

次世代医療機器の分野

体内埋め込み型能動型機器分野 [高機能人工心臓]
ナビゲーション医療分野 [手術ロボット]
再生医療分野 [心筋シート]
体内埋め込み型医療機器分野 [生体親和性インプラント]
テラーメイド医療用診断機器 [DNAチップ]

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図2 ●次世代医療機器評価指標検討会の5分野

チェック項目を明らかにしていこうと考えています。

ただ、この分野はまだ確立した評価法がありませんし、未知のリスクや有効性が出てきて、確認の手技手法も新しく出てくるのではないかと思います。そこで、現時点でどこまで確認できるのかを研究者の皆さまにもお考えいただき、あまり硬直した指針にならないように、その時点での最新の科学の目で評価していただけるようになっていきたいと思っています。あまり画一的な方法をガイドラインとして示す必要はないと思っています。むしろ技術の進歩の方が早いので、その時々で最適な評価をしていくように心がけなくてはいけないと思います。

2005年11月に、EUは再生医療に対して新しい規制を提唱しました。まだファイナルにはなっていませんが、これまではEU全域としての規制がなかったため、産業化を推進するためにも明確な安全性評価の体制をとるべきだという議論が出て今回の提唱に繋がったとされています。米国でもFDAが1990年代後半から枠組みを作り始めて安全性の評価を進めています。きちんとした安全性の評価体制があつてこそ世の中に認知されるし、社会の役に立っていくのだと思います。

土屋 もう1つのトピックは、ISO150

という外科用インプラントの国際標準化技術委員会があるのですが、そこでは整形外科から循環器までの色々な用具に対して、個別にどういったスタンダードを作るかと議論しています。ここにWG11という組織工学製品のワーキンググループ(WG)がありますが、現在日本より、WGからサブコミTEE(SC)への格上げの提案をしています。このSCを作ればその下にいくつもWGが作れますし、また日本が幹事国になれる。そういった国際的な場に日本の標準文書をたくさん持っていけば、世界との調和が取れて、製品化を進めやすくなると思います*2。

次世代医療機器の展望

佐木 平成17年から厚生労働省は経済産業省と連携して次世代医療機器評価指標検討会/医療機器開発ガイドライン評価検討委員会を設置しました。5つの分野(図2)について開発の推進と審査の迅速化を目指して新たなガイドラインをつくり、それを通知していくという試みで、審査WGを土屋先生に推進していただいています。ただ、やはり次世代医療機器もまだ評価指標が固まっているわけではありません。

*2: 追加情報

インタビュー後、日本の提案が可決され、TC150WG11はSC7に格上げとなった。

で、評価をしてくださる先生方も方法論が難しい分野だと思います。その内容が硬直したものになってしまうと、逆に開発を阻害するようなことにもなるので、緩やかな評価指標を世の中にお示しして、開発や審査の参考になるようなものを示していく必要があるのだと思います。

—あまり自由度があり過ぎると、現場が不安に思うことはないのでしょうか？

佐木 実際の審査を行っている医薬品医療機器総合機構では、相談制度を設けています。例えば、治験の前のプロトコールの内容についての相談であるとか、申請に当たって必要となる試験の相談などです。医療機器の相談制度自体は平成16年からスタートしていますが、まだまだ活用しにくいところがあるので、色々な相談のコースをつくって、開発の各段階で活用いただきたいと思っています。事前に審査サイドとディスカッションしてから申請に持ってきていただければ、審査が非常にスムーズにいくのではないかと思います。FDAでもやはり承認申請の前の段階、あるいは治験の前の段階でかなり企業とコンサルテーションを行っていて、申請から承認までの期間を短くすることができたと言われていています。

土屋 心筋シートの次世代評価指標作成事業審査WGであった話なのですが、細胞の生存率が例えば80%以上でないといけないという限定的な指標であった場合、それならば70%はだめなのか？という問題が出てきます。何か多少混ざっていたとしても、それが別の作用をしているかもしれません。器官は色々な細胞のミクスチャー

ーで構成されています。フィーダー細胞の役割をしたり、サイトカインを出していたり、複合化しているのです。そういう意味で、明確な数値は避け、今の知見に基づいて科学的・合理的な説明ができればいいということにしたいと思います。実際 FDA では、具体的な数値が設定されていたためにある製品の開発が止まっているという話も聞いています。

それから材料開発という意味では、従来の医療機器は工業製品を使ってきました。ステンレスやチタンなどです。しかし今は、色々な生体成分を作れるようになった。そしてそれがどういうふうに反応するかのメカニズムがわかる時代になってきました。だからこそ、今までのように、製品を作ってから厚生労働省に申請するために安全性試験をするという流れではなく、最初に材料をセレクションする過程で安全性評価を踏まえることが大切になります。なぜ安全性が低いのがメカニスティックにわかれば、今度はそれを有効性に置き換えるにはどうすれば良いかと次のステップが考えられるようになります。我々はその過程を経て新しいセラミクスを開発し、国際特許にも出しています。

新たな医療機器の可能性

一次世代医療機器検討会では5つのテーマが進められていますが、今後さらに新しい6つ目のテーマが出てくる可能性はあるのでしょうか？

俵木 検討すべきテーマがあれば5つで終わりということにはならないと思います。

土屋 この間すごいと思ったのは、国立循環器病センターの杉町 勝先生のバイオニック臓器のお話でした。杉町先生の発想は、先端を行っておられるという感じがしましたね。あのような研究を我々が協力して上手く軟着陸させることができればと思っています。

それから先ほども言いましたように、ペースメーカーなどは日本も非常に技術があるので、日本の企業が日本人に合ったより良いものを出していただきたいと思います。企業間で連携して進めないと、これからの医療機器開発は進まないと思います。プラスチックだけを入れるのではなく、その治療部位に薬を投与した方がさらに効果上がるのは、どなたでも理解しやすいですね。逆に、薬を体内に大量に投与しても目的の部位に行き着く量は少ないですが、医療機器だとまさにその部位に効率良く入れることができる。そう言った意味で、これからはコンビネーションの医療機器が増えてくると思いますし、薬の業界がもっと参入する必要があると感じています。じつは世界的にもコンビネーション医療機器は進められていて、ISO の中にもコンビネーション WG ができつつあります。また、先日のバイオマテリアル学会の会場で、Johnson&Johnson 社の方に未来志向バイオマテリアルの世界戦略を発表していただきましたが、あらゆるものに薬と細胞のコンビネーションを使うと講演されていました。彼らは現在、心筋梗塞時のステント治療で遅発性に生じる血栓を溶かすために、さらに抗血栓剤を2段構えで入れることを考えているそうです。また、合金

を使うのであればノンアレルギーの金属をコーティングすれば良いのではないかなど、様々な改良や開発品ができてくると思いますし、今まさにその転換期を迎えていると感じています。

俵木 5分野だけではないさらに新たな次世代の医療機器開発に向けて、今後も評価指標を作る必要があると思います。何を評価すればいいのかという指標を作っていくことが、患者さんの手元に有用な医療機器を早くお届けすることに繋がるのではないのでしょうか。

土屋 次世代医療機器の会議を始めてから、非常に医療機器の研究開発が進んでいるように感じます。コミュニケーションは非常に大切ですね。

俵木 やはり情報や認識の共有は重要だと思います。開発する方々も、相手は何を考えているのかな、どこまでやればいいのか、と手探りの状態で進めるのは相当大変なことですし。皆がこういうことを心配しているのだ、という共通認識を持って進めていっていったらと思います。

土屋 いま、半年に1回くらい新しい技術が出ています。最近も、間葉系幹細胞が無血清培地で培養できるという厚生労働科学研究費の再生医療研究班の成果が報告されました。これを製品化するには、製造工程から様々な手段に従ってやらなくては行けませんので、小さい会社だけではなく大手の製薬企業にも協力して進めてもらう必要があります。産業と行政と研究の連携が、これからさらに大きく変わっていくと思います。

—貴重なお話をありがとうございました。

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A Novel Non-Destructive Method for Measuring Elastic Moduli of Cultivated Cartilage Tissues

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Keywords: Mechanical Property, Bulk Modulus, Volume, Pressure, Human Articular Chondrocyte, Cultivated Cartilage

Abstract. Accurate measurement of the mechanical properties of artificial or cultivated cartilage is a major factor for determining successive regeneration of defective soft tissues. In this study, we developed a novel method that enabled the bulk modulus (k-modulus) to be measured nondestructively using the relationship between volume and pressure of living soft tissues. In order to validate this method we estimated the bulk modulus of soft silicone rubbers using our new method and a conventional method. The results showed a 5 ~ 10% difference between the results obtained with the two methods. Our method was used subsequently to measure the mechanical properties of cultivated cartilage samples (collagen gel type), that had been incubated for four weeks in the presence or absence of human articular chondrocytes (HACs). Our experiments showed that cultivated cartilage tissues grown in the presence of HACs had a higher bulk modulus (120 ± 20 kPa) than samples grown without HACs (90 ± 15 kPa). The results indicated that our novel method offered an effective method for measurement of volume changes in minute living soft tissues, with the measurements having a high degree of accuracy and precision. Furthermore, this method has significant advantages over conventional approaches as it can be used to rapidly and accurately evaluate the strength of soft tissues during cultivation without causing damage to the specimen.

Introduction

Recently, artificial cartilage, obtained by cultivation with human articular chondrocyte (HACs) and mesenchymal stem cell (MSCs) on collagen scaffolds, has provided a method with the potential to regenerate damaged articular cartilage [1, 2]. In order to achieve successfully regeneration of damaged soft tissues, it is well established that biomechanical function and the biological construct of the artificial tissues play an important role in the tissue engineering [3-5]. For this reason, many experimental approaches have been used to assess the mechanical properties of factitious soft tissues. Some researches recently proposed a method for measuring mechanical properties with micro-needles or other specially designed devices [6-8]. However, using these approaches it has proved very difficult to accurately assess the mechanical properties of biological materials, such as skeletal muscles, cartilage, or other soft tissues including artificial tissues. This is due to the technical difficulties associated with non-destructive measurements, in addition to the irregular geometries of living tissues. In spite of these practical limitations, mechanical assessments are crucial when determining the maturity of cultivated soft tissues for transplantation and to ensure the success of regenerative medicine. We therefore developed a novel method that enabled the bulk modulus of elasticity (modulus of volume elasticity) to be measured rapidly, using the relationship

between volume and pressure of living soft tissues. This method was then applied to measure the elastic modulus of cartilage cultivated for four weeks on collagen scaffolds with and without human articular chondrocyte (HACs).

Materials & Methods

Theory and Experiment. Figure 1 shows a diagrammatic illustration of our device, which was used in a pressure pot under different air pressures. The system was composed of Chambers 1 and 2, a pressure sensor, an A/D converter and an air compressor. The sample was placed in Chamber 1, with Chamber 2 acting as a reference for the device. The pressure sensor detected the difference in pressure (ΔP) produced when a pressure change occurred in Chamber 1. The volume change (ΔV) was expressed as an electronic signal using a FFT (Fast Fourier Transform) analyzer as shown in Fig. 1-(A). In order to calculate the relationship between volume (V) and pressure (P), we used the classical equation of the relationship between volume and pressure [6]. If the volume of the sample changed (V_x) in chamber 1, the equation (1) can be written as:

$$\Delta P_1 - \Delta P_2 = \frac{n P_1 \Delta V_1}{V_1 - V_x} - \frac{n P_2 \Delta V_2}{V_2} \quad (1)$$

where P_1 , V_1 are the pressure and volume in chamber 1, respectively, and P_2 , V_2 are the pressure and volume in chamber 2, respectively. n represents the number of the polytropic index. When the pressure increases, ΔP is inserted into the equation (1). The relationship between pressure and volume in this system can be rewritten as equation (2).

$$\Delta P_1 - \Delta P_2 = \frac{n(P + \Delta P)\Delta V}{V_1 - V_x} - \frac{n(P + \Delta P)\Delta V}{V_2} = n(P + \Delta P)\Delta V \left(\frac{1}{V_1 - V_x} - \frac{1}{V_2} \right) \quad (2)$$

The bulk modulus is calculated and converted by the following equation (3), (4).

$$k = P / \varepsilon_v \quad (3)$$

$$k = E / 3 (1 - 2\nu) \quad (4)$$

where k , P , ε_v , E , and ν are the bulk modulus, pressure, volume strain, elastic modulus, and poisson ratio, respectively.

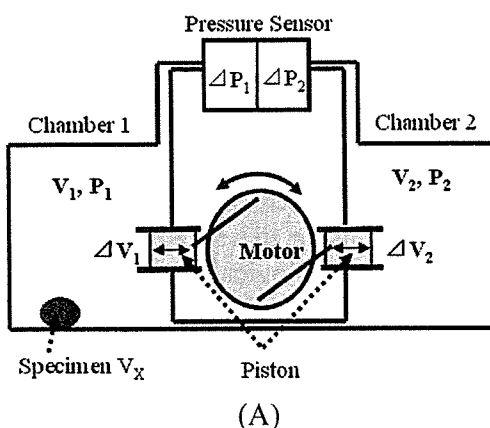


Fig. 1 Schematic diagram (A) and photograph (B) of the novel testing device using the principle of the relationship between pressure and volume changes

Validation of New Testing Method. Before calculating the bulk modulus of the samples, the relationship curves between the known volumes and pressure changes between 0 ~ 120 kPa in this system were first defined using stainless steel balls. Soft silicone rubber, which had similar mechanical properties to human soft tissues, was then used to validate our specially-designed device. The elastic modulus of the silicone rubbers was measured by two methods: 1) the conventional dynamic elastic modulus (E') test and 2) our method. In the dynamic test, the silicone samples were loaded with a cyclic strain of amplitude 0.5% at a frequency of 0.5 ~ 100 Hz by Rheogel-E4000 (UBM Co., Japan). The dynamic elastic moduli at 1 Hz were converted to bulk moduli using equation (4). The two bulk moduli calculated using the conventional dynamic test and our method were then compared using the statistical t-test ($p < 0.05$).

HACs and Culture Methods. Two types of the cultivated cartilage grown with HACs (+) and without HACs (-) on collagen scaffolds were used to measure the bulk modulus. The artificial cartilages were prepared by the following process [2]. HACs of the knee joint were commercially obtained from BioWhittaker (Walkersville, USA) and cultured in chondrocyte growth medium (Walkersville, USA). After the collagen scaffold (BD Science, USA) was placed in a 24-well tissue culture micro-plate (Corning, USA), high-density micromass cultures were started by seeding 4×10^4 HACs in 20 μL of medium onto the collagen scaffold. After a 2 h attachment period in a 5% CO_2 incubator at 37°C, 1 mL of culture medium was added to each well. The cultures were then incubated for a further 4 weeks with the medium being changed twice weekly. Figure 3-(A) shows samples of the cultivated cartilages on the collagen scaffolds grown with and without HACs.

Results & Discussions

In order to validate this method, we measured the bulk modulus of soft silicone rubber using our proposed method and the conventional method. The results were then compared. The results obtained using the novel method (409 ± 14 kPa) compared favorably with those obtained using the conventional biomechanical measurements (417 ± 22 kPa). As shown in Table 1, this result represented a mere 5 ~ 10% difference between the two methods, thereby confirming the accuracy of our new method. We found no significant difference between the two measuring methods in two types of silicone ($p > 0.05$).

We then applied our method to measure the bulk moduli of cultivated cartilage samples incubated for four weeks in the presence or absence of HACs. Figure 2 shows the relationship between volume and pressure changes of the cultivated cartilages. The volume of the cultivated cartilages was decreased with increasing pressure. As shown in Fig. 3-(B), cultivated cartilage tissues grown in HACs had a higher bulk modulus (120 ± 20 kPa) than samples grown without HACs (90 ± 15 kPa). This difference between the two samples of cultivated cartilage was statistically significant ($p < 0.05$). These findings indicated that growing cells such as HACs could increase the mechanical property of cultivated cartilages. Although the results showed a lower elastic modulus than that reported for normal human articular cartilage of 0.3 ~ 1.5 MPa, the lower mechanical property of an initial artificial cartilage would be suitable for assimilation around normal living cartilage [8,9].

Table 1 Comparison of the elastic moduli calculated using elastic modulus and our novel method

| Samples | Bulk Modulus by Conventional Method | Bulk Modulus by Our Novel Method |
|-----------------------|--|-------------------------------------|
| Silicone gel (n=3) | 176 ± 34 [MPa] | 120 ± 20 [MPa]* |
| Silicone rubber (n=3) | 417 ± 22 [kPa] | 409 ± 14 [kPa]** |

*, **: were converted with $\nu = 0.48$ and $\nu = 0.42$

Conclusions

On the basis of these results, it can be concluded that our novel method offers an effective method for measurement of the biomechanical properties of artificial or cultivated soft tissues as well as living soft tissues. The method has a high degree of accuracy and precision. In addition, the method can be used for rapid and accurate evaluation of changes in strength of soft tissues during cultivation without causing damage to the specimen.

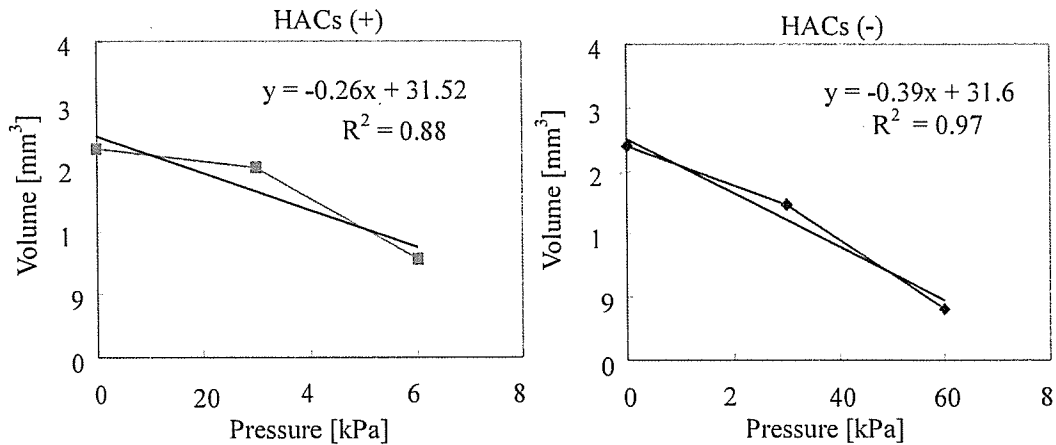


Fig. 2 Comparison of volume changes in relationship to changes in pressure in two types of cultivated cartilages grown with HACs (+) and without HACs (-)

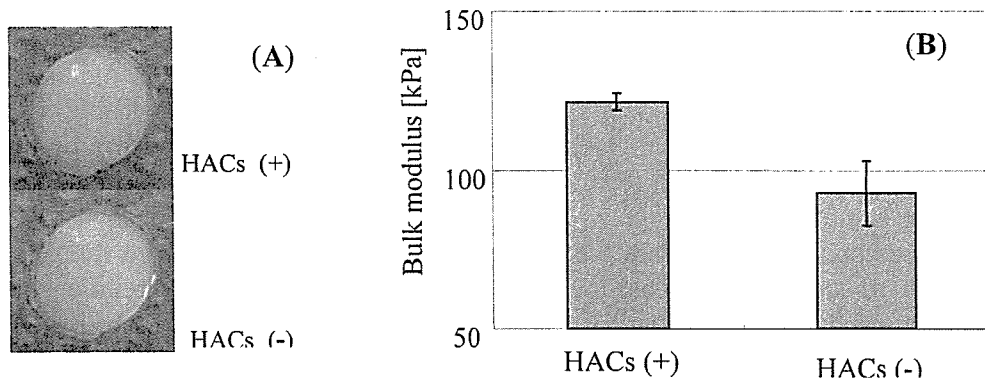


Fig. 3 Samples (A) and bulk moduli (B) of the cultivated cartilages grown with HACs and without HACs on collagen scaffolds for four weeks

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Effects of sulfated hyaluronan on keratinocyte differentiation and Wnt and Notch gene expression

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Abstract

Sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan (Hya), has high activity on and biocompatibility with cells. When normal human epidermal keratinocytes (NHEKs) were incubated in dishes coated with SHya, cell proliferation was increased in a dose-dependent manner. The expression levels of keratin 1 and loricrin mRNAs, as detected by real-time RT-PCR, were increased significantly. The expressions of Wnt mRNAs, which play important roles in cell proliferation and differentiation, were modulated. Wnt4 and Wnt6 mRNA expressions were increased compared to controls, while expression of Wnt5a was similar to the control and that of Wnt7a mRNA was decreased. In addition, the expression of Notch mRNAs, which play a critical role in keratinocyte differentiation, were affected. Notch3 mRNA was increased significantly, while Notch1 mRNA was decreased compared to controls, and expression of Notch2 was similar to that of control. These results suggested that a SHya-coated scaffold might be useful for regulating cell activity in tissue engineering.

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Keywords: Sulfated hyaluronan; Normal human epidermal keratinocyte; Differentiation; Wnt; Notch

1. Introduction

Normal human epidermal keratinocytes (NHEKs) are usually cultured on 3T3 mouse feeder-layer cells [1]. Several biomaterials have been developed from other animals or humans, but they carry the risk of infection from prions and viruses [2,3]. In contrast, biomaterials from microorganisms carry lower risks of infection, and the development of a semi-synthetic material promoting cell activity will enable safer cell culture.

Several types of polysaccharides for culturing NHEKs have been studied [4,5]. Hyaluronan (Hya) is a negatively charged glycosaminoglycan that is a major component of the extracellular matrix (ECM) [6,7]. Hya plays important roles in cell adhesion, migration, proliferation, and differentiation [7–9]. Park and Tsuchiya reported that a Hya-coated surface is capable of enhancing gap junctional

intercellular communication (GJIC) and differentiation or cell growth [10,11]. Sulfated polysaccharides, such as heparin or heparin sulfate, stabilize some growth factors, resulting in enhancement of their effects [12], and promote their mitogenic activity [13]. Sulfated hyaluronan (SHya), a semi-synthetic material composed of Hya and a sulfate group [14] can be synthesized using Hya extracted from microorganisms; therefore, it has a lower infectivity and a lower risk of containing virus-induced carcinogens.

It has been reported that Hya and chondroitin sulfate A enhanced chondrogenesis of human mesenchymal stem cells [15,16]. We hypothesized that SHya has the potential to function as a biomaterial promoting keratinocyte differentiation because it has been reported that SHya stimulates cell activities [14,17]. Analysis of the effects of SHya on cell differentiation and intercellular signaling will provide the information allowing construction of biomaterials of greater usefulness for tissue engineering.

Wnts are secreted glycoproteins that bind Frizzled receptors and play a critical role in the process of cell differentiation in the canonical pathway. In the canonical

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pathway, beta-catenin stabilized by the binding of Wnt proteins to Frizzled receptors stimulates TCF/LEF transcription. A mouse keratinocyte cell line with decreased Wnt4 expression showed a more malignant morphology and was less differentiated [18], Wnt6 was required for epithelialization of the segmental plate mesoderm [19], and Wnt7a promoted cell proliferation by activation of Rac-GTPase and beta-catenin [20].

Notch receptors play a crucial role in determination of cell fate. Notch1 signaling plays an essential role in regulation of mouse keratinocyte differentiation [21]. In mouse keratinocytes, Notch1 activation suppressed Wnt4 activity mediated by the cyclin/CDK inhibitor p21 (WAF1/Cip1) [22]. Notch3 was required for the differentiation of vascular smooth muscle cells and T-cells [23,24].

In a previous study, we demonstrated that a Hya coating promoted several cell functions better than a Hya-supplemented one [25]. Therefore, in this study, we investigated the effects of a SHya coating on keratinocyte differentiation.

2. Materials and methods

2.1. Sulfated hyaluronan

SHya was prepared by the method reported previously [4]. A solution of 2% Hya120 (molecular weight, 1.2×10^6) solution in *N,N*-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd., Osaka, Japan)

was mixed with trimethylamine (TMA)-SO₃ complex (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) and stirred for 24 h at 60°C. The reaction mixture was then diluted, neutralized, and precipitated by adding a large quantity of acetone (Wako Pure Chemical Industries). The precipitate was dissolved in distilled water and dialyzed against distilled water. The molecular weight of SHya was 2.0×10^5 , and the degree of substitution (D.S.) of SHya was 1.0, as determined by the chelate titration method [26] (Fig. 1). Moreover, the effectiveness of sulfation was also demonstrated by FT-IR analysis. The IR spectrum of SHya exhibited two absorption bands at 1240 and 820 cm⁻¹ due to S=O and SO₃⁻ stretching, respectively.

2.2. Cell culture

NHEKs isolated from neonatal human foreskins (Cambrex Bioscience, Walkersville, MD, USA) were cultured with K-110 Type II medium (Kyokuto, Tokyo, Japan) supplemented with 2% whole bovine pituitary extract, 50 IU penicillin G, 50 µg/ml streptomycin, and 0.03 mM CaCl₂ (low-calcium condition) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Keratinocyte differentiation was induced in 0.20 mM CaCl₂ medium (high-calcium condition). The medium was exchanged for a fresh one every 2 days.

2.3. Preparation of SHya-coated culture dishes and well plates

Both 35 and 100 mm polystyrene dishes (Iwaki, Funabashi, Japan) and 24-well plates (Corning, Corning, NY, USA) were coated with SHya dissolved in distilled water at a final density of 0.4 or 0.8 mg/cm². The SHya-coated dishes and plates were dried under a sterile airflow at room temperature for 8 h.

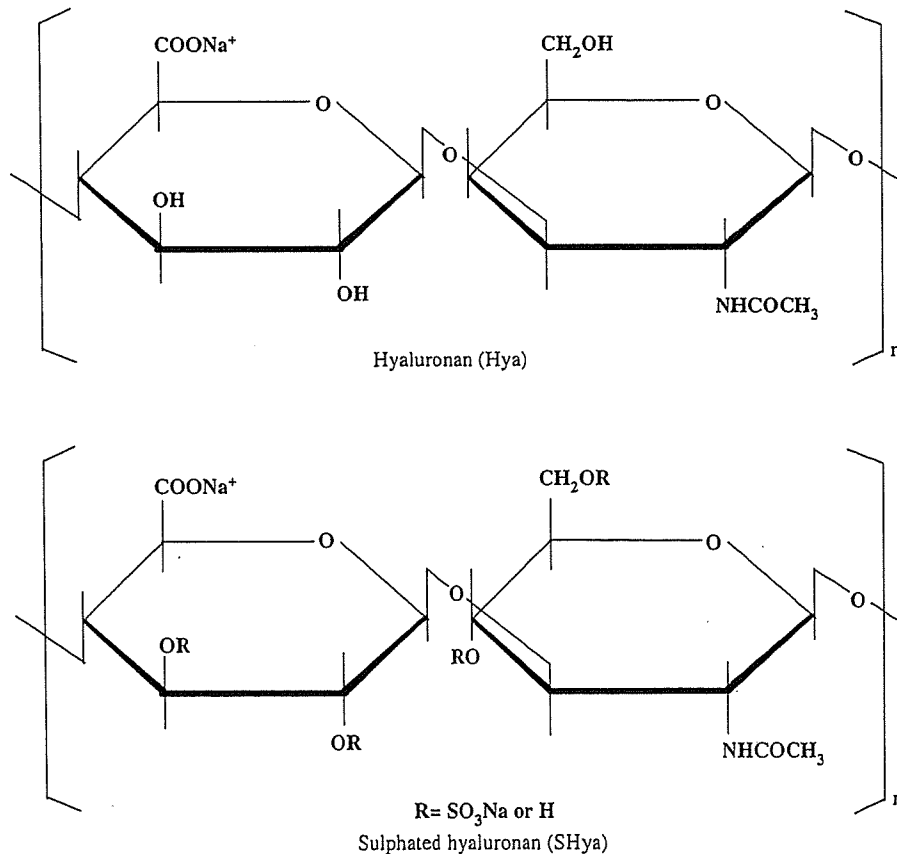


Fig. 1. The structures of hyaluronan and sulfated hyaluronan. Sulfated hyaluronan (SHya) is composed of Hya and a sulfate group. The molecular weight of SHya is 2.0×10^5 , and the degree of substitution of SHya was 1.0.

4. Determination of cell numbers by crystal violet assay

NHEKs were seeded at 1.0×10^4 cells/cm² in wells of 24-well plates coated with various concentrations of SHya (0, 0.4, and 0.8 mg/cm²) and incubated in the high-calcium condition (0.20 mM) for 5 days. After washing with Ca²⁺, Mg²⁺-free phosphate-buffered saline [PBS(-)], cells were exposed to 0.4% crystal violet (Wako) in methanol for 15 min. NHEKs were washed with PBS(-) three times and destained with 500 μ l of methanol for 20 min. Then, absorbance was read at 590 nm using a plate reader.

2.5. Quantitative real time RT-PCR

NHEKs were seeded at 1.0×10^4 cells/cm² in various concentrations on SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm²) and incubated in the high-calcium condition (0.20 mM) for 5 days. Cells were washed with PBS(-) three times, and total RNA was extracted from NHEKs using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into 10 μ l cDNA using an ExScript RT reagent kit (Takara Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Aliquots of the cDNA were used as templates for PCR analysis using a Lightcycler system (Roche, Mannheim, Germany). PCR amplification was performed in a total volume of 20 μ l including 1 μ l of RT reaction, 10 μ l of SYBR Premix Ex Taq (Takara), and 0.4 μ M of each primer. The PCR reaction was performed as follows: 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 65 °C for 15 s. The PCR primers were purchased from Takara Co., Ltd. The PCR primer sequences for amplification of keratin 1 were forward primer 5'-AGATCACTGCTGGCAGACATGG-3', and reverse primer 5'-TGATGGACTGCTGCAAGTTGG-3'. The PCR primer sequences for amplification of lorincrin were forward primer 5'-TCATGATGCTACCCGAGGTTG-3', and reverse primer 5'-CAGAACTAGATG CAGCCGGAGA-3'. The PCR primer sequences for amplification of Wnt4 were forward primer 5'-CCAGCAGAGCCCTCATGAAC-3', and reverse primer 5'-TCCACCTCAGTGGCACCATC-3'. The PCR primer sequences for amplification of Wnt6 were forward primer 5'-CTG GAATTGCTCCAGCCACA-3', and reverse primer 5'-GCAGTGAT GGCGAACACGA-3'. The PCR primer sequences for amplification of Wnt7a were forward primer 5'-GCCCGACTCTCATGAACTTG-3',

and reverse primer 5'-CCTCGTTGACTTGTCTTGGAGCA-3'. The PCR primer sequences for amplification of Notch1 were forward primer 5'-TGCGAGGTCAACACAGACAG-3', and reverse primer 5'-GTG TAAGTGTGGGTCCGTCCAG-3'. The PCR primer sequences for amplification of Notch2 were forward primer 5'-TGAACACTGGGTC GATGATGAAG-3', and reverse primer 5'-AGCGATGGTGTCC TACGGATG-3'. The PCR primer sequences for amplification of Notch3 were forward primer 5'-TGATGGCATGGATGTCAATGTG-3', and reverse primer 5'-CAGTTGGCATTGGCTCCAGA-3'. The PCR primer sequences for amplification of GAPDH were forward primer 5'-GCACCGTCAAGGCTGAGAAC-3', and reverse primer 5'-ATGG TGGTGAAGACGCCAGT-3'. Each sample was tested in triplicate.

2.6. Statistical analysis

Significant differences between groups were evaluated with Student's *t*-test. Mean differences were considered significant when $p < 0.01$. Three samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

3. Results

3.1. Adhesiveness of NHEKs to SHya

Normal human dermal fibroblasts showed low adhesion to Hya-coated surfaces [9,10]. In order to assess the adhesion of NHEKs to SHya-coated surfaces, NHEKs were seeded on SHya- and Hya-coated surfaces (0, 0.4, and 0.8 mg/cm²). Fig. 2 shows that NHEKs adhered to SHya-coated surfaces as well as to an uncoated dish, but they did not adhere to the Hya-coated dishes.

3.2. Effect of SHya coating on keratinocyte proliferation

NHEKs were seeded on SHya-coated dishes (0.4 or 0.8 mg/cm²) and incubated in a high-calcium condition

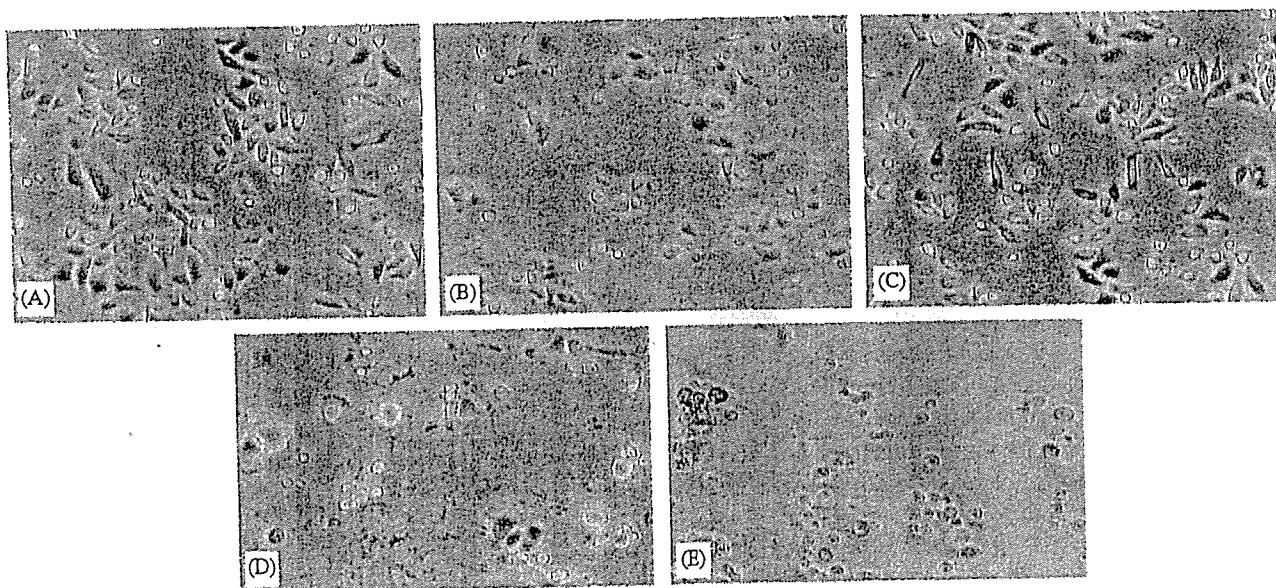


Fig. 2. The adhesion of NHEKs to SHya-coated surfaces. NHEKs were seeded at 1.0×10^4 cells/cm² onto SHya- and Hya-coated 24-well plates. Then NHEKs were incubated with non-coated (A), 0.4 mg/cm² SHya-coated (B), 0.8 mg/cm² SHya-coated (C), 0.4 mg/cm² Hya-coated (D), and 0.8 mg/cm² Hya-coated (E) wells for 16 h.

(0.20 mM) for 5 days. The cell proliferation assay showed that the SHya coating suppressed keratinocyte proliferation remarkably in a dose-dependent manner (Fig. 3).

3.3. Effect of SHya coating on keratin1 and loricrin expression

To ensure that the SHya coating promoted keratinocyte differentiation, we detected the relative expression levels of differential marker mRNA by real-time RT-PCR. After NHEKs were incubated with SHya coating in a high-calcium condition (0.20 mM) for 5 days, the expression level of keratin1 mRNA on NHEKs was increased more than eight-fold compared to the control (Fig. 4A) and that of loricrin mRNA was increased in a dose-dependent manner (Fig. 4B).

3.4. Effect of SHya coating on Wnts expressions

The expression levels of Wnt4 and Wnt6 mRNA on NHEKs incubated with SHya coating in the low-calcium

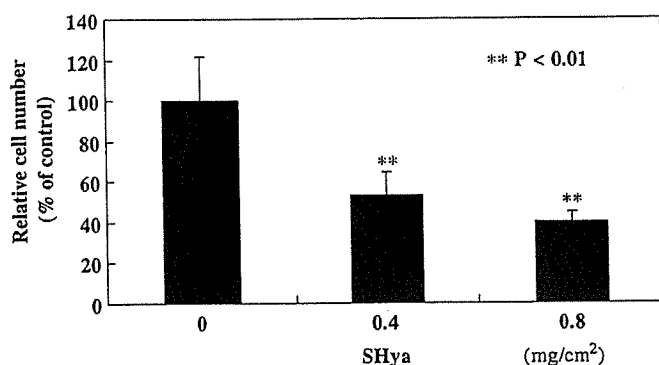


Fig. 3. Suppressive effect of SHya on keratinocyte proliferation. NHEKs were seeded at 1.0×10^4 cells/cm² densities onto non-coated (A), 0.4 mg/cm² SHya-coated (B), and 0.8 mg/cm² SHya-coated (C) 24-well plates and cultured for 5 days. Then numbers of NHEKs were determined by crystal violet assay. Each value is expressed as the mean \pm SD. ** $P < 0.01$ compared to control.

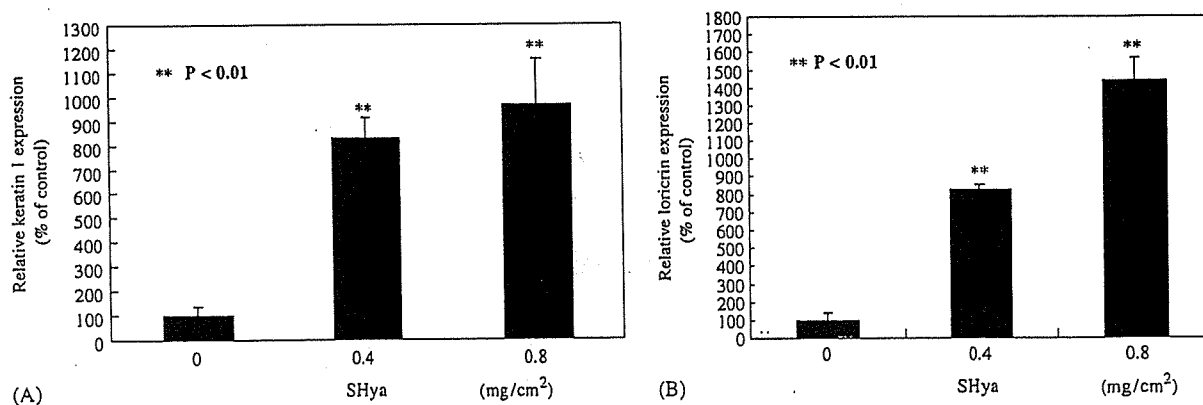


Fig. 4. The expression levels of keratin1 and loricrin mRNA of NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm² onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm²) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression levels of keratin1 and loricrin mRNA. Effect of SHya on the expression level of (A) keratin1 mRNA, and (B) loricrin mRNA. Each value is expressed as the mean \pm SD. ** $P < 0.01$ compared to control.

condition were measured (Fig. 5A and B); the expression level of Wnt5a of NHEKs incubated with SHya coating was decreased to 95% of the control (Fig. 5C) and that of Wnt7a mRNA to about 40% of the control (Fig. 5D).

3.5. Effect of SHya coating on Notch expressions

The expression level of Notch1 mRNA on NHEKs incubated with SHya coating was decreased about 75% compared to the control, and that of Notch2 mRNA was similar to the control (Fig. 6A and B). However, the expression level of Notch3 mRNA was increased about eight-fold compared to the control (Fig. 6C).

4. Discussion

Several studies have suggested that SHya interacts with cells [4], but the effect of SHya on cell differentiation and intercellular signaling was not clear. We demonstrated that a SHya coating promoted keratinocyte differentiation and modulated the expression levels of Notch and Wnt mRNAs.

In this study, the expression levels of Wnt4 and Wnt6 on NHEKs incubated with SHya were increased. A mouse keratinocyte cell line with a deficit of Wnt4 expression showed less differentiation [18]. Wnt6 regulated epithelization [19], suggesting that the SHya-induced upregulation of Wnt4 and Wnt6 is associated with the regulation of keratinocyte differentiation. Wnt7a promoted cell proliferation in corneal epithelial cells during wound healing [20], suggesting that SHya down-regulated Wnt7a expression, resulting in the enhancement of keratinocyte differentiation. The activation of beta-catenin, a downstream factor of Wnt signaling, contributes to keratinocyte differentiation [27]. A sulfated proteoglycan-induced Wnt-11 expression in mouse kidney cells, and sulfated polysaccharides were required in Wnt signaling in mouse kidney cells [28,29]. Therefore, it was suggested

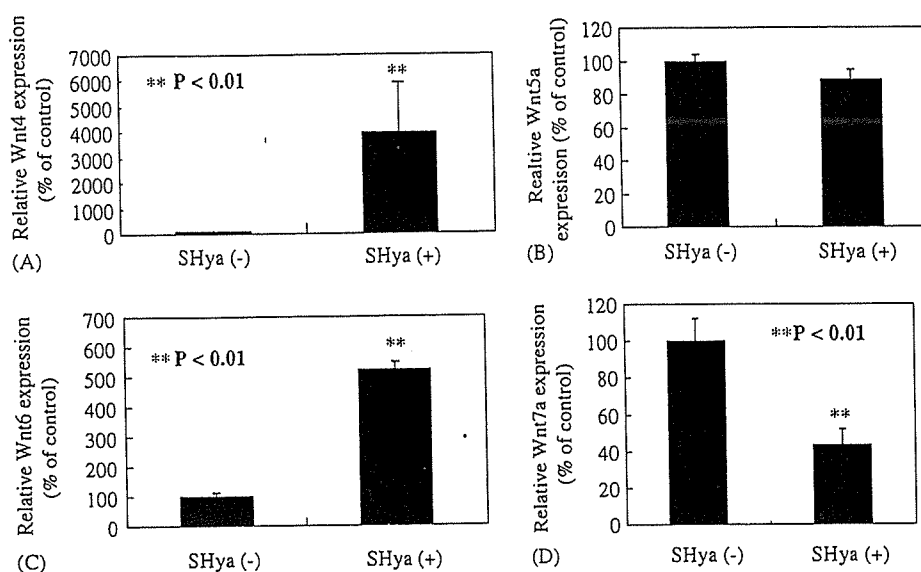


Fig. 5. The expression level of Wnt mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm² onto SHya-coated 60 mm dishes (0 and 0.8 mg/cm²) and cultured in medium with 0.20 mM calcium for 20 h. Then RNA was extracted, and real-time RT-PCR was performed to determine the expression level of Wnt mRNA. Effect of SHya on the expression level of (A) Wnt4 mRNA, (B) Wnt5a mRNA, (C) Wnt6 mRNA, and (D) Wnt7a mRNA. Each value is expressed as the mean \pm SD. ***P* < 0.01 compared to control.

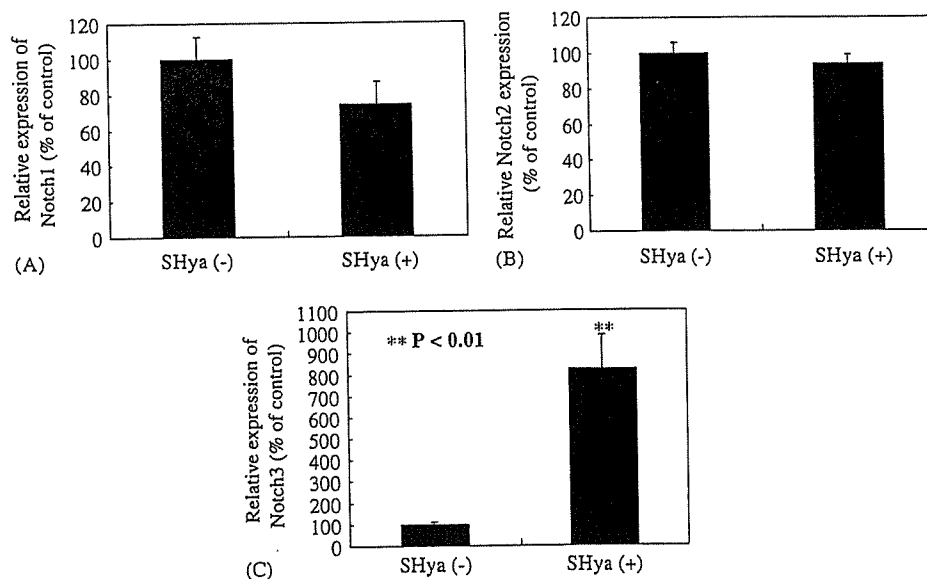


Fig. 6. The expression level of Notch mRNA in NHEKs incubated with SHya coating. NHEKs were seeded at 1.0×10^4 cells/cm² onto SHya-coated 60 mm dishes (0, 0.4, and 0.8 mg/cm²) and cultured in medium with 0.20 mM calcium for 5 days. Then RNA was extracted, and real-time RT-PCR was performed to measure the expression level of notch mRNA. The effect of SHya on the expression level of (A) Notch1 mRNA, (B) Notch2 mRNA, and (C) Notch3 mRNA. Each value is expressed as the mean \pm SD. ***P* < 0.01 compared to control.

that SHya modulated Wnt signaling leading to beta-catenin activation.

In mouse keratinocytes, Notch1 is associated with the regulation of cell differentiation via p21, and Notch1 activation down-regulates Wnt4 expression [21]. Notch1 is required in keratinocyte differentiation and in the regulation of Wnt expression [24]. Activation of Notch receptors induces an increase in its own expression level by a positive feedback mechanism [24]. The expression of Notch1 in NHEKs incubated in SHya-coated dishes was decreased

compared to that of the control. It was suggested that the decrease in Notch1 expression triggered by SHya induced the increase of Wnt4. The expression level of Notch3 mRNA in NHEKs incubated with SHya coating was increased, suggesting that SHya interacted with Notch3, particularly resulting in the modulation of Wnt expression (Fig. 7). Notch3 is required for the differentiation of vascular smooth muscle cells or T cells [23,24]. The role of Notch3 in keratinocyte differentiation triggered by elevation of the extracellular calcium-ion concentration may not

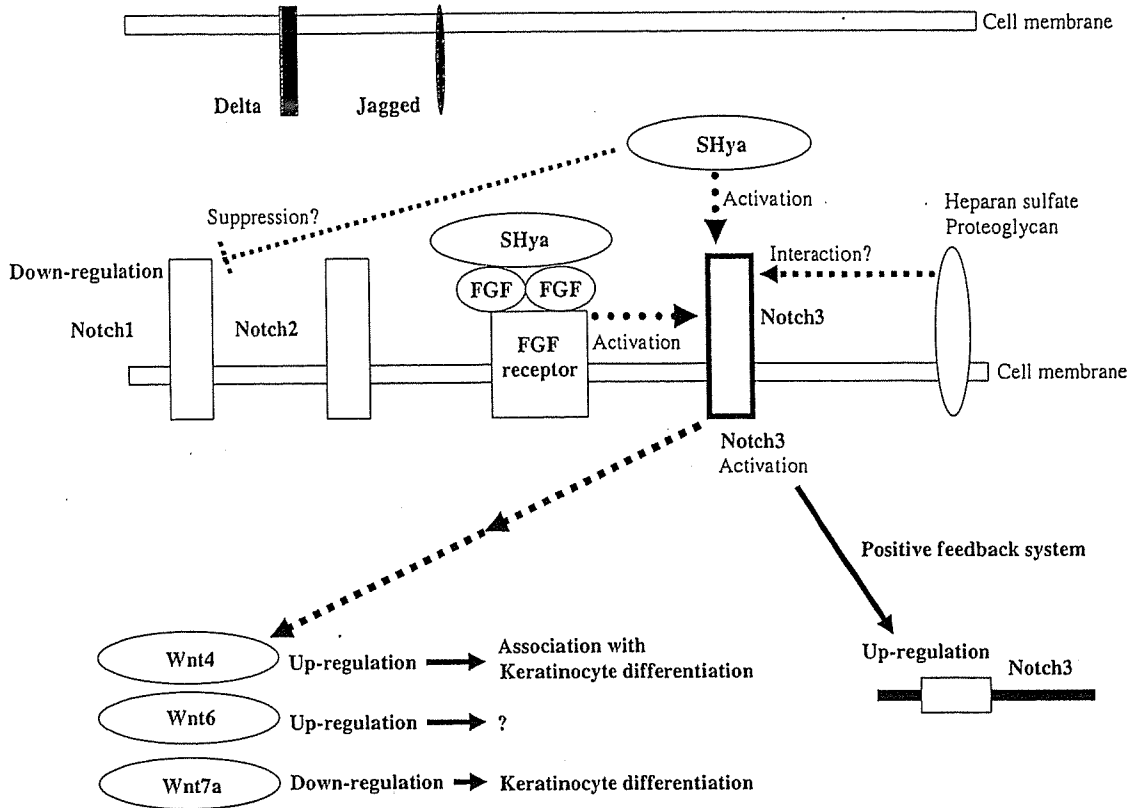


Fig. 7. Diagram of the effect of SHya on Wnt expression via Notch3.

be important, but Notch3 may be activated by SHya, leading to a modulation of intracellular signaling and enhancement of keratinocyte differentiation. Notch3 may be required for interaction with the sulfate groups of sulfated polysaccharides.

Notch1 activation stimulates p21 via the RBP-J kappa transcription factor, resulting in growth arrest, keratinocyte differentiation, or a decrease of Wnt4 expression [25]. Therefore, SHya might activate p21 via Notch3 activation, leading to the modulation of Wnt expression.

The differentiation of NHEKs incubated in SHya was better than that in Hya (data not shown). This suggests that the introduction of sulfate groups into Hya may be a key factor in the enhancement of keratinocyte differentiation.

Normal human dermal fibroblasts showed very low adhesiveness to Hya-coated surfaces because of the anionic surface of Hya [10,11]. However, NHEKs showed very high adhesiveness to SHya-coated surfaces. This suggested that the introduction of sulfate groups into Hya may change the property of its surface, resulting in a high adhesiveness. Sulfated polysaccharides such as heparin and heparan sulfate enhance the stabilities of some growth factors or adsorption of them by the cell membrane, resulting in the enhancement of cell differentiation [13]. The sulfate groups of SHya may bind to and stabilize cationic growth factors, and stabilized growth factors may neutralize the negative charge of the SHya surface, resulting in high cell attachment. Further, it was reported

that fibroblast growth factors (FGF) receptors interact with the Notch signaling pathway [30,31]. Therefore, it was suggested that SHya binds and stabilizes FGF to activate FGF receptors leading to stimulation of Notch3 and intercellular signaling.

5. Conclusion

This study demonstrated that a SHya coating promoted keratinocyte differentiation triggered by an elevated extracellular calcium ion concentration. Furthermore, SHya modulated Wnt expressions and increased the expression level of Notch3 mRNA. These results suggest that Notch3 may be an important target for the regulation of cell differentiation. SHya may be a useful biomaterial to regulate Wnt signaling in tissue engineering. This study provides new information that clarifies the interaction between sulfate groups and Notch families. Studies are in progress to clarify the roles of these modulations of *Notch* and *Wnt* genes in keratinocyte differentiation.

Acknowledgments

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特集：分子イメージング

原子間力顕微鏡 (AFM) による 蛋白質のイメージング

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原子間力顕微鏡 (AFM) による 蛋白質のイメージング

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Protein imaging by atomic force microscopy

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Abstract

Atomic force microscopy (AFM) has been used for imaging of non-conductive surface using a cantilever with a sharp probe to mediate the atomic force interaction between the probe and substrate. The application of AFM for the imaging of protein including transmembrane protein has been studied and revealed their single molecular structure on a nanometer scale. Especially for the transmembrane proteins that lack of 3D structural information obtained by X-ray crystallography, AFM imaging has significant advantages. Since the imaging is capable in the aqueous solution, the obtained images are expected to provide information that reflects structures found in the living cells. Additionally, the force curve measurement for intra- or inter-molecular non-covalent interaction such as protein folding or ligand-receptor interaction will be explained.

Key words: atomic force microscopy, protein imaging, recombinant P2X₂ receptor

1. 蛋白質の構造解析

生体の主要構成成分の一つである蛋白質は、生体内での有機化合物の代謝(酵素など)、情報伝達(受容体など)、組織骨格形成などにかかわる機能性分子であり、その機能に関連する構造の解析は現在の化学の分野で最もホットな分野の一つである。一般にアミノ酸配列を決定する一次構造解析は、繁用法があり、その結果から、 α -ヘリックスや β -シートなどの二次構造が推定される。しかし生体内での蛋白質の機能に大

きくかかわっている三次元構造の決定は困難な点が多い。

現在行われている蛋白質三次構造決定法としては、X線結晶構造解析法と核磁気共鳴(nuclear magnetic resonance: NMR)分光法などがあげられる。X線解析法は解析対象とする蛋白質の単結晶を調製し、X線の照射回折を行い、三次元的画像を得るもので、解像度が非常に高い(Åレベル)という利点がある。特に近年の放射光を用いた測定法の開発により、解析される蛋白質の数は飛躍的に伸びた。しかし、本法は単

¹カリフォルニア大学サンタバーバラ校 ²国立医薬品食品衛生研究所薬理部 ³同療品部

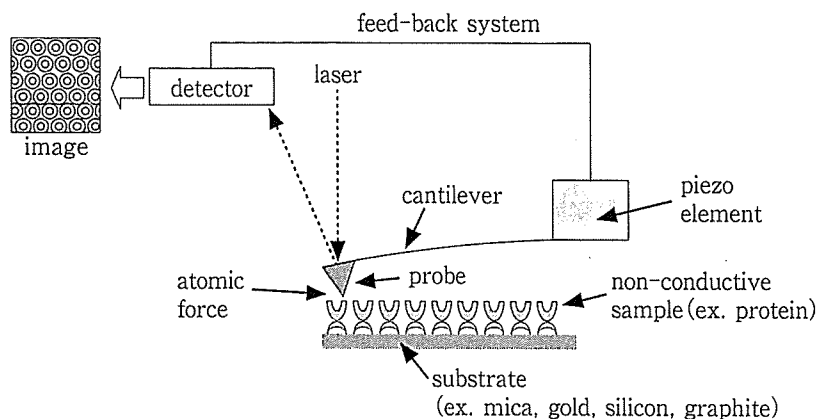


図1 原子間力顕微鏡(AFM)の模式図

結晶の調製が困難である難溶性の蛋白質の解析にはあまり向いておらず、また、得られる構造が結晶格子内における分子構造であるため、動的な構造ではなく、活性型の蛋白質の構造との相関には不明な点が残る。後者のNMR法は溶液中での測定法であり、生体内の蛋白質の挙動に近い情報を求めることが期待され、また、核オーバーハウザー効果(nuclear overhauser effect: NOE)などの手法を用いると、核の間の空間的距離の情報が得られ立体的構造に関する解析も可能である。ただし、蛋白質をNMRで測定して得られるスペクトルは、非常に複雑で解析が難しく、 ^{15}N -enrichした試料の調製が必要になる場合もある。また、試料としては高濃度溶液が必要であるため、難溶性あるいは微量しか発現していない蛋白質の解析にはあまり向いていない。

2. なぜ原子間力顕微鏡(AFM)で蛋白質の画像化を行うのか？

原子間力顕微鏡(AFM)を用いた蛋白質の解析の目的は蛋白質の大まかな構造と機能を知ることである。現在のところ、AFMの解像度はX線解析に遠く及ばない(大まかに言って1/10程度である)が、AFMを用いることの利点を以下にあげる。

- (1) 試料の量が微量で済む。
- (2) 高濃度溶液や単結晶の調製が不要である。
- (3) 溶液中測定も可能であり、より生体内環境に近い条件下での測定が可能である。

(4) 経時的測定モードによりダイナミックな構造情報が得られる。

(5) 単一分子測定が可能である。

3. AFM からどんな情報が得られるのか？

a. 装置の概略とイメージング

AFMが最初に発表されたのは、1986年にPhysical Review Letters誌に掲載されたIBM-チューリヒの研究者達の報告¹⁾においてである。同研究グループはその数年前に、走査型トンネル顕微鏡(scanning tunneling microscope: STM)を発表し、原子像の画像化に成功しているが(1986年ノーベル物理学賞受賞)、トンネル電流を利用したSTM²⁾が電導性のサンプルの測定のみ適用されるのに対し、AFMは非電導性のサンプルの測定も可能であり、DNAや蛋白質などの生体高分子の新しい解析手法として、威力を発揮することが期待されている。

機器の基本的な構造は、主に以下の4つの部分からなる(図1)。

- (1) 試料の表面を走査する微細な探針(probe)
- (2) 探針と試料表面との間に働く原子間力を増幅する微小な板ばね(cantilever)
- (3) 探針および板ばねの高さや位置を調節する圧電(ピエゾ)素子
- (4) 板ばねの変位を測定するレーザー光と、その反射を検出するフォトダイオード検出器

試料と原子間力を介して直接相互作用するのは板ばねに結合した探針の先端で、その位置制御は微細な圧電素子の動きによって行われ

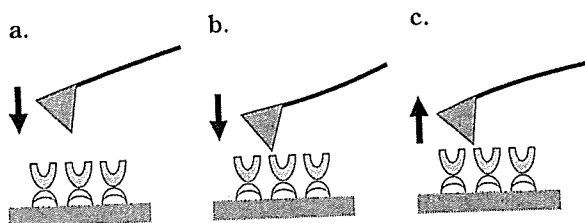


図2 AFM 探針の試料の相互作用と板ばねのそり
 a: 試料へ接近. b: 試料へ接触と、板ばねのそり.
 c: 試料からの離脱と原子間力による板ばねのそり.

る(図2). 試料表面にナノメートルレベルの距離で接近した板ばねは探針と試料との間に生じる原子間力に応じてそり(deflection)などの変位を起こす. そこにレーザー光を照射し、生じる反射光を四分割フォトダイオードディテクターで検出し、その結果に基づきサンプルのトポグラフィカルな情報を画像化するのである. 具体的な蛋白質のイメージングの例は、4. 測定法の項に後述する.

b. アンフォールディング

蛋白質の機能に大きくかわる三次元構造は、蛋白質のフォールディング(折りたたみ)により形成されるが、その機構に関しては未知の部分が多い. しかし、BSEに代表されるように蛋白質のミスフォールディングが疾患に関連している例が知られるようになり、フォールディングに関する基礎的知見は、疾患の検出あるいは診断に重要な役割を果たすことが期待される.

ミュンヘン大学のGaubらのグループは、膜貫通型蛋白質の一つバクテリオロドプシンを用いて、AFMによるアンフォールディングの実験を行った³⁾. バクテリオロドプシン分子が大量に発現した*Halobacterium salinarum*の紫膜を劈開したばかりのマイカ表面上にマウントし、そこへAFM探針を接触させた後引っぱり上げると、フォースカーブに繰り返しパターンがみられた(図3). フォースカーブ上にピークが生じた距離と、フォールディングしているアミノ酸残基の長さは一致しており、ピークは蛋白質のフォールディングパターンを示していると考えられた.

c. 分子間相互作用の測定 (chemical force microscopy)

ハーバード大学のLieberらは、AFM探針先端を特定の有機化合物で修飾し、対象とする試料表面の官能基を選択的に検出しようとするchemical force microscopyを報告した⁴⁾. 彼らは、金で表面をコートしたシリコンナイトライド探針上に親水基(-COOH)あるいは疎水基(-CH₃)を末端に有するアルキルチオール分子で自己組織化膜(self assembled monolayer: SAM)を形成させる方法でAFM探針の修飾を行い、フォースカーブ測定を行った(図4). その結果、CH₃/CH₃, CH₃/COOHおよびCOOH/COOH対での相互作用の違いをフォースカーブで検出することに成功した.

同様に、SAMで修飾したAFM探針を用いて、分子のキラリティーの判別⁵⁾、超分子ホストゲストコンプレックスの結合能の測定⁶⁾などが報告されている. Gaubらのグループは、このchemical force microscopyをレセプター対リガンドの結合能測定に応用し、ストレプトアビジンとビオチンの結合能をフォースカーブとして測定した. この手法を種々のレセプター-リガンド対に応用することで、将来的にはごく微量の蛋白質を用いたドラッグスクリーニング法として有用となる可能性も考えられる(図5).

4. 測定法(各論)

a. 試料の調製法

AFM測定において、最も要となるところが、試料の調製法である. 測定結果の良し悪しは、この試料調製法によるといっても過言でない. AFMは基本的にナノメートルレベルでの表面解析である. したがって、測定する試料はできるだけ平坦なものである必要がある. 調製した試料の凸凹が激しいと、単分子レベルでの測定を行う解像度が望めなくなるからである.

基板としては、マイカ、単結晶金、グラファイトなどの原子レベルで平坦なものを用いる. マイカ、グラファイトは、劈開したばかりのものを用い、金は蒸着後高温でアニールし単結晶表面を形成したものを用いる. ここに目的の蛋

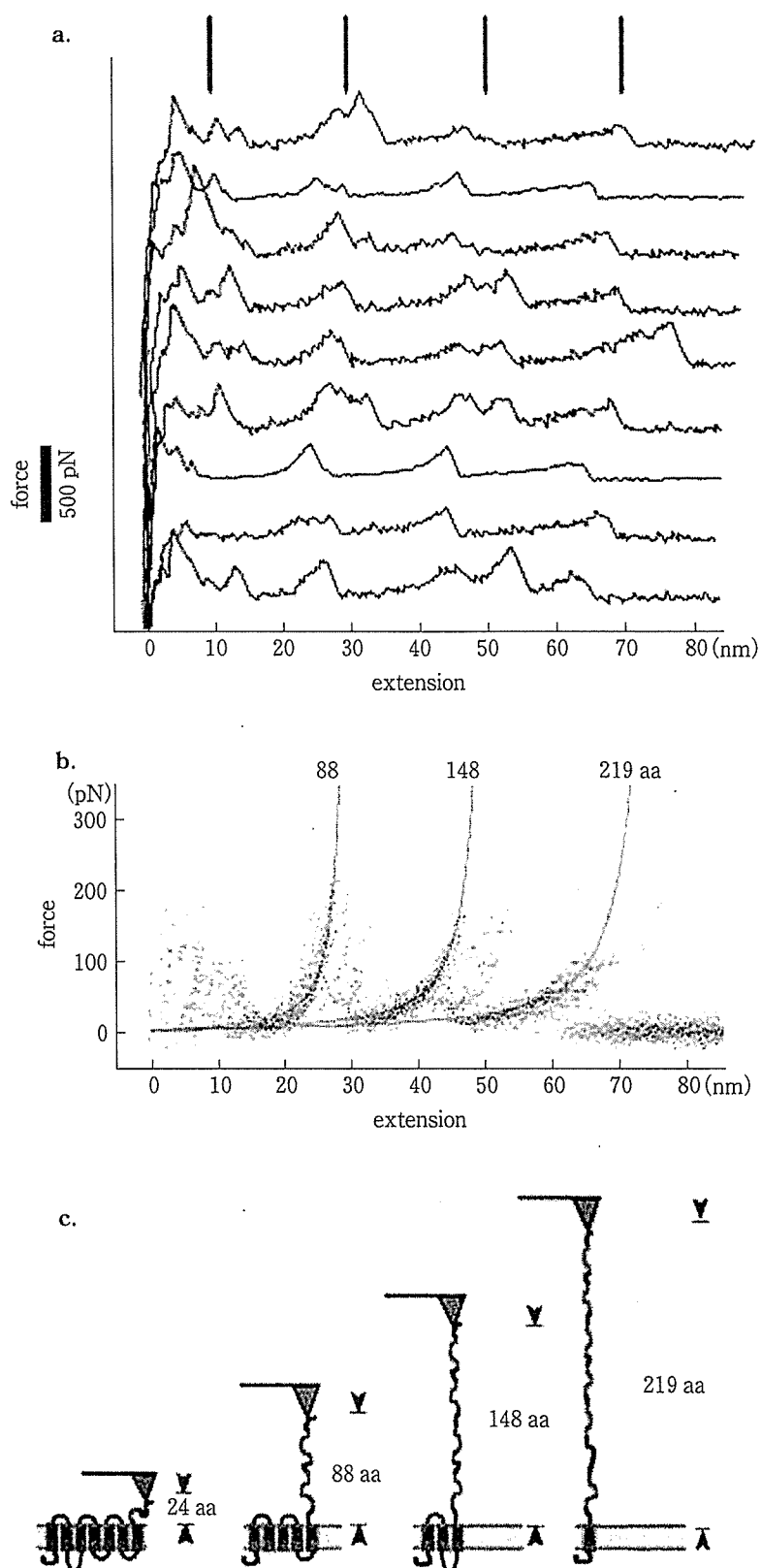


図3 AFMを用いた蛋白質アンフォールディングの実験

細胞膜中の膜蛋白質にAFM探針を接触(-1nN, 1秒間)させ、C末端をAFM探針の先端に接着させる。それを引っ張ると、フォースカーブが生じ、カーブ上のピークまでの距離は、予測されているフォールディングパターンのアミノ酸残基の長さとも一致する。

(文献³⁾より引用)

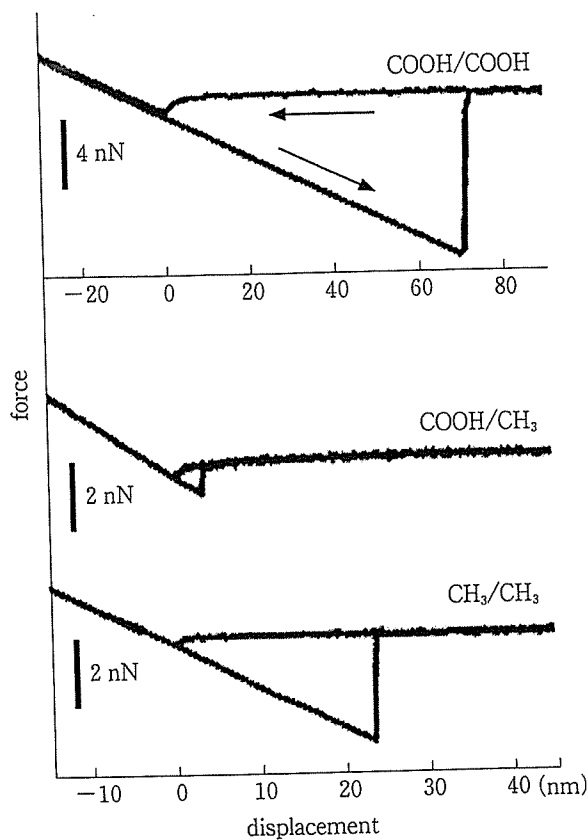


図4 末端に親水基あるいは疎水基を有する分子の自己組織化膜で修飾したAFM探針によるフォースカーブの測定 (文献⁹⁾より引用)

白質を単分子膜として不動化させる。測定する蛋白質は、nativeなものを用いる場合もあれば、精製したものを用いる場合もある。膜結合型蛋白質では、細胞膜上に蛋白質が大量に発現していれば、それを直接測定することも可能であるが、精製した蛋白質を基板上に不動化したうえで測定する場合もある。測定は、溶媒を乾燥させたうえで行う場合と、溶液中でそのまま行う場合がある。いずれにしても超純粋な溶媒を用い、ほこりなどが基板に吸着しないような環境下で試料調製を行う必要がある。図6に調製法の異なる試料のイメージングの例を示す。乾燥した試料の大気中測定では蛋白質の凝集がみられたが、水溶液中での測定では、分散した蛋白質が単分子レベルで観測された。緩衝溶液中での測定では、蛋白質の再構築がよく観察され、チャンネル孔らしき構造も観測された⁷⁾。

b. 測定

1) 測定モード

基本測定モードには、コンタクトモード、タッピングモード、ノンコンタクトモードの3種類があり(図7)、測定する試料に応じてモードを選択する必要がある。コンタクトモードは、探針の先端と試料を接触させながら測定する方法で、探針と試料の接触により生じる板ばねのそりをフィードバックシステムで一定に保つように設定し、電気的信号をもとに画像化する。スキャン速度を上げることができる、比較的凹凸の大きい試料の測定にも適しているなどの利点があるが、直接接触することにより試料に損傷が生じる可能性があるため、ソフトな試料(特に生物学的試料)の測定にはあまり適していない。これに対し、タッピングモードと、ノンコンタクトモードは一定の周波数で振動させた板ばねを用いた測定法である。タッピングモードでは、探針が試料に最も近づくときに軽く試料に接触するが、ノンコンタクトモードでは、全く接触しない。試料と探針が接近すると、相互作用により振動幅に変化が生じるが、これらのモードではフィードバックシステムにより振動幅を一定に保つよう調節し、その調節に必要な電気的信号をもとに画像化を行う。双方とも試料と探針の接触が少なく、ソフトな試料の測定に適している。

2) カンチレバー

AFMの解像度は探針の先端に依存する。すなわち、高分解能な測定のためには、探針の先端がなるべく微細であることが重要である。この微細加工が可能であること、更に、高い共振周波数を得られるという理由から、シリコン、シリコンナイトライドなどの素材が現在汎用されている。また、前述のchemical force microscopyなどの応用測定に用いられる、金コート済みの探針も市販されている。

更に高い解像度を達成するためには、探針の先端が非常に鋭利であることが求められ、単分子であることが理想である。より微細な探針素材としてはカーボンナノチューブを用いる研究も行われている⁸⁾。

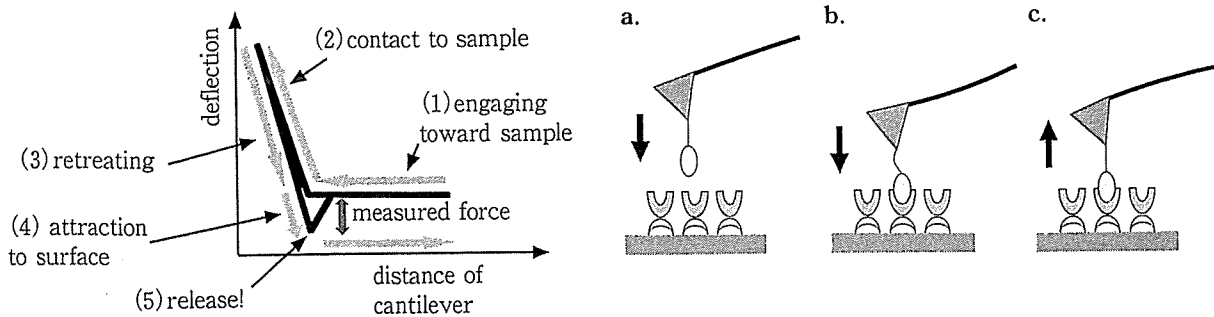


図5 Chemical force spectroscopyを用いたレセプターとリガンド間の分子間相互作用の実験
 a: 試料に接近. b: 試料に接触. c: 相互作用(フォースとして現れる).

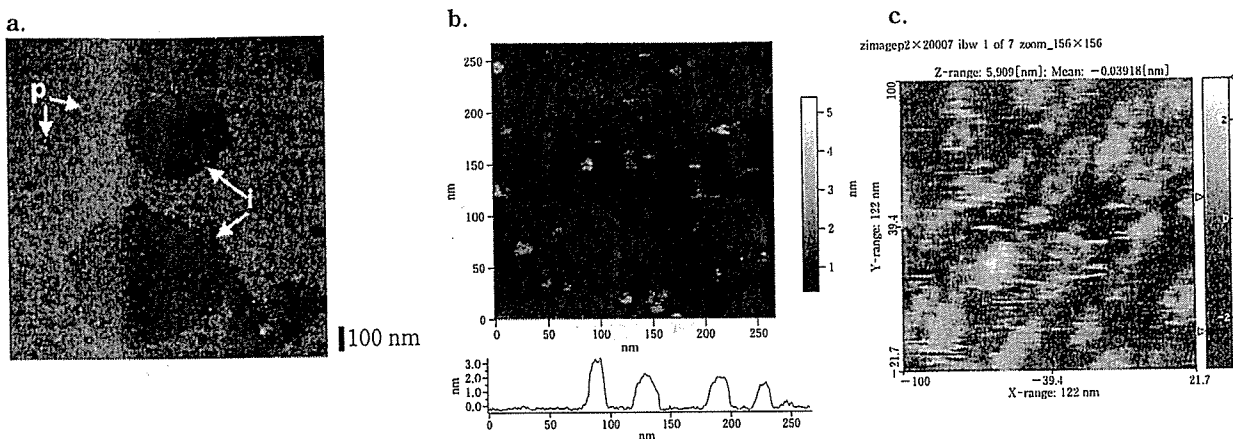


図6 膜蛋白質 P2X₂受容体のAFMによるイメージング

- a: 蛋白質水溶液をマイカ上に滴下後、乾燥させたサンプルを測定した画像。蛋白質が島状に凝集している状態が見える。
- b: 蛋白質水溶液をマイカ上に滴下、そのまま水中で測定した画像とその断面図。蛋白質が単分子状に分散している状態が見える。
- c: 蛋白質の緩衝溶液をマイカ上に滴下、そのまま緩衝液中で測定した画像。蛋白質が再構築し、チャンネル状の構造を取っている状態が見える。

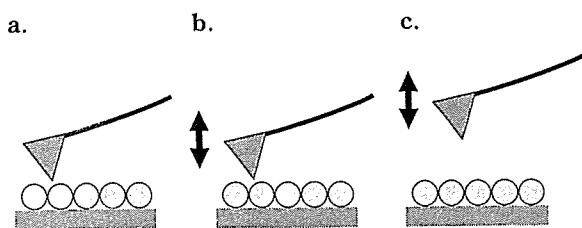


図7 AFMの測定モード

- a: コンタクトモード. b: タッピングモード.
- c: ノンコンタクトモード.

3) 膜結合型蛋白質のイメージングの例

難溶性で単結晶の作成が難しい膜蛋白質のイメージングにおいて、AFMが最も威力を発揮する。ドレスデン工科大学のMüllerらの研究グループは、膜蛋白質のAFMイメージングを

精力的に行っている。その成果の例を図8に示す。

図8は、Cx26 Hela細胞に多量に発現したコネキシン26分子を精製後、マイカ上にマウントして緩衝液中でAFM測定を行ったものである⁹⁾。コネキシン分子の細胞外部分がサブ分子レベルで観測されている。コネキシン分子が六量体でチャンネル孔を有する様子がわかる。図8-aはカルシウムイオン非存在下、bは存在下での測定で、カルシウムイオンの添加により蛋白質のコンフォーメーションが変化し、チャンネルの入り口の直径が1.5nmから0.6nmへと小さくなることが観測された。

図9は脂質二重膜中に埋包させた膜蛋白質