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論文内容要旨

Several lines of evidence have indicated that mechanical tension in adherent cells works as a strong regulator of cellular functions such as structure remodeling, proliferation, differentiation, and apoptosis. The tension in cells is produced passively by external loading and actively by actin stress fibers (SFs) inside the cells. SFs are a contractile cytoskeletal structure made of proteins, mainly, nonmuscle myosin, actin, and α-actinin. It is increasingly becoming clear that SFs, responsible for the generation of the tension, play critical roles in mechano-sensing and the resultant cellular adaptation. To quantitatively consider how the tension is borne or produced in SFs and even transmitted in the cytoplasm to induce the cellular functions, knowledge of their biomechanical characteristics such as the viscoelastic and contractile properties is crucial. However, compared with increasing reports on those properties of the whole cells, the properties intrinsic to individual SFs remain poorly understood.

In this thesis, I wish to reveal the basic biomechanical properties of individual SFs isolated from cells. To accomplish this purpose, several necessary techniques and apparatus were herein newly developed, which include isolation of functional or contractile SFs from cells and a versatile tensile tester for evaluation of the biomechanical properties. More importantly, the mechanical properties obtained here would have a universal value in biology or more specifically in mechanobiology (i.e., a relatively new discipline that highlights the critical role of mechanical factors in biological phenomena), because SFs are considered to be an essential factor in sensing and adapting to mechanical environment as will be described below. Based on the measurement results, I discussed how our understating of the mechanism of the mechano-sensing could be enhanced. The present thesis consists of five chapters, and the subjects of each are summarized in the following.

In Chapter 1, I summarized accumulated knowledge regarding the SF structure, molecular basis, relation with signaling molecules, biomechanical properties, and both their evident and possible functions. As there is noteworthy similarity in molecular components between SFs and striated myofibril, particular attention was focused here on the difference between the poorly characterized subcellular structure of current interest and the better characterized muscular structure. Based on the

known natures, I discussed here why SFs are important in cellular function with a mechanobiological perspective and mentioned my motivation for the present study. The discussion is then moved on more specific topic on the mechanical properties of SFs, the main theme of this thesis. SFs are unstable within the cytoplasm, and constantly change their structure depending on the mechanical as well as biochemical circumstances. This is one of the biggest differences from the nature of the myofibril, which usually has a much slower molecular turnover rate compared to that of SFs and is therefore very stable. Most probably due to this fact that would prevent us from conducting comprehensive measurements of the unstable structure, detailed assessment of the mechanical properties of SFs had been difficult to approach. As described in later chapters, there is a useful solution that could overcome this difficulty, the use of isolated SFs as a sample for measurement. I could succeed here in obtaining and physically manipulating contractile SFs by suitably isolating them from cultured cells, and those functional samples were used for experiment throughout this study. Finally, the objectives of the present study were described, which particularly aimed at obtaining biomechanical properties of individual SFs and related techniques necessary for the measurements.

In Chapter 2, a versatile tensile tester was developed for measuring dynamic mechanical properties of SFs isolated from cells. First, the detail of the method for obtaining sample SFs was described, which allowed isolation of individual SFs from cultured vascular smooth muscle cells that contained nonmuscle isoforms of actin and myosin. The isolation is a modified technique that was originally developed elsewhere by other researchers, but several key techniques that enabled the author to successfully conduct the unexplored experiments were also included and emphasized. The novel apparatus, tensile tester, was designed to allow a variety of measurements, namely strain rate dependency, stress relaxation properties, and creep properties. As the capability of the tester thus covers essentially all kinds of dynamics mechanical properties, the system is applicable even to evaluation of contractile behavior of SFs although it was not touched on in this chapter. These versatile measurements were made possible by employing a particular physical manipulator using a piezo-driven fine glass needle and a visual-based feedback control system. Demonstration of viscoelastic measurements was carried out for SFs isolated from cells to present the required performance. The most unique feature of the system is a capability of simultaneous measurement of the viscoelastic properties together with possible structural changes of the sample tested. This structural change could be detected in the current system by monitoring the entire view of the sample placed in a projected horizontal plane. With a help of certain fluorescent markers suitably bound to the sample, therefore, local strain distribution along the stretched/contracted direction could be obtained during the testing, thereby potentially providing the relationship between the dynamic mechanical properties and basis for the macroscopic natures at the substructure level.

In Chapter 3, the contractile properties of SFs were explored. The contraction of SFs was induced by giving a suitable amount of MgATP (1 mM) to the sample isolated from cells with essentially the same method described in the earlier chapter. One of the surprising findings here was that such contracted SFs keep practically repeatable contractility. Even after the SFs

contracted in the MgATP-dependent manner and shortened in length, SFs exhibited almost reversible contractions when they were stretched back using a glass manipulator. Importantly, it seemed that SFs were not able to contract when they were stretched to a length longer than their original length. Instead, SFs virtually behaved as an elastic material in the high strain range. The putative elastic element in SFs responsible for the surprising behavior is nonmuscle titin, which has been recently reported to exist even in fibroblasts and cultured smooth muscle cells, because striated muscle titin isoform is known to play a force-bearing role at high strain range to keep the integrity of the actomyosin unit structure (i.e., sarcomere), similar to what I found in the current observation. The magnitude of contractile force was also measured using a glass needle. The contractile force and external loading onto the contracting SFs (that is actually equivalent in magnitude to the contractile force) gradually developed when MgATP was administered. The time rate of change in the force or the velocity of the contraction decreased monotonically as time proceeded with increase in external loading, indicating that the Hill's relation previously found in skeletal muscle (i.e., an inverse relationship between the contraction velocity and loading) holds true for the nonmuscle SFs. Another remarkable finding was that SFs produced a larger contractile force near the original length rather than a shortened length that was reached after a certain extent of contraction. Considering these together, the current results suggested that there is an optimal structural strain (or, in plainer word, sarcomere distance if an analogy to striated myofibril was used) for SFs in terms of generation of contractile force. Given that dynamics of adherent cells is dominated by nonmuscle actomyosin or SFs under suitable regulation by related signaling molecules, the findings obtained in this chapter will be a basis for understanding the unsolved mechanisms of phenomena in mechanobiology such as the ability of sensing force, rigidity, and geometry that adherent cells possess in order to adapt to the surrounding environment.

In Chapter 4, viscoelastic properties of SFs, another important biomechanical property, were investigated. With the use of the tensile tester, first, the effect of strain rate on the tensile properties was evaluated with or without MgATP. It is known that, without MgATP, myosin firmly attaches to actin, a condition referred to as the rigor state. One finding was that even in the rigor state where actomyosin and other possible structural components such as α-actinin would work as force-bearing components but no active contraction occurs, a striking viscoelasticity was observed. When stretched at a rate faster than a quasi-static condition, larger tension was developed in single SFs even at the same degree of strain. In addition, I found that stress generated by stretching decreased gradually with time to a certain level higher than the original value and reached a plateau even when the initial stretching was kept unchanged. Relaxation of stress, a typical feature of viscoelastic materials, was thus seen in the rigor state where no active movement in the structural components should be present. Given that the stress magnitude at the plateau was higher than that at the initial or no-stressed condition, SFs in the rigor state could be modeled with the standard three-element viscoelastic model in which an elastic spring and a viscoelastic damper are placed in parallel and the complex component is placed in series with another elastic spring. The time constant of the stress relaxation was on the order of 1–10 sec, which would not be negligible in quantitative comparison between biochemical and mechanical influences on

temporal responses of cells. Regardless of the magnitude of strain rate, the stiffness that is the ratio of force to strain increased on average as stretch proceeded. This behavior in the rigor state seemed to be in the opposite of that in the presence of an almost physiological concentration of MgATP (1 mM), in which SFs apparently became softer in stiffness as strain proceeded to a high level possibly enough for shifting the major stress-bearing component from actomyosin to nonmuscle titin. This unexpected result may be consistent with the observation in Chapter 3 that demonstrated that SFs were not able to contract at a length longer than the original length probably because of a shift of the effective structural component to nonmuscle titin. The rigor complex of actomyosin is thought to bear stress without definite unfolding of the protein conformation, whereas skeletal titin is known to unfold with external stretching and elongate considerably. The former that will raise resistance to deformation may exhibit strain-induced stiffening, whereas the latter softening. Thus, I thought the specific substructure of SFs constructed from actomyosin and most likely nonnuscle titin as well was attributable to the difference in the curve shape, as the current viscoelastic tensile test was initiated from the original length. Although more thorough measurements are needed, the present data could thus lead to better understanding the process of intracellular force transmission together with implications of the mechanical role of SFs in the mechanical behavior of living cells.

In Chapter 5, this thesis is concluded.

The present work has thus provided interesting and important data on the biomechanical properties of SFs, and novel views based on the uncovered experimental findings were proposed. As virtually no data has yet appeared that clarified dynamic mechanical properties of individual SFs, the data presented in Chapters 3 and 4 will be useful in all related fields, such as interpretation of experimental results and as constitutive models for computational biology. Regarding possible improvement of the present research, the author would like to extend the experiment in Chapter 4 to draw more solid conclusion. A possible path for future research is to consider the ultra-microstructure of native SFs that elicits the macroscopic mechanical properties. SFs locate deep in the cytoplasm, and hence a transmission electron microcopy (TEM) will be needed to observe the fine structure. There is actually limitation in conventional TEM to obtain the whole view along the longitudinal direction. To overcome this, the knowledge of the SF isolation technique shown in Chapter 2 could be available, which provides functional SFs that could be mounted in a single plane facilitating detailed observation and that are exposed directly to solution, advantageous for replica EM. With these possible advances, the relationship between macroscopic mechanical behavior and ultra-microstructure such as the number of associated critical molecules and spacing between sarcomeric units could be elucidated. This will contribute to revealing more clearly the role of SFs or nonmuscle actomyosin in cellular mechano-sensing and adaptation, which, in the author's opinion, is one of the most important as well as interesting and exciting points in mechanobiology.

論文審査結果の要旨

細胞内に発達する収縮性線維状構造体であるアクチンストレスファイバは，その両端を細胞外と細胞内を物理的に結合する焦点接着斑と接続した状態で存在している。焦点接着斑には，細胞生理を調節するセンサー分子が多数凝集しており，力が作用することで活性が制御されているという報告がなされている。センサー分子の出力はセンサー分子周囲の力のバランスによって決定されると考えられていることから，細胞内部からの力としてのストレスファイバの能動的な収縮特性，および細胞外部からの力を受容する際の支持要素としてのストレスファイバの力学特性を明らかにすることは，力によって調節される細胞生理の理解にとって重要な課題である。著者は細胞からストレスファイバを単離し，微小力学操作可能な実験系を確立することで，ストレスファイバの収縮特性および粘弾性特性を初めて明らかにしている。本論文は，これらの成果をまとめたものであり，全編5章からなる。

第1章は緒論であり，本研究の背景，目的および構成を述べている。

第2章では，ストレスファイバの単離方法，および粘弾性特性を計測するための装置の開発について述べている。その結果，ストレスファイバの収縮能を維持，かつ顕微鏡観察下で力学操作可能な状態で単離可能であることが示されている。また，装置の開発により，ひずみ速度一定制御試験，緩和試験，クリープ試験などの粘弾性試験を実施でき，なおかつ，同時にストレスファイバの内部構造変化を蛍光観察することも可能である。これは，ストレスファイバの力学特性を明らかにするために有益な成果である。

第3章では，ストレスファイバの収縮特性の計測について述べている。その結果，繰り返し伸長，短縮刺激負荷時の収縮挙動から，等尺性収縮の維持には構成分子の取り込みは重要ではないことが示されている。また，ストレスファイバには筋原線維と同じような等尺性収縮力とサルコメア様長さとの関係が存在していることや，作用・反作用の法則に従って収縮力を発揮できることが示されている。これは，細胞の基質硬さ感知機序の解明などに繋がる非常に重要な成果である。

第4章では，ストレスファイバの粘弾性特性の計測について述べている。その結果，アクチン-ミオシン間結合状態に依存したひずみ速度依存性の張力-ひずみ関係，および緩和試験において，初期ひずみ負荷時にアクチン-ミオシン間結合状態に依存した初期張力を生じることが示されている。また，ストレスファイバの粘弾性特性には，タイチン様タンパク質の寄与がなければ説明できないことが示されている。これは，ストレスファイバの構造と粘弾性特性との関係を理解する上で，重要な知見である。

第5章は結論である。

以上要するに本論文は，ストレスファイバの力学特性を計測し，細胞の基質硬さ感知機序や，内部構造の力学的な関わりを示したものであり，バイオロボティクスおよび生体機械工学の発展に寄与するところが少なくない。

よって，本論文は博士(工学)の学位論文として合格と認める。