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Estimation for the elasticity of vascular endothelial cells on the basis of atomic force microscopy and Young's modulus of gelatin gels

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Summary

The initial Young's modulus of vascular endothelial cells (HUVECs) was studied with atomic force microscopy (AFM) in comparison with gelatin gels. The cross-sectional area of gelatin gels in contact with a tip of the cantilever was estimated from combination of force-displacement curves for glass plate and the Young's modulus of the gels obtained by a tensile tester. The cross-sectional area of vascular endothelial cells in contact with a tip of the cantilever was assumed identical to that of the 10 wt% gelatin gel. Thus, the elasticity of vascular endothelial cells on gelatin-coated glass was estimated as $5x10^3$ to 10^6 Pa at points of 0.1 to 3 μm in height between a nucleus and extended lamellipodia of cells, where the edge region resulted in lower elasticity. The numeral is concluded to be in good agreement with the values reported on cells.

Introduction

Blood vessels in contact with blood flow are covered with monolayer of vascular endothelial cells. The cells are exposed with shear stress of *ca*. 0 to 20 dyne/cm^2. Too high stress to the cells follows impairment of vascular walls, and causes rapid thrombus formation, i.e., blood coagulation. Information on the elasticity of the cells is important to design vascular endothelial cell-plated artificial blood vessels which should be tolerable under shear stress.

Depending not only on shear stress [1] but also on the cell movement [2], cells are reported to change the shape with varying the elasticity. The shape of cells under shear stress is resistant and supported mainly by polymers of cytoskeleton protein [3], i.e., f (filamentous)-actin with actin-associated proteins and of microtubules formed by tubulin polymerization.

Thus, the mechanical response of cells seems related to diverse and fundamental cell behavior [4] such as adhesion, migration, proliferation, and differentiation as well as pathological behavior. Here we try to determine the elasticity of vascular endothelial cells in comparison to that of gelatin gels with atomic force microscopy (AFM). That is, the cross-sectional area of vascular endothelial cells in contact with a tip of the cantilever may be regarded as the same area in the case of gelatin gels owing to their resembled elasticity.

Experimental

Materials

Gelatin powder samples (derived from porcine skin, Sigma) were dissolved in distilled water at concentrations of 5, 7, and 10 wt% at 45°C. The solutions were poured into a metal mold to make disk-shape samples. The solutions in the mold of 11.1 mm in radius and 5.97 mm in height were gradually cooled to *ca*. 20°C and then cooled down further at 4°C. These samples were used for compression tests by using a tensile tester. For AFM measurements, gelatin gels were prepared on a cover glass (Matsunami, 15 mm x15 mm) at *ca*. 10°C for the AFM measurements. Each cover glass was used after being immersed in ethyl alcohol and then rinsed sufficiently with distilled water.

Cell Culture

Human umbilical vein endothelial cells were obtained according to the method reported by Jaffe [5]. The cells were identified as HUVEC from the cobblestone-like shape and the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate-labeled acetylated low density lipoprotein (DiI-Ac-LDL; Biomedical Technologies), as described in our previous paper [6]. The cells were cultured at 37°C under 5% CO_2 in 1% gelatin-coated tissue culture polystyrene dishes (TCPS, Corning and Falcon). Medium 199 (Nissui), 15% fetal bovine serum (FBS, Whittaker Products), heparin (90 μg/ml, Opocrin), endothelial cell growth suppliments (ECGS, 45 μg/ml, Collaborative Research), L-glutamine (8mM, Wako Pure Chemicals), potassium benzyl penicillin (170 U/ml, Meiji Seika), streptomycin sulfate (85 μg/ml, Meiji Seika) and gentamycin sulfate (34 μg/ml, Schering-Plough) were contained in a culture medium. The medium was changed three times a week. The HUVECs in the confluent state were treated with 0.25% trypsin (Wako Pure Chemicals) containing 0.02% ethylenediamine tetraacetic acid for 60 sec.
HUVECs cultured on gelatin-coated cover glass in TCPS dishes were measured in phostate buffered saline (PBS) or serum-free medium 199 for the AFM measurement. Those PBS and medium 199 were warmed beforehand at 40°C, and became cooled gradually at room temperature *ca.* 25°C. The HUVECs from passages 10 to 19 were used for this work.

Measurements

The compression tests of the disk samples were made to obtain a stress-strain curve using a tensile tester RTM-500 (Orientec Corporation). Young's modulus (E) was estimated from the initial slope of the stress-strain curve. An AFM SPA300HV (Seiko Instruments) was used in the contact mode. The size of silicon nitride cantilevers (SN-AF01, Seiko Instruments) used were 100.0 μm in length. The pyramidal shaped tip of 2.90 μm in height and of a nominal spring constant (k_0) of 0.09 N/m was used. The actual value of the force constant was set as a ratio of the displayed force value (F) to the displacement of the sample scanner (Δd) using a solid body (here, glass plate) as a specimen in water, giving k_0 =0.0860 N/m on an average. The obtained value was in good agreement with the nominal spring constant, meaning that Δd becomes almost identical to the deflection of the tip ($\Delta d'$).

Two types of the scanners, 20 μm for gels and 50 μm for the cells, were scanned at 0.25 Hz. In the AFM measurements for the gels and cells, the surface observation as well as the estimation of the apparent force constant (*k*) were made.

Results and discussion

Stress (σ)-strain (ε) curves of gelatin gels are shown in double-logarithmically in Figure 1. Yong's modulus of gelatin gels was extrapolated to the numeral of σ at logε=0, and listed on Table 1. Gelatin gels of 7 wt% and 10 wt% had similar hardness, while the modulus of the 5 wt% gel was smaller than those of both gels.

For the plots of the force *F* against the scanner displacement Δ*d*, Hook's law holds as long as Δ*d* is small. In measuring the force-displacement curves for soft materials such as gelatin gels, the original thickness varies when the force is applied to the sample. That is, the sample is compressed with an extended cantilever and then rebound with retracted one. In this case, *F* acting on the gel sample is given by

$$F = k\,\Delta d = S_0\,E(\Delta d - \Delta d')/d_0 \qquad (1)$$

The cross-sectional area S_0 equivalently is written by

$$S_0 = k\,d_0 / \{E(1 - \Delta d'/\Delta d)\} = k\,d_0/\{E(1 - k/k_0)\} \qquad (2)$$

Here, *k* is the apparent spring constant for the gel samples obtained from the relation between *F* and Δ*d*, Δ*d*' being the deflection of the tip as defined before. The quantity d_0 is the thickness of the specimen determined by AFM measurements, *E* being obtained from the compression test using the tensile tester. The calculated values of S_0 are listed in Table 1. The quantity S_0 is a kind of virtual cross-sectional area in order that *E* of the gels by AFM coincides with that determined by compression tests.

Figure 2 shows AFM images for HUVECs C1 (a) and C2 (b). The horizontal cross-sectional profiles of the cells were obtained at various positions expressed by

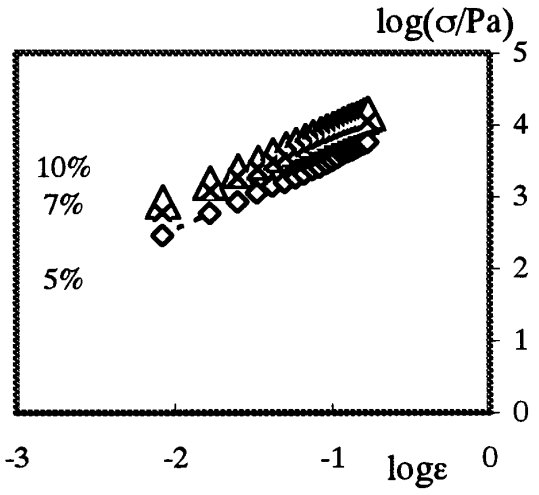
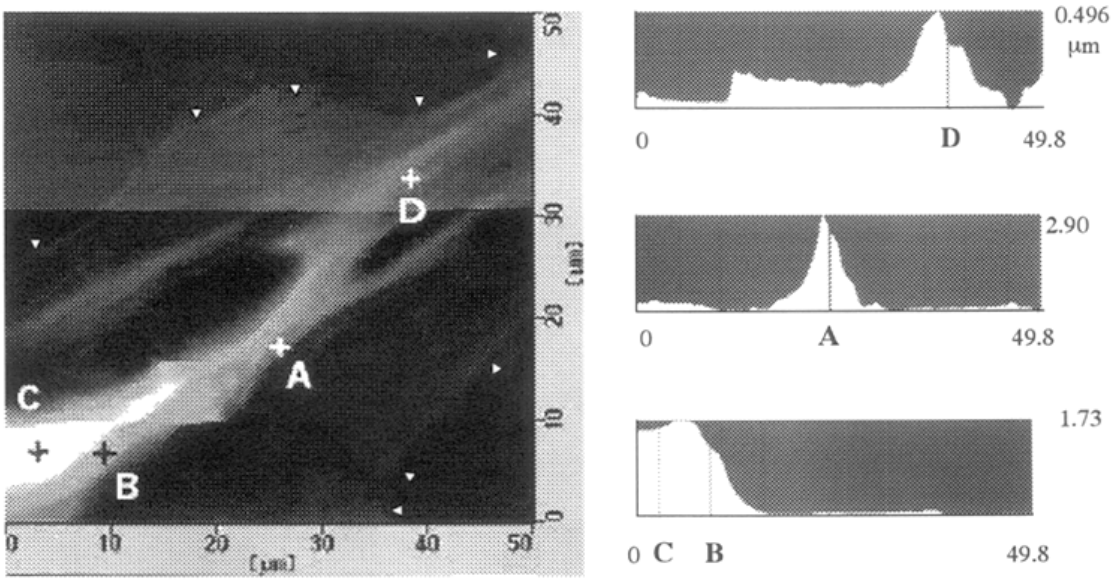


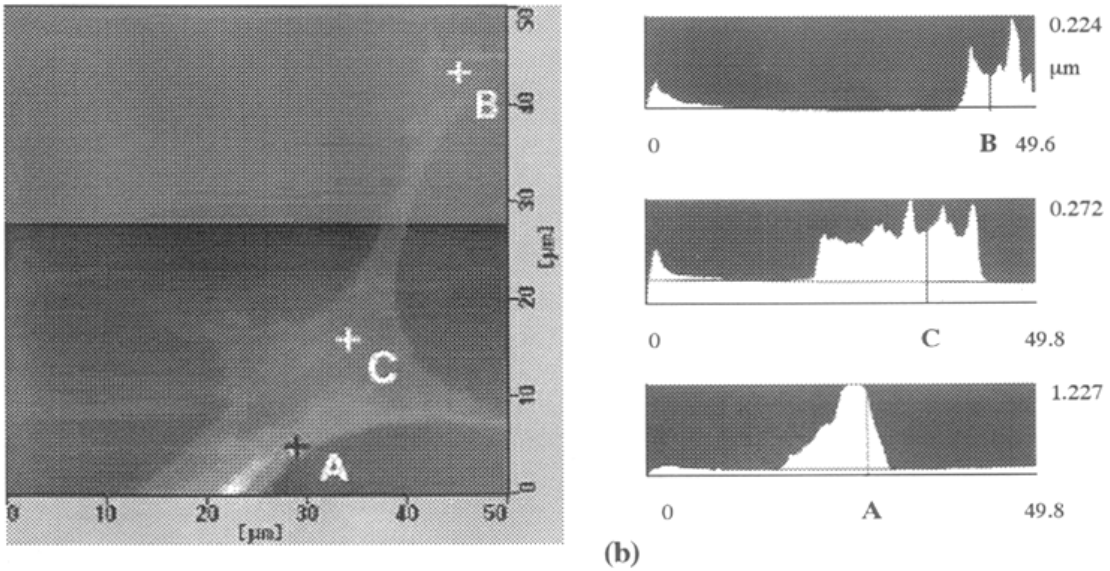
Figure 1. Log-log plots of compressive stress (σ) against strain (ε) for 10, 7, and 5% gelatin gels

Table 1. Initial Young's modulus (E) and initial contact area (S_0) with cantilever tips to gels

Samples	Concentrations	E_0 (Pa)	Average of S_0 (m^2)
Gelatin gel	10 wt%	$8.1x10^4$	$1.3x10^{-13}$
Gelatin gel	7 wt%	$6.5x10^4$	$0.8x10^{-13}$
Gelatin gel	5 wt%	$3.1x10^4$	$1.0x10^{-13}$



(a)



(b)

Figure 2. AFM images (50x50 μm) and cross-sectional profiles of HUVECs, C1 (a) and C2 (b)

alphabetic capitals (Figure 2), where force curves were taken. An example of force curves for HUVEC is shown in Figure 3 with one of those for 10% gelatin gels. Force curves for the cells were depicted as concave downward compared with gelatin gels. The initial slope of each force curve was obtained against extending and retracting force at various positions for samples. The numerical data of the initial Young'

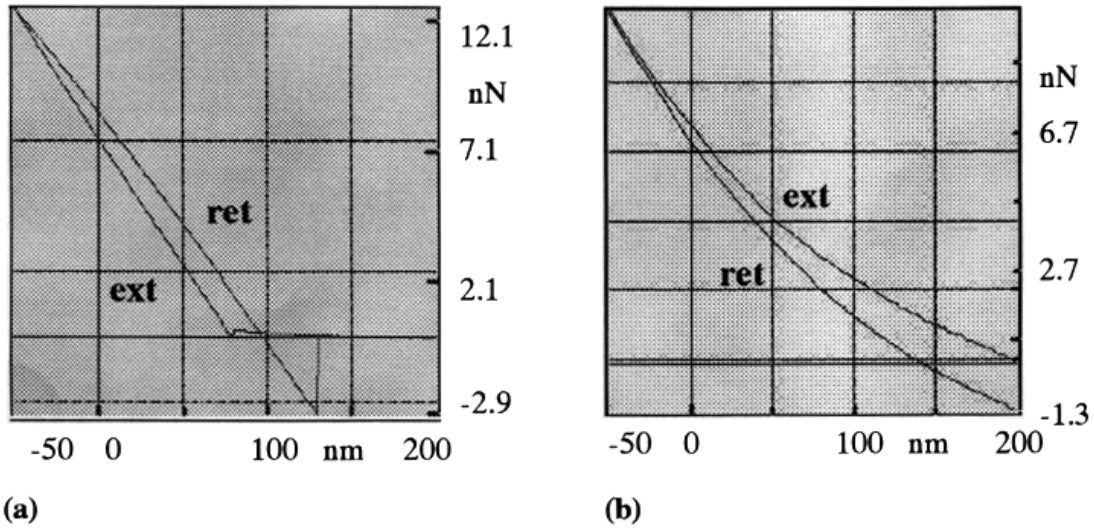


Figure 3. Stress-strain curves for 10 wt% gelatin gels (a) and HUVEC (b) in liquid.

Table 2. Elasticity of vascular endothelial cells at various positions. AFM measurement was carried out in medium199 except for the cell C5 in PBS.

Cells: positions	Initial Young's modulus (x10^4 Pa)		Thickness	Elapsed	Data
	Extending	Retracting	d_0, μm	time, h	No.
C1 –A	23±4	20±9	1.43	2.22-2.47	3
-B	46±6	22±3	2.12		3
-C	114±34	57±8	2.65		3
-D*	8.9±0.6*	6.1±0.4*	0.36*		2
C2.-A	98±30	66±10	1.00	1.43-1.68	4
-B*	6.5±0.8*	5.6±0.2*	0.087*		2
-C	6.2±0.9	4.1±0.3	0.136		2
C3 -A	22±2	16±3	1.67	3.45-3.65	4
-B	16±3	7.4±1.3	0.928	4.5-4.7	3
C4 -A	4.9±1.4	1.9±0.9	1.51	1.33-1.53	6
-B	0.50±0.2	0.31±0.1	0.418	2.5-2.7	4
C5	8.97	3.02	3.09	1.03	1

* explained in the text.

modulus, thickness of the cells (d_0), elapsed time from taking out from a CO_2 incubater until AFM measurements are summarized in Table 2. The position identified in the table corresponds to that in Figure 2.

The cell C1 (Figure 2 a) appears to extend remarkably the lamellipodia whose frontal edge is marked with arrow heads. The region above the lines as seen at 31 and 10 μm (Figure 2 a) and at 27 μm (Figure 2 b) may be influenced by disturbance bits hooked on the tip of a cantilever. In this case, data estimated in the regions is less reliable. Therefore, the data obtained in the regions are marked with asterisks in Table 2.

A gelatin gel of 10 wt% led to the most similar value in the elastic modulus to that of HUVECs. So, the value S_0 of 10 wt% the gel was adopted to estimate the elasticity of the cells, which are summarized in Table 2. Generally speaking, the numerals of the elasticity calculated from the extending force to the cells are slightly higher than those obtained from the retracting force. Data obtained from the extending force where the tip starts to contact with cells are considered to evaluate more properly the initial Young's modulus owing to complex systems such as biological cells. The deformation of the cells under force should result from the physical and chemical changes of cytoskeltons influenced by each mechanical displacement and movement of a number of cell-compartments such as endoplasmic reticulum, Golgi apparatus, mitochondrias, peroxisomes, centrosomes as well as nucleus.

Our mechanical measurement for HUVECs was unable to be carried out under physiological conditions at 37°C under 5% CO_2. The elapsed period from taking out from a CO_2 incubater to the AFM measurement is written in Table 2. It is noted that the cell C3-B maintained the hardness even after *ca*. 5 hours under our experimental conditions, while the elasticity at the position C4-B was extremely low compared with other cells. The reason is unknown; probably there was low level contamination by microorganisms and less biodegradation since the sample chamber for the AFM measurement was closed from the outside. It may be owing to different activity of phosphorylation-related enzymes in individual cells. The strength of cytoskeltal proteins appear to become lower as the result of dephosphorylation and depolymerization in f-actin and microtubules.

The position C4-B was located more peripherally than the position C4-A. Moreover, the elasticity at the position C1-C, C2-A, and C3-A close to nuclei is higher than that at positions of C1-D, C2-B and C2-C, and C3-B in the peripheral and edge region of the respective cell. As for the whole cell the initial Young's modulus may be concluded to be 10^6 to $5x10^3$ Pa as seen from Table 2.

Other results reported follow; From the AFM measurement of HUVECs in growth medium and HEPES buffer, the elastic modulus was determined to be 7,220 Pa over the nucleus, 2,970 Pa in proximity of the nucleus, and 1,270 Pa on the cell body near the edge [7]. The elastic modulus of human platelets adhered on glass in Tyrode buffer was estimated to be in a range of 10^3 to $5x10^4$ Pa measured in the frequency range of 1-50 Hz by AFM [8]. According to Rotsch *et al*. [2], the protruding and leading edge of motile fibroblasts was thinner and softer than the stable edge. The former elasticity was estimated to be 300-5,000 Pa, while that of the latter stable edge was presumed to be *ca*. $1.2x10^4$ Pa. Their three groups applied Hertzian model [9], assuming elastic deformation of cells. Thoumine and Ott [10] applied a three-element Kelvin viscoelastic model to cell rheology on chick fibroblasts, and thus calculated two elastic moduli of the cells in the range of 600-1,000 Pa. In the case of bacteria, *Listeria monocytogenes*, the elastic tails consisting of actin filaments were measured

with optical tweezers of laser beams to be in a range of 10^3-10^4 Pa in Young's modulus [11]. Thus our data for the elasticity of HUVECs are in good agreement with reported numerals.

Force applied should be susceptible to elastic deformation on a cell depending on the elasticity of the cell sites in a certain cell state. With respect to the cell motility the moving and leading frontal edge appears softer owing to weak cell adhesion to substrates [12]. The cell adhesion is closely related to development of stress fibers consisting of f-actin bundles [1] and formation of focal adhesion contact [7], which appear to increase stiffness and elasticity of cells. In addition, the movement [13] of actin filaments toward a nucleus from the focal contacts seems to contribute the elasticity of the cell. Relation between cell activity and rheology should be studied further.

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