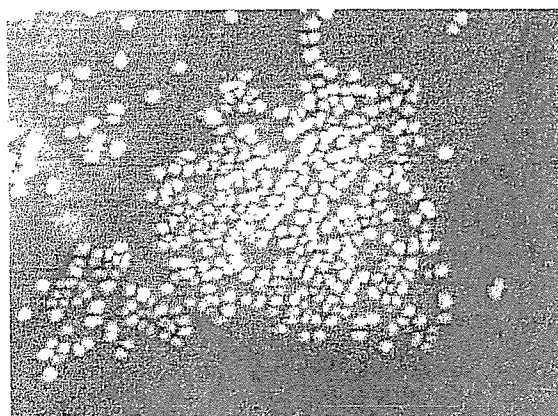


Figure 3. SEM image of 6: the sample was prepared by spin coating of the dispersed solution of 6 (entry 4, Table 1) in water.

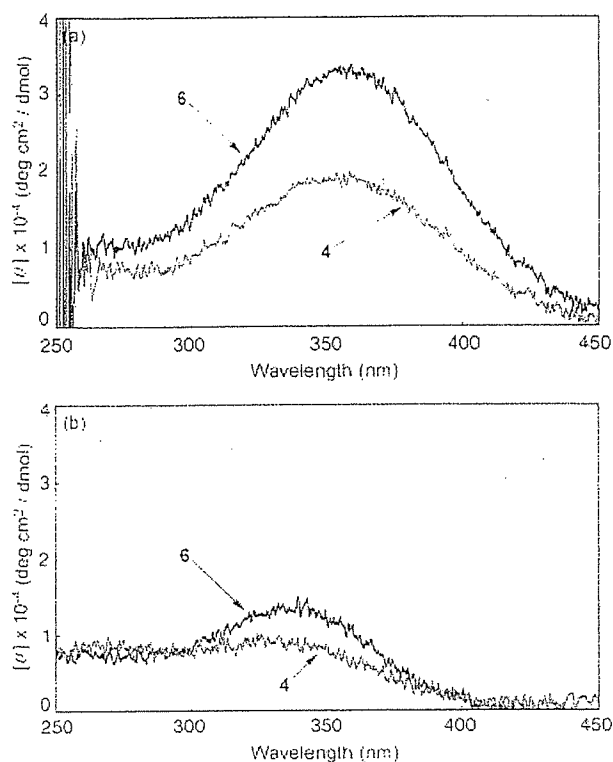
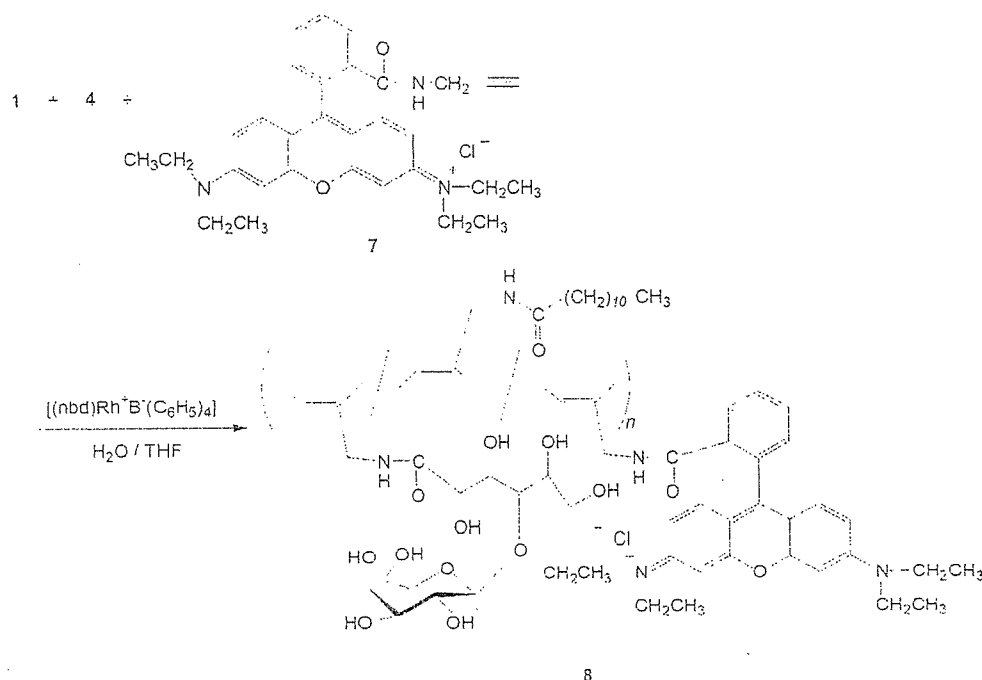


Figure 4. CD spectra of **6** (entry 5, Table 1, unit ratio: 1:5 = 1.0:0.26) and **4** (unit ratio: 1:3 = 1.0:0.39) in DMSO (a) and water (b) ($c = 0.2 \text{ mmol} \cdot \text{L}^{-1}$).

Figure 4a, b), which has the less bulky hexanoyl groups. The CD spectra of **4** show weaker Cotton effects than those of **6**, indicating the stabilization of the helical conformation as a result of the bulkiness of the pendant groups.

Cellular Uptake of Amphiphilic Copolymer **8**

To evaluate cell uptake of the copolymer by fluorescence microscopy, the rhodamine B dye moiety was introduced into the amphiphilic copolymer. First, an *N*-propargylamide derivative **7** having a pendant rhodamine B moiety was prepared by condensation of *N*-propargylamine hydrochloride with rhodamine B in the presence of a condensing agent (1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride) in methanol. Then, the isolated **7** was copolymerized with **1** and **5** under conditions similar to those described above (Scheme 3). Although **7** did not have homopolymerizability by Rh catalyst, the unit from **7** was slightly incorporated into the resulting terpolymer by the copolymerization. The existence of the rhodamine B moiety in the obtained terpolymer **8** was confirmed by appearance of the signals due to methyl protons of $\text{N-CH}_2\text{CH}_3$ as well as the aromatic protons in the ^1H NMR spectrum of the product. However, the intensities of the signals were too weak to determine the exact content of the dye moiety in the copolymer by the integration ratio. For comparison, hydrophilic copolymer **9** was synthesized by copolymerization of monomer **1** with monomer **7** using Rh catalyst (Figure 5).



Scheme 3. Terpolymerization of **1**, **5**, and **7**.

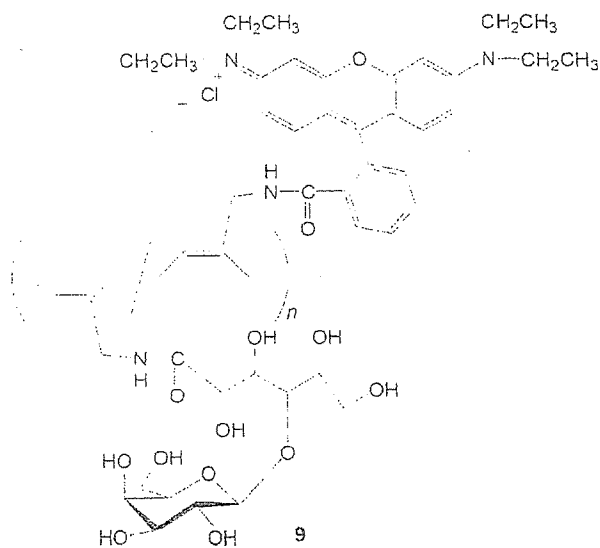


Figure 5. Structure of copolymer 9.

The cell uptake of terpolymer **8** was studied by culturing human aortic endothelial cells (HAECs) in a culture medium containing the terpolymer **8**. Ten milligrams of **8** was suspended in 10 mL of culture medium EGM-2 and stirred for 3 h at room temperature. The suspension of **8** was sonicated at 25 W and 40 kHz for 5 min in an ultrasonic bath. The sonication was repeated twice. The obtained suspension was filtered through membrane filters

with the pore sizes of 0.45 μm and 0.2 μm for sterilization. An aqueous solution of polymer **9** was prepared by following the above procedure. To evaluate the concentration of **8**, a calibration curve (data not shown) was obtained by using the aqueous solution of **9** ($1 \text{ mg} \cdot \text{mL}^{-1}$) as a standard polymer sample to relate concentration to fluorescence intensity. The concentration of **8** was estimated to be $0.09 \text{ mg} \cdot \text{mL}^{-1}$ by using the calibration curve. The DLS measurement revealed that the polymer aggregates (nanoparticles) of **8** have a mean diameter of $114.9 \pm 32 \text{ nm}$ in a culture medium containing 10% bovine serum. HAECs were exposed to the polymer aggregates of **8** while they were cultured in the culture medium containing the amphiphilic polymer **8**. After the prescribed period of culture, 1, 6, or 24 h, the HAECs were fixed in 10% formaldehyde neutral buffer solution for microscope observation. Figure 6 shows phase contrast (a), fluorescence (b), and merged (phase contrast + fluorescence) (c) images of HAECs after 24 h of incubation. The merged image demonstrates that red fluorescent light of rhodamine B was emitted from the sites where HAECs were located. This indicates that the polymer aggregates of **8** were incorporated into HAECs. The fluorescence images at each time of incubation are shown in Figure 7. The fluorescence images get brighter with the incubation time. To quantitatively evaluate cell uptake of nanoaggregates, fluorescence intensity per image (1360×1024 pixels) was determined by integrating the brightness at each pixel of the fluorescence image using image analysis software. The

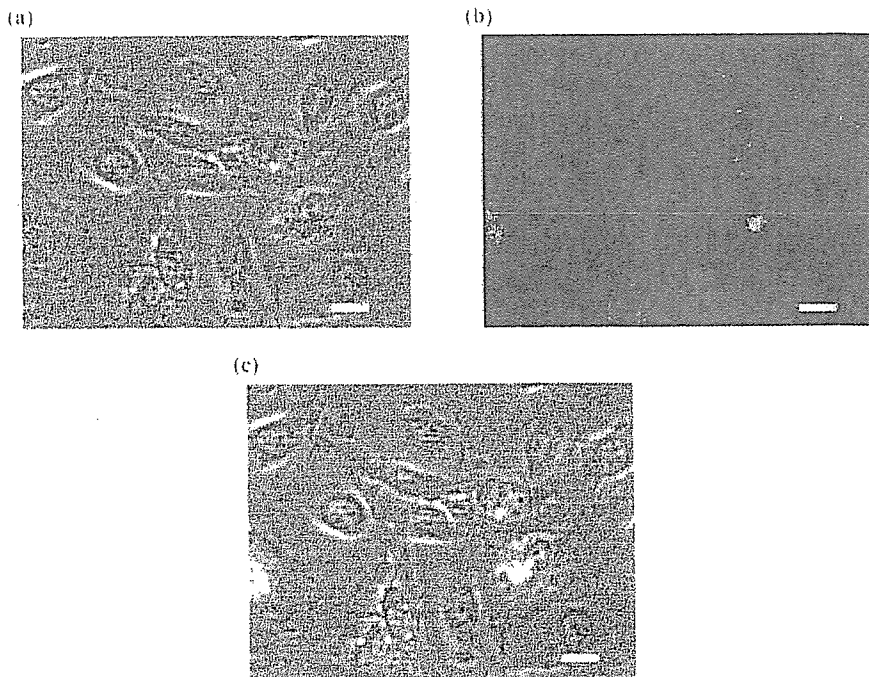


Figure 6a–c. Localization of rhodamine B-labeled copolymer **8** in human aortic endothelial cells. Phase contrast image (a), fluorescence image (b), and merged image of (a) and (b). Bars: 20 μm .

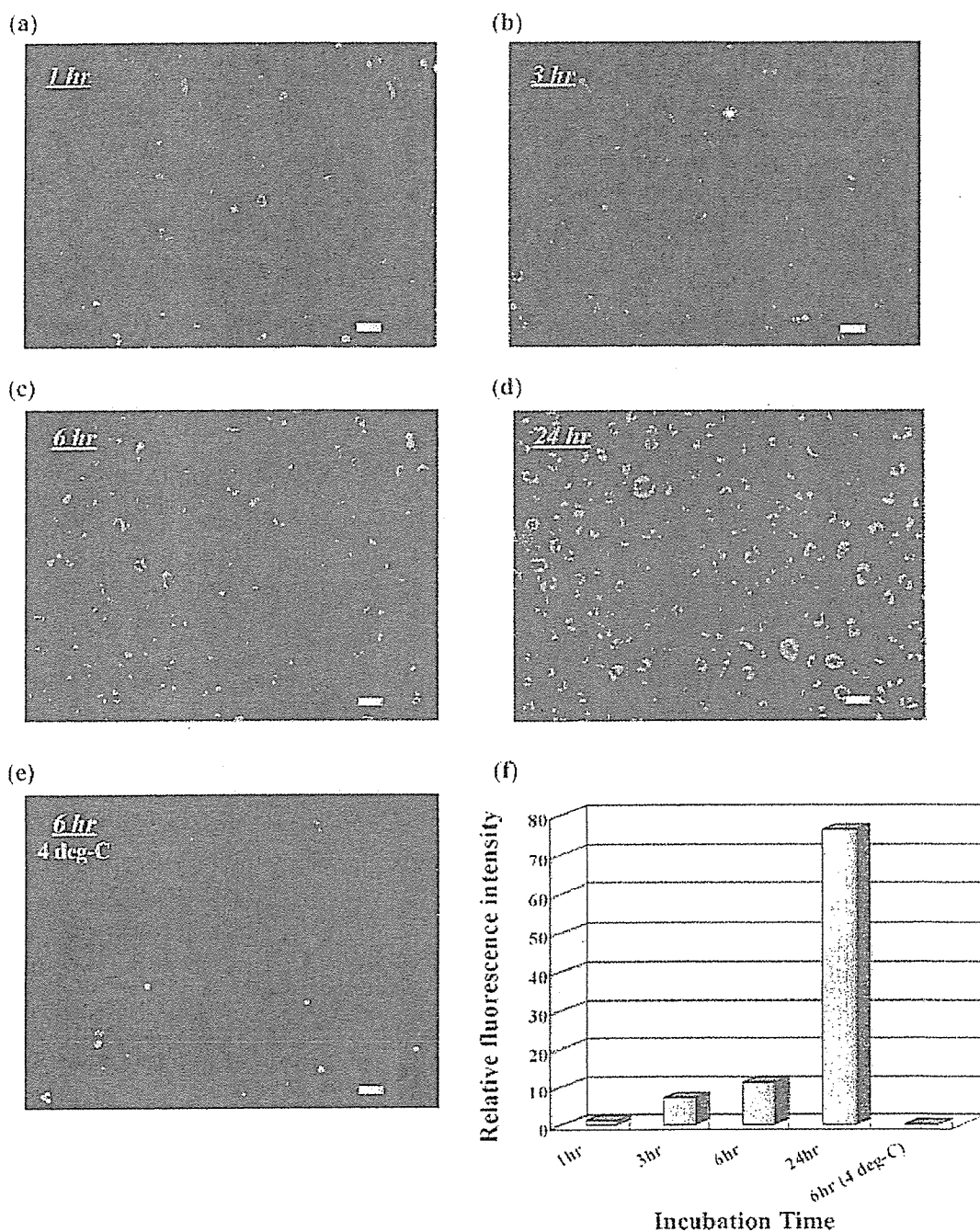


Figure 7a–f. Uptake of rhodamine B-labeled copolymer 8 by human aortic endothelial cells: incubation time, 1 h (a), 3 h (b), 6 h (c), 24 h (d) (incubation at 37 °C), and 6 h (incubation at 4 °C) (e). Bars: 20 μ m. The uptake is quantitatively represented as relative fluorescence intensity in the bar graph (f).

fluorescence intensity was normalized in a ratio of the fluorescence intensity at each incubation time to that at 1 h of incubation. The ratio was termed as the relative fluorescence intensity. The time course of the relative fluorescence intensity is shown in Figure 7f and indicates that HAECs incorporated progressively more nanoaggregates of 8 during the incubation time. The relative

fluorescence intensity was 7-fold at 3 h, 11-fold at 6 h, and 76-fold at 24 h of incubation. In contrast, the relative fluorescence intensity dropped considerably and was only 12% of the control level (1 h at 37 °C) when HAECs were exposed to the nanoaggregates of 8 at 4 °C for 6 h. The fact that the temperature triggered a dramatic decrease in the relative fluorescence intensity suggests that the nanoag-

gregates are incorporated into HAECs by endocytosis. Endocytosis is known as a cellular process that is coupled with temperature-dependent metabolic activities.^[18] Furthermore, it is known that some receptors and membrane microdomains of endothelial cell are involved in endocytosis.^[19] However, it is not clear which of the endocytic pathways is responsible for the uptake of the nanoaggregates. This issue is now under investigation.

Conclusion

In this study, we investigated synthesis of the amphiphilic poly(*N*-propargylamide) **6** containing both sugar residues (hydrophilic part) and long alkyl chains (hydrophobic part) in order to develop a novel nanoaggregate based on the self-organization of amphiphilic polymers with rigid backbone. The desired polymer was prepared by copolymerization of the two *N*-propargylamide monomers **1** and **5** having a galactose residue and a lauryloyl group, respectively, catalyzed by a Rh complex. The GPC, ¹H NMR, SEM, and DLS analyses of the resulting copolymers indicated formation of the nanoparticles in water. The formation of the one-handed helical conformation of the copolymer in both DMSO and water was confirmed by the CD spectra. An amphiphilic poly(*N*-propargylamide) containing fluorescent dyes was newly designed to evaluate cell uptake of nanoparticles of the amphiphilic copolymer by fluorescence microscopy. The *N*-propargylamide monomer **7**, having a rhodamine B dye moiety, was prepared and copolymerized with **1** and **5**. Human aortic endothelial cells (HAECs) were cultured in a medium containing the fluorescent-dye-labeled amphiphilic copolymer. Cell uptake of the copolymer was confirmed by red fluorescence emission from each of the HAECs. Progressive uptake was observed during the incubation period. When the cell culture experiment was conducted at 4 °C, the fluorescence intensity of the red emission was considerably lowered. This indicates that the cell uptake is inhibited at 4 °C and that this uptake process should occur in an endocytic pathway rather than by simple adsorption to the plasma membrane of HAECs. We are now synthesizing a fluorescent-dye-labeled hydrophilic copolymer of monomer **1** with monomer **7** to study preferential cell uptake of the nanoaggregates of the amphiphilic copolymer. In the future, we anticipate that nanoparticles

will be able to be preferentially endocytosed into cells rather than monomeric chains of water-soluble copolymer. If this does occur, the nanoparticles of the amphiphilic copolymer will be a promising nanocarrier for drug delivery.

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Postexercise VO_2 “Hump” phenomenon as an indicator for inducible myocardial ischemia in patients with acute anterior myocardial infarction

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Abstract

Objectives: At exercise testing with respiratory gas analysis in patients with inducible myocardial ischemia, we have occasionally observed abnormal transient oxygen uptake (VO_2) components with a characteristic “Hump”-shaped morphology early after exercise, which may serve as an index for inducible ischemia. We examined this hypothesis in patients with anterior q-wave myocardial infarction in whom the accuracy to identify ischemia by exercise ECG is limited.

Design: From patients with acute anterior q-wave infarction but without clinically overt heart failure who underwent pre-discharge exercise testing, we examined patients with (Group-I, $n=30$) and without (Group-N, $n=29$) inducible ischemia. To identify “Hump”, postexercise VO_2 (up to 4 min) standardized for peak VO_2 was exponentially fitted with use of peak VO_2 and VO_2 of 90–240 s, yielding “expected VO_2 ”. “D-curve” was obtained by subtracting “expected VO_2 ” from measured VO_2 .

Results: Although exercise-induced ST depressions more frequently appeared in Group-I (27%) than in Group-N (3%, $p<0.05$), the prevalence was low. D-curve peaked later ($p<0.01$) and its value was greater ($p<0.05$) in Group-I than in Group-N. When “Hump” was defined to be present if D-curve peaked ≥ 40 s and its peak value $\geq 15\%$, it was far more frequently found in Group-I ($n=17/30$) than in Group-N ($n=1/29$, $p<0.01$). Thus, “Hump” could diagnose inducible ischemia with a sensitivity of 57% and a specificity of 97%.

Conclusions: Although not highly sensitive, postexercise VO_2 “Hump” with its peak occurring around 60 s after exercise is a specific marker for inducible ischemia. The identification may be useful, particularly in patients with limited accuracy of exercise ECG such as those with q-wave anterior infarction.

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Keywords: Exercise test; Respiratory gas analysis; Myocardial ischemia; Oxygen uptake

1. Introduction

In patients after acute myocardial infarction, the evaluation of inducible myocardial ischemia is important in the subsequent management, [1–3]] however, the diagnostic accuracy of exercise ECG is known to be limited in those patients [4–8]]. This is particularly crucial in patients with q-wave anterior infarction, in whom exercise-induced ST-

segment depression would be often obscured by the presence of q-wave in the precordial leads.

Exercise testing with respiratory gas analysis is most often performed for evaluating the functional capacity and predicting prognosis in patients with heart failure, however, we have conducted the test in a considerable number of these post-infarct patients (approximately 200 tests/year) in our institute for more than 10 years [9]. Although the concomitant use of respiratory gas analysis is conducted mainly for the same purpose as above, postexercise oxygen uptake (VO_2) kinetics may provide useful information for detecting inducible ischemia in these patients. In practice,

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we have occasionally observed abnormal components with a characteristic “Hump”-shaped morphology in the early portion of the postexercise VO_2 decay in some of patients with evidence of inducible ischemia. The mechanism is unclear, however, it is conceivable that this phenomenon may be caused by enhanced stroke volume following resolution of ischemia during exercise, that is presumably responsible for delayed recovery of postexercise systolic blood pressure in patients with ischemia [10–15].

Abnormal VO_2 kinetics after exercise has been reported in patients with heart failure due to left ventricular dysfunction [16–18]. However, to our knowledge, no studies have examined the significance of the abnormal VO_2 kinetics after exercise occurring in association with inducible ischemia. We thus examined the diagnostic utility of this phenomenon (“Hump”) as an indicator for inducible ischemia in patients after acute anterior q-wave infarction without clinically overt heart failure.

2. Methods

2.1. Study population

From the consecutive inpatients with acute anterior q-wave myocardial infarction but without overt heart failure who underwent both pre-discharge exercise testing with respiratory gas analysis (within 3 weeks after the onset of infarction) and coronary angiography (approximately 4 weeks after the onset), we recruited the study population as follows. As a control group, we selected 29 patients (Group-N) who had no significant (>50% luminal diameter narrowing) stenosis in the coronary arteries at angiography, although 83% ($n=24/29$) of these patients had received percutaneous coronary intervention (PCI) during the acute phase of their infarction. It was assumed that this group did not have inducible ischemia. In 30 patients with abnormal coronary arteries, exercise thallium-201 scintigraphy (single-photon emission computed tomography; SPECT) showed reversible perfusion defects corresponding to the anatomic lesions demonstrated (Group-I). Of these patients, 43% ($n=13/30$) had received PCI during the acute phase, and at subsequent angiography 22 were left with single, five with double, and three with triple vessel disease (Table 1). All patients with Group-I subsequently received revascularization with either percutaneous transluminal angioplasty ($n=26$) or coronary artery bypass graft surgery ($n=4$).

We excluded patients with primary lung disease, orthopedic difficulties that precluded maximal exercise, arterio-sclerotic obliteration and significant arrhythmias including atrial fibrillation. The patients with VO_2 plateau or leveling off (defined as an increase in VO_2 of less than 50 ml/min) around at peak exercise were also excluded ($n=2$), because the present study aimed to evaluate the significance of abnormal VO_2 kinetics (“Hump”) only seen in the recovery period.

Table 1
Patients' characteristics

	Group-I (N=30)	Group-N (N=29)	p value
Sex (M/F)	26/4	22/7	NS
Age (years)	64.8	61.1	NS
LV EF (%)	39.7	43.9	NS
History of prior MI	7(23%)	2(7%)	NS
PCI therapy	13(43%)	24(83%)	<0.01
Coronary artery disease			
SVD	22(73%)	-	
DVD	5(17%)	-	
TVD	3(10%)	-	
Medication			
beta-blocker	13(43%)	6(21%)	NS
Ca antagonist	15(50%)	18(62%)	NS
Nitrate	21(70%)	10(34%)	<0.05
Digitalis	0(0%)	2(7%)	NS

Values are expressed as mean \pm SD.

LVEF, left ventricular ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention; SVD, single vessel disease; DVD, double vessel disease; TVD, triple vessel disease.

Left ventricular ejection fraction (LVEF) was similar between the two groups (Table 1). There were no significant differences in sex, age, and history of prior myocardial infarction. The use of cardiovascular drugs was similar in the two groups except for nitrate, and it was neither altered nor withheld for the exercise test. All patients gave informed consent for the study.

2.2. Exercise testing

Symptom-limited exercise testing with respiratory gas analysis was performed on an upright bicycle ergometer in a ramp fashion. After a 2-min rest, exercise was begun with a 1-min warm up at 0 W at 60 rpm, followed by 15 W incremental loading every 1 min. ECGs (V_1 , V_5 , a V_F) and heart rate (HR) were monitored throughout the testing, while recording hardcopies of 12-lead ECG every 1 or 2 min. HR and blood pressure (BP) measured by a conventional cuff sphygmomanometer were recorded at rest, at 1-min intervals during exercise, and 1, 2, and 4 min into the recovery period. All patients stopped exercise because of dyspnea and/or leg fatigue, and there was no patient in whom exercise was stopped because of angina, marked ST-segment depressions or fall of blood pressure. Patients were asked to stop pedaling soon after exercise (up to 10 s) to avoid the possible influence of cool-down exercise on recovery VO_2 kinetics.

Expired gas was measured on a breath-by-breath basis at rest, during the exercise, and recovery period (at least up to 4 min) with a respiromonitor AE280 (Minato Medical Electronics, Osaka, Japan). The system was carefully calibrated before each study. VO_2 , carbon dioxide production (VCO_2), and minute ventilation (VE) were stored in a computer hard disk every 6 s for later analysis.

We identified a significant ST-segment depression induced by exercise according to the following criteria: (1) a horizontal or downsloping ST-segment displacement at J-point ≥ 0.1 mV (2) up-sloping ST-segment displacement at 80 ms after the J-point ≥ 0.15 mV in at least 3 consecutive beats at peak exercise. A significant ST-segment elevation was defined as an upward shift of the ST-segment ≥ 0.1 mV at the J-point compared with the resting level.

2.3. Exercise SPECT

The test was performed with symptom-limited bicycle exercise. At near-maximal exercise, thallium-201 was intravenously injected and the patient was encouraged to exercise for another 1 min. SPECT images were obtained at 15 min (initial images) and 4 h (delayed images). The images were assessed by two experienced physicians unaware of the patient clinical information. Thallium-uptake was classified as normal, mildly, moderately or severely reduced, or absent. A reversible defect was defined when the classification improved by at least one category from the initial to delayed image.

2.4. Data analysis

By using our custom-made software, we evaluated abnormal manifestations (“Hump”) in early postexercise VO_2 decay (Fig. 1). To characterize “Hump”, we performed the following procedures, assuming that postexercise VO_2 decay would normally (i.e., without inducible ischemia) follow an approximately exponential curve and that

“Hump” would be expressed by the components that was not fitted by this approximation. We first standardized the time-series of VO_2 data following exercise up to 4 min for peak VO_2 . The curve was monoexponentially fitted with use of peak VO_2 and continuous VO_2 data over the period of 90–240 s; i.e., the data from 6 to 90 s were excluded from the fitting because we had observed abnormal components in this period. Nonlinear least-squares fitting was made assuming a monoexponential model: $V = Z_0 \times e^{-t/\mu} + Z_{\infty}$ (where V is the standardized VO_2 data, Z_0 is the initial standardized VO_2 above Z_{∞} , μ is the time constant, t is time after the termination of exercise and Z_{∞} is the asymptote to which standardized VO_2 decay).

The fitted curve was termed the “expected VO_2 curve” in this study (Fig. 1). To characterize “Hump” phenomenon, we obtained the “D-curve” by subtracting the expected VO_2 curve from actually measured VO_2 curve. In the D-curve that was a function of time (every 6 s) after exercise, we determined the peak value (D_{max}) in amplitude and the elapsed time at the time point of D_{max} (T_{max}). These two indices were compared between the two groups.

Systolic BP and HR at rest, at peak exercise and at the recovery period of 1, 2, and 4 min after exercise were also analyzed.

2.5. Statistical analysis

Values are expressed as mean \pm SD. Between-group differences for unpaired values were analyzed by Student's t test and by the Mann-Whitney U test when appropriate. Repeated measures of analysis of variance were used to

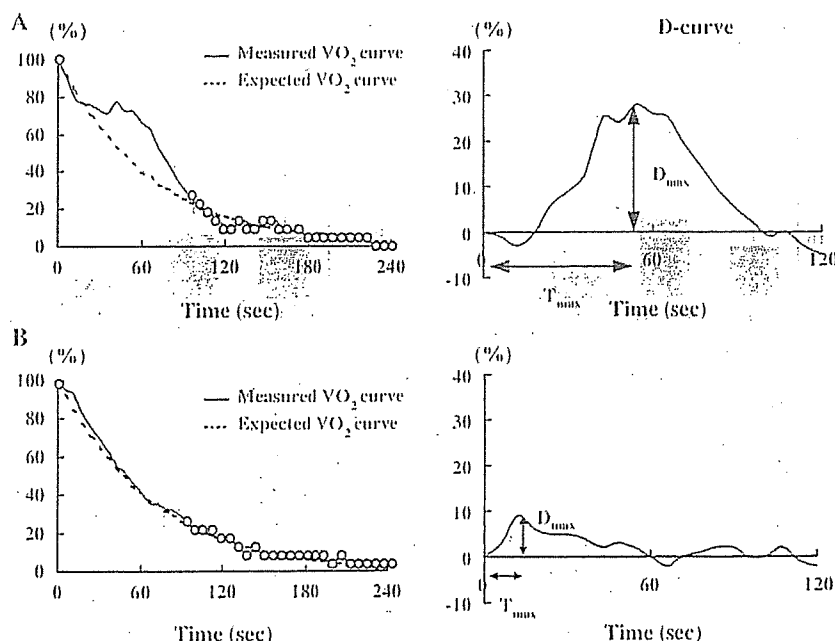


Fig. 1. Representative examples of postexercise VO_2 decay (left panel) and the derived D-curve (right panel) of two patients. Illustrated in the upper panels (A) are graphs for a patient of Group-I, and the lower (B) depict those for a patient of Group-N. After VO_2 decay curve standardized for peak VO_2 was exponentially fitted with use of peak VO_2 and VO_2 over the period of 90–240 s (“expected VO_2 curve”, broken line), we obtained D-curve by subtracting “expected VO_2 curve” from actually measured VO_2 curve. In the D-curve, D_{max} (peak value) and T_{max} (time to peak) were estimated. VO_2 = oxygen uptake.

Table 2
Exercise variables

	Group-I (N=30)	Group-N (N=29)	p-value
Rest HR (bpm)	70±12	75±17	NS
Rest SBP (mm Hg)	128±26	149±16	NS
Duration of exercise (s)	510±66	553±87	<0.05
Exercise-induced angina	3(10%)	0(0%)	NS
Peak HR (bpm)	137±27	147±24	NS
Peak SBP (mm Hg)	174±25	178±25	NS
ECG change			
ST elevation	14(47%)	16(55%)	NS
ST depression	8(27%)	1(3%)	<0.05
Peak WR (watt)	113±19	123±22	NS
Peak VO ₂ (ml/min)	1179±192	1335±278	<0.05

Values are expressed as mean±SD. HR, heart rate; SBP, systolic blood pressure; VO₂, oxygen uptake; WR, work rate.

compare the values during recovery period. When this test was significant, the Newman–Keuls post hoc test was performed for multiple comparisons. Difference in categorical variables was analyzed by chi-square analysis. A *p*-value ≤0.05 was considered statistically significant. Receiver operating characteristics curves (ROC) were used to assess the ability of T_{\max} and D_{\max} to diagnose inducible ischemia [19].

3. Results

3.1. Exercise testing results

Table 2 shows exercise parameters for Group-I and Group-N. There were no significant differences in rest HR,

rest systolic BP (SBP), peak HR or peak SBP between the two groups. Although peak work rate was similar between the two groups, the duration of exercise was shorter and peak VO₂ was lower in Group-I than Group-N (*p*<0.05, both). However, these 2 parameters were of little help for differentiating the two groups; for instance, there was considerable overlap in peak VO₂ between the groups. Only 3 patients of Group-I complained of anginal symptoms after exercise.

As for ECG parameters, the frequency of a significant exercise-induced ST-segment elevation was comparable between Group-I and Group-N (47% and 55%, respectively). Despite the presence of inducible ischemia, a significant ST-segment depression was found in only 8 of 30 patients (27%) in Group-I, although it was more frequently observed in Group-I than in Group-N (27% vs. 3%, *p*<0.05).

3.2. Comparison of postexercise VO₂

To evaluate the overall difference in postexercise VO₂ decay (D-curve) between the 2 groups, we averaged the value of D-curve over every 30 s in the first 2 min of the recovery period (Fig. 2, upper two panels). As a result, the magnitude was significantly greater in the period of 30–60 s than in the period of 0–30 s in Group-I (13.7%±8.2% vs. 8.7%±5.1%, *p*<0.01, Fig. 2A), whereas such a difference was not observed in Group-N (11.0%±5.2% vs. 10.2%±5.0%, NS, Fig. 2B).

To characterize the “hump” phenomenon in D-curve, the peak value (D_{\max}) and its elapsed time (T_{\max}) were compared between the groups. T_{\max} was significantly longer in Group-I than in Group-N (42.6±14.6 vs. 31.2±13.2 s,

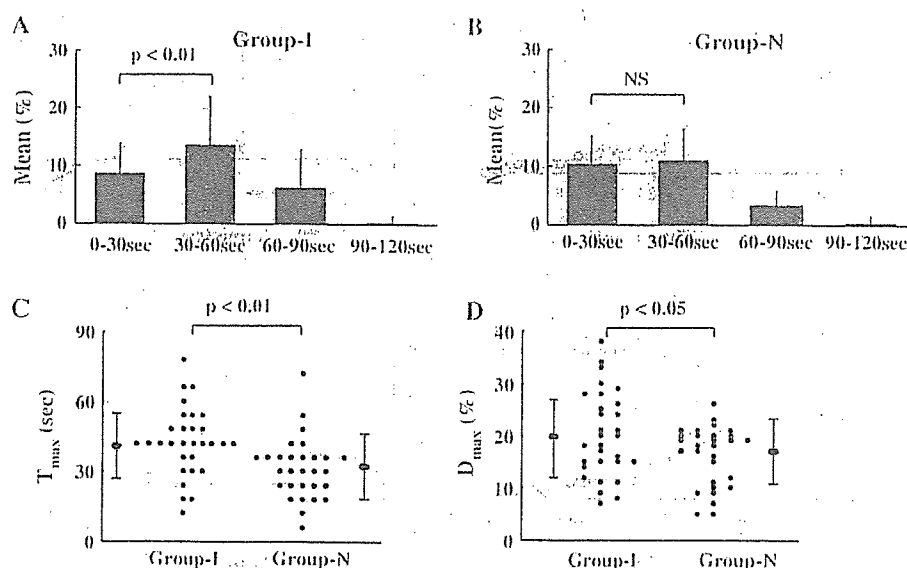


Fig. 2. The time-course changes in the D-curve for the first 2 min of recovery (upper panel) and comparisons of T_{\max} (C, lower left panel) and D_{\max} (D, lower right panel) between Group-I and Group-N. The D-curve values averaged over every 30 s were shown as bar graphs for Group-I (A, upper left panel) and Group-N (B, upper right panel). The mean for 30–60 s was greater than that for 0–30 s in Group-I (*p*<0.01), but not in Group-N. T_{\max} was longer in Group-I than in Group-N (*p*<0.01), and D_{\max} was greater in Group-I than in Group-N (*p*<0.05).

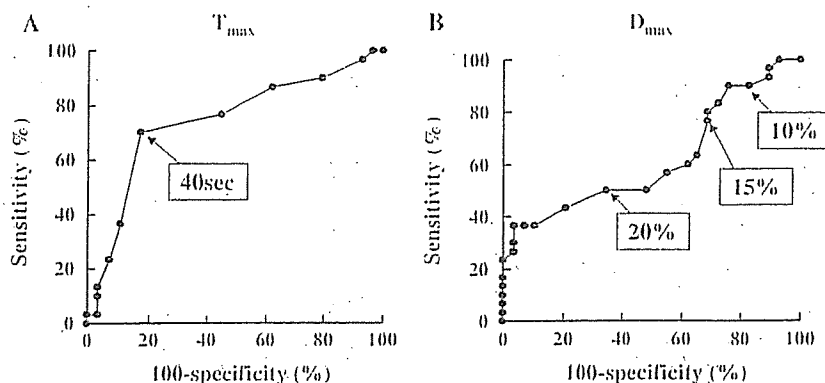


Fig. 3. ROC curves describing the ability for the diagnosis for inducible ischemia using T_{\max} (A, left panel) and D_{\max} (B, right panel).

$p < 0.01$, Fig. 2C), and D_{\max} was also significantly greater in Group-I than in Group-N ($20.1\% \pm 8.1\%$ vs. $16.2\% \pm 5.7\%$, $p < 0.05$, Fig. 2D).

We used ROC analysis to determine the optimum cutoff values of these two parameters for identifying Hump phenomenon, i.e., inducible ischemia. When ROC analysis was conducted separately for T_{\max} (Fig. 3A) and D_{\max} (Fig. 3B), we could easily recognize 40 s as the optimum cutoff for T_{\max} in differentiating the two groups, whereas such an optimum point was not found for D_{\max} . Since it is considered that the combination of the two optimum cutoff values, each of which was determined by a separate ROC analysis, would not necessarily have the highest discriminative power, ROC analysis of T_{\max} was repeated for a given D_{\max} , while shifting D_{\max} every 1% from 0% to 40%. As a result, when assuming that the best cutoff was defined as the point with highest sum of sensitivity and specificity, the combination of $D_{\max} \geq 10\%$ and $T_{\max} \geq 40$ s (from 37 to 41 s, because temporal resolution was 6 s) could most accurately discriminate Group-I from Group-N (sensitivity 67%, specificity 90%, accuracy 78%, Table 3). When $D_{\max} \geq 15\%$ was applied (Fig. 4), the specificity was increased to 97%, although the sensitivity decreased to 57%.

3.3. Comparison of postexercise SBP

Although resting and peak SBP were not different between the two groups, Group-I had a higher SBP than Group-N at 2 and 4 min of recovery (both $p < 0.05$, Fig. 5A).

Furthermore, among patients of Group-I, patients with “Hump”, defined as $T_{\max} \geq 40$ s and $D_{\max} \geq 15\%$, had a higher SBP at 1 and 2 min of recovery than those without “Hump” (both $p < 0.05$, Fig. 5B). A significant decrease in

SBP from peak exercise was found at 1 min of recovery in patients without “Hump”, but not in those with “Hump”.

4. Discussion

Although exercise-induced ST-segment depression is a cardinal index to identify inducible myocardial ischemia on exercise testing, the diagnostic accuracy by the standard ST criteria is limited in post-infarct patients [4–8]. In our population consisting of patients after acute anterior q-wave myocardial infarction, ST-segment depressions during exercise appeared in only 27% of patients with inducible myocardial ischemia (Group-I), that was comparable to the incidence of ST depression reported previously [6–8]. The most likely explanation for the low sensitivity of ST depression in these patients is that exercise-induced ST elevation over q-wave leads, related to left ventricular asynergy, would mask the appearance of ST depression [5,20]. In patients with anterior q-wave myocardial infarction, the vector of ST-segment depression, which most frequently appears in the left precordial leads of V_4 to V_6 ,

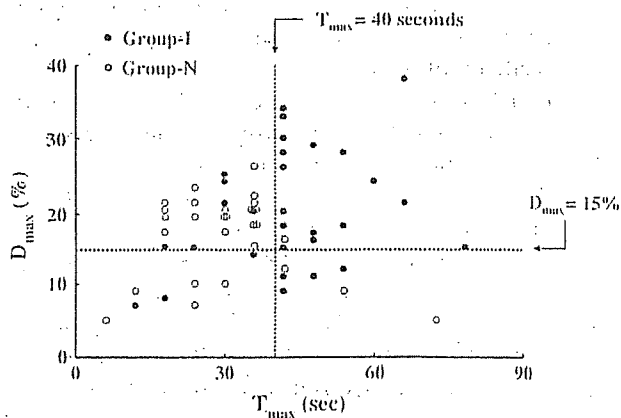


Fig. 4. Scatterplots showing D_{\max} plotted against T_{\max} for each patient. Approximately half (57%) of patients of Group-I (closed circles) were distributed in the limited area of $T_{\max} \geq 40$ s and $D_{\max} \geq 15\%$, whereas only one patient in Group-I (open circle) was distributed in this area. Using this criterion, we could diagnose the presence of inducible ischemia (Group-I) with a sensitivity of 57% and a specificity of 97%.

Table 3
Diagnostic accuracy of “Hump” for identifying inducible ischemia (Group-I)

T_{\max} (s)	≥ 40	≥ 40	≥ 40
D_{\max} (%)	≥ 10	≥ 15	≥ 20
Sensitivity	67% (20/30)	57% (17/30)	37% (11/30)
Specificity	90% (26/29)	97% (28/29)	100% (29/29)
Accuracy	78%	76%	68%

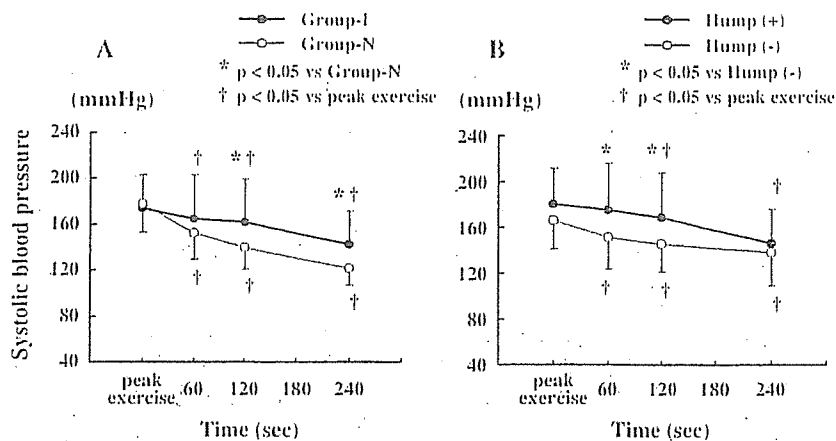


Fig. 5. Comparisons of systolic blood pressure time-course after exercise between Group-I and Group-N (A, left panel), and between the Group-I patients with and without “Hump” phenomenon (B, right panel).

may be electrically canceled by the opposite vector of ST elevation occurring in association with ventricular asynergy.

It is generally accepted that exercise-induced ST-segment elevation over post-infarct q-wave leads occurs in association with severe left ventricular asynergy, however, several studies indicated that exercise-induced ST-segment elevation might occur due to induced ischemia [21,22]. In the present study, we observed a similar prevalence of ST-segment elevation of ≥ 0.1 mV in Group-N (55%) and Group-I (47%, NS), suggesting that this index alone is of no use for identifying ischemia. We cannot exclude the possibility that ST-segment elevation might be caused by ischemia in some patients of Group-I, because three Group-I patients presented both exercise-induced ST-segment elevation and depression.

On the other hand, the present study has shown that abnormal transient VO_2 components after exercise, “Hump” phenomenon defined by our method described above, is a useful indicator for inducible ischemia in patients with acute anterior infarction. When the combination of $T_{\text{max}} \geq 40$ s and $D_{\text{max}} \geq 15\%$ were used for the definition of “Hump”, we could diagnose inducible ischemia with sensitivity of 57%, specificity of 97%.

Abnormal postexercise VO_2 kinetics has been reported previously in patients with cardiocirculatory disorders. Hayasida et al. and other authors reported that the recovery of VO_2 was prolonged in patients with left ventricular dysfunction and that the time-course of VO_2 decay after exercise was closely related to exercise capacity [16–18,23–27] and prognosis [28]. Delayed energy store recovery in the skeletal muscle [22,24] and prolonged decrease in cardiac output [23,25–27,29] are considered to be involved in the genesis of this abnormal VO_2 recovery.

To our knowledge, there have been no published studies specifically examining the significance of exercise-induced ischemia on the postexercise abnormal VO_2 kinetics. Abnormal components of our interest, i.e., “Hump”, is characterized by a transient convex bulge in the limited portion of VO_2 decay at around 1 min. The occurrence

seems to appear not immediately but soon after the termination of exercise, generally lasting approximately 1 min. Previous reports estimated abnormal VO_2 kinetics by estimating the whole VO_2 decay with use of temporal parameters such as half time, [18,24,26] time constant [16,23,25] or cumulative area [28]. Since these measures are clearly unsuitable for our purpose, the non-exponential components (i.e., D-curve), that were derived by subtraction assuming that the abnormal components would be superimposed upon the monoexponential VO_2 decay, were compared between Group-I and Group-N. As a result, in Group-I, the mean value averaged over 30–60 s was greater than that averaged over 0–30 s, whereas such a difference was not found in Group-N (Fig. 2, upper two panels). Furthermore, the D-curve peaked later and its maximal value was greater in Group-I compared with Group-N (Fig. 2, lower two panels). Thus, the group difference of the D-curve with respect to the amplitude and temporal profile enabled us to identify the presence of inducible ischemia by the criterion shown in Table 3.

It was somewhat unexpected that some of patients without inducible ischemia (Group-N) showed a sizable amount of non-exponential components in the very early period of recovery up to 30 to 40 s. Our expectation was that non-exponential components in Group-N would be negligibly small, because the VO_2 decay curve should be closely fitted by the monoexponential model. This discrepancy indicates that postexercise VO_2 decay is not necessarily monoexponential in shape, and may be more precisely fitted by other mathematical models such as a sigmoidal model, even in the absence of inducible ischemia, although simple noise inherent in the measurements might be also related to the components. Impairment of LV function due to infarction and peripheral dysfunction caused by the immobilization (deconditioning effects) in the acute phase of myocardial infarction would contribute to the loss of the rapid fall of VO_2 immediately after exercise.

Several studies indicated that, in patients with inducible myocardial ischemia, an abnormal systolic blood pressure

response is observed not only during exercise but also during the recovery phase; a lesser decrease in systolic BP in the early recovery period, [10–15] which was in agreement with our results that the prolonged time-course of the postexercise decrease in systolic BP was seen in Group-I. It was also reported that, in these patients, stroke volume during exercise progressively decreased according to the development of severe ischemia, and it did not decrease but rather increased during the early period of recovery. [29–31] Although this paradoxical increase following exercise may occur either by a decrease in peripheral vascular tone after exercise [29,30] or by an improvement of LV function following the resolution of induced ischemia, [31], dynamic changes in the former factor are unlikely to transiently occur. The fact that, in Group-I, systolic BP at 1 min of recovery did not significantly decrease from peak exercise only in patients with “Hump”, but not in patients without “Hump” reasonably suggests the direct role of enhanced stroke volume soon after exercise on the occurrence of “Hump”.

The exact mechanism responsible for “Hump” is speculative, however, a recent study by Belardinelli et al. may provide a clue to the mechanism [32]. They indicated that exercise-induced ischemia resulting in a reduction in stroke volume decreases the increase rate of VO_2 to work rate increase (i.e., $\Delta\text{VO}_2/\Delta\text{WR}$) after the ischemic threshold. This reduction in $\Delta\text{VO}_2/\Delta\text{WR}$ would produce some amount of abnormal oxygen deficit (that is, extra-oxygen deficit), which might be paid back soon after exercise when the cardiac performance recovers. We consider that this process may be “Hump”, transiently appearing on the limited portion of the early recovery of VO_2 decay. It should be noted that a reduction in $\Delta\text{VO}_2/\Delta\text{WR}$ during exercise was difficult to discern by visual inspection in any patient in our patients including those manifesting “Hump”, probably because of a large spontaneous variations in VO_2 during exercise.

As described previously, it is possible that a mono-exponential curve used for deriving the non-exponential components is not be the most appropriate model for identifying “Hump”. No single model may be suitable for fitting of the postexercise VO_2 decay, because the morphology of postexercise VO_2 decay curve considerably varied between individuals, probably due to the varying extent of impairment of LV function and the status of conditioning. Nevertheless, our method could identify “Hump” with a reasonable sensitivity and high specificity. Further investigation is necessary to ascertain this issue.

In conclusion, postexercise VO_2 “Hump” phenomenon, with its peak occurring around 60 s after exercise, seems to be a useful marker for inducible myocardial ischemia. The identification of this phenomenon may be more useful, particularly in patients with limited diagnostic accuracy of exercise ECG such as those with anterior myocardial infarction.

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Crystal structures of VAP1 reveal ADAMs' MDC domain architecture and its unique C-shaped scaffold

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ADAMs (a disintegrin and metalloproteinase) are sheddases possessing extracellular metalloproteinase/disintegrin/cysteine-rich (MDC) domains. ADAMs uniquely display both proteolytic and adhesive activities on the cell surface, however, most of their physiological targets and adhesion mechanisms remain unclear. Here for the first time, we reveal the ADAMs' MDC architecture and a potential target-binding site by solving crystal structures of VAP1, a snake venom homolog of mammalian ADAMs. The D-domain protrudes from the M-domain opposing the catalytic site and constituting a C-shaped arm with cores of Ca²⁺ ions. The disintegrin-loop, supposed to interact with integrins, is packed by the C-domain and inaccessible for protein binding. Instead, the hyper-variable region (HVR) in the C-domain, which has a novel fold stabilized by the strictly conserved disulfide bridges, constitutes a potential protein–protein adhesive interface. The HVR is located at the distal end of the arm and faces toward the catalytic site. The C-shaped structure implies interplay between the ADAMs' proteolytic and adhesive domains and suggests a molecular mechanism for ADAMs' target recognition for shedding.

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Introduction

ADAMs (a disintegrin and metalloproteinase) or MDC (metalloproteinase/disintegrin/cysteine-rich) proteins comprise an emerging class of mammalian metalloproteinases with potential regulatory roles in cell–cell and cell–matrix adhesion and signalling (Becherer and Blobel, 2003; Seals and Courtneidge, 2003; White, 2003; Blobel, 2005). To date, over 30 ADAMs have been identified in a variety of species from fission yeast to human. Roughly, half of these are believed to

function as active metalloproteinases and thus to constitute major membrane-bound sheddase that can proteolytically release cell-surface-protein ectodomains including growth factors and cytokines, their receptors and cell adhesion molecules. For example, ADAM17 (TACE, TNF- α converting enzyme) releases many cell-surface proteins including TNF- α precursor (Black *et al.*, 1997; Moss *et al.*, 1997) and ADAM10 (kuzbanian), which dictates lateral inhibition of *Drosophila* neurogenesis (Rooke *et al.*, 1996), releases Notch ligand Delta (Qi *et al.*, 1999) and Notch itself (Pan and Rubin, 1997). With regard to cellular interactions, fertilin α and β (ADAM1 and ADAM2, respectively) have been identified as sperm surface molecules essential for fertilization (Primakoff *et al.*, 1987; Blobel *et al.*, 1990, 1992) and meltrin α (ADAM12) is implicated in myogenesis (Yagami-Hiromasa *et al.*, 1995). ADAMs have been associated with numerous diseases including arthritis, Alzheimer's disease, and cancer (Duffy *et al.*, 2003; Moss and Bartsch, 2004). ADAM33 has been genetically linked with asthma (Van Eerdewegh *et al.*, 2002). ADAMs uniquely display both proteolytic and adhesive activities on the cell surface, however, most of their physiological targets and the adhesion mechanisms remain unclear.

Disintegrins are small proteins (40–90 aa) isolated from snake venom typically with an Arg-Gly-Asp (RGD) recognition sequence on an extended loop (disintegrin-loop) that inhibit platelet aggregation via integrin binding (Huang *et al.*, 1987; Calvete *et al.*, 2005). ADAMs are unique among cell surface proteins in possessing a disintegrin (D-) domain and thus it has been suggested that integrins might be common receptors for ADAMs (Blobel *et al.*, 1992; Evans, 2001; White, 2003). However, the RGD sequence in the ADAMs' disintegrin-loop is usually replaced by XXCD and therefore, its adhesive potential has been controversial. Both the ADAMs' D- and cysteine-rich (C-) domains are involved in the protein–protein interactions (Myles *et al.*, 1994; Almeida *et al.*, 1995; Zolkiewska, 1999; Iba *et al.*, 2000; Gaultier *et al.*, 2002; Smith *et al.*, 2002), however, the details of the interactions have remained elusive. This is because high-resolution structures have been available only for isolated domains (Maskos *et al.*, 1998; Orth *et al.*, 2004; Janes *et al.*, 2005) and no structural information has been available for the C-domain of the canonical ADAMs. To clarify the molecular mechanisms of target recognition for shedding by and of cellular adhesion via ADAMs, elucidation of the atomic structure of the ADAMs' MDC domains is indispensable.

To obtain structural data on an ADAM family member, we exploited the fact that hemorrhagic P-III snake venom metalloproteinases (SVMPs) share the ADAMs' MDC architecture (Jia *et al.*, 1996; Evans, 2001; Fox and Serrano, 2005). Most ADAMs possess additionally, EGF-like, transmembrane and cytoplasmic domains and therefore are primarily membrane-associated, whereas SVMPs are secreted. Vascular apoptosis-inducing protein-1 (VAP1) is a disulfide-bridged

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homodimer P-III SVMP isolated from *Crotalus atrox* venom (Masuda *et al*, 1998, 2000). VAP1's stability and intrinsic two-fold symmetry enabled us to solve the crystal structures at 2.5-Å resolution. The structure reveals the residues that are important for stabilizing the MDC architecture are strictly conserved throughout the primary structure among all the known ADAMs. Therefore, the present structure represents the general architecture of ADAMs' MDC domains and provides insights into the molecular mechanism of the ADAMs' target recognition.

Results

Structure determination

VAP1 yielded crystals readily, and initial phases were determined by molecular replacement method using the structure of P-I SVMP, acutolysin-C (1QUA) (Zhu *et al*, 1999) as a starting model. Although the initial model, with 99 identical residues out of 197, represented less than 50% of the total molecule, two distinct local noncrystallographic two-fold symmetry (NCS) operations (see below) allowed us to completely model the whole molecule. The native structures were determined from the crystals with two distinct space groups, $P2_12_12_1$ and $P4_12_12$, both at 2.5-Å resolution (Table I).

Orthorhombic crystals were used for inhibitor soaking and the GM6001 ((3-(*N*-hydroxycarboxamido)-2-isobutyl-propionyl-Trp-methylamide)-bound structure was determined at 3.0-Å resolution (Table I). In either crystal forms, the asymmetric unit contained one dimer molecule. The four monomers in the two crystal forms have almost identical structures, except for slight variations in their domain orientations, terminal residues, surface loops and active-site GM6001-binding region.

MDC architecture

The MDC architecture of VAP1 is shown in Figure 1A and B. The metalloproteinase (M-) domains in the dimer are related by NCS such that their active sites point in opposite directions and an intermolecular disulfide bridge is formed between symmetry-related Cys365 residues (Figure 1A). The M-domain is followed by a disintegrin (D-) domain that is further divided into D_s - and D_a -domains (see below). The D_s -domain protrudes from the M-domain close to the Ca^{2+} -binding site I (see below) opposing the catalytic site. The D-domain forms a C-shaped arm, together with the cysteine-rich (C-) domain, with its concave surface toward the M-domain. There are no direct interactions between the arm and the M-domain. Notably, the distal portion of the C-domain comes close to

Table I Data collection and refinement statistics

	Native (orthorhombic)	Native (tetragonal)	GM6001-bound
	$P2_12_12_1$	$P4_12_12$	$P2_12_12_1$
<i>Data collection</i>			
Space group			
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	86.7, 93.3, 137.7	93.9, 93.9, 244.8	86.3, 91.4, 136.0
α , β , γ (deg)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	50–2.50 (2.59–2.50)	50–2.50 (2.59–2.50)	50–2.95 (3.06–2.95)
R_{merge}^a	0.072 (0.369)	0.084 (0.380)	0.072 (0.367)
$I/\sigma I$	14.4 (2.9)	18.7 (7.1)	12.6 (4.3)
Completeness (%)	99.4 (98.8)	99.7 (99.6)	99.9 (99.4)
Redundancy	3.91	12.7	4.95
<i>Refinement</i>			
Resolution (Å)	50–2.50 (2.59–2.50)	50–2.50 (2.59–2.50)	50–2.95 (3.06–2.95)
No. of reflections	38874	38786	23295
R_{work}^b/R_{free}^c	0.212/0.258	0.229/0.269	0.208/0.264
<i>No. of atoms</i>			
Protein	6558	6513	6558
Zn ²⁺	2	2	2
Ca ²⁺	4	4	4
Co ³⁺	1	1	1
<i>N</i> -acetyl glucosamine	56	42	56
GM6001			56
Water	205	165	35
<i>B-factors</i>			
Protein	44.9	51.2	55.4
Zn ²⁺	40.9	41.6	46.4
Ca ²⁺	43.5	52.4	49.3
Co ³⁺	35.5		56.8
<i>N</i> -acetyl glucosamine	69.8	65.1	75.8
GM6001			78.6
Water	39.8	41.5	37.0
<i>R.m.s deviations</i>			
Bond lengths (Å)	0.0052	0.0080	0.0038
Bond angles (deg)	1.18	1.39	0.92

^a $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th intensity measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is its average.

^b $R_{work} = \sum (||F_{obs}| - |F_{calc}||) / \sum |F_{obs}|$.

^c R_{free} = *R*-value for a randomly selected subset (5%) of the data that were not used for minimization of the crystallographic residual. Highest resolution shell is shown in parenthesis.

For each data set, single crystal was used for measurement.