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Effect of External Stimuli on Cell Adhesion Dynamics

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Summary

Adhesion of cells to biomaterials or extracellular matrix (ECM) is a fundamental process in tissue engineering devices and biotechnological process, as well as in organogenesis and tissue maintenance. Anchorage-dependent cells adhering on surface modified with adhesive ligands will spread and undergoes cytoskeleton rearrangement. In turn, the cellular phenotypes of adherent cells on biomaterials are directly correlated with the biophysical responses of cells. However, the lack of integrated bio-analytics for probing the cell-substrate interface poses significant obstacles in the engineering analysis of cells on biomaterial or ECM surface. Based on the principle of confocal reflectance interference contrast microscopy (C-RICM), our group has successfully determined several key biophysical/biomechanical parameters of cells on model ECM. In this thesis, I apply the unique biophysical approaches on the elucidation of the effect of GFP-actin expression, Hepatitis B virus and cytoskeleton-targeting drugs on the biophysical behavior of HepG2 cells on a model ECM.

In the first part of this work, our results show that the evolution of adhesion contact of HepG2 cells upon integrin-collagen or fibronectin interaction is impaired by GFP-actin expression. Also, GFP-actin expression reduces the initial rate of cell deformation. Interestingly, the steady-state adhesion energy of HepG2 cells remains unchanged and increases on fibronectin- and collagen-coated substrate, respectively, upon. In addition, GFP-actins diffusively concentrate in the cytoplasmic cortex while adhesion contact evolves and cell spreads. Kinetics analysis on the adhesion contact formation demonstrates the intricate interplay between cytoskeleton property and ECM proteins in cell adhesion.

In the second part of this study, I focus on exploring the adhesion contact dynamics and cytoskeletal remodeling of HepG2 cell in response to HBV infection on model ECMs. Our results demonstrate that HBV infection affects the reorganization of microfilaments, the interaction of HBX and vinexin, and vinculin gene expression, leading to alteration of cell morphology and delay of focal adhesion formation. Also, the lag time of adhesion contact evolution and initial adhesion energy of HepG2 cell infected with HBV is significantly increased and reduced, respectively. The initial cell deformation rate on collagen- and fibronectin- coated surfaces is unaltered and reduced, respectively, upon HBV infection. Intricate interplay between GFP-actin expression, HBV infection and adhesion dynamics has been revealed herein.

In the third part of my work, the kinetics of cell detachment and actin dynamics of live HepG2 cells following the addition of cytochalasin D (Cyto-D), latrunculin A (Lat-A), and doxorubicin (Dox) is probed with confocal reflectance interference contrast microscopy (C-RICM) and fluorescence confocal microscopy. The results show that the reduction in two dimensional spread area of HepG2 cells is 10.5 %, 15.4 % and 21.9 % under the influence of 5 μ M of Lat-A, Cyto-D and Dox, respectively. Also, C-RICM demonstrates that the recession of adhesion contact against time of cell seeding upon the addition of the three drugs. In addition, the initial cell detachment rate and extent of change in degree of cell deformation (a/R) are dependent on both the drug types and concentration. Lastly, oscillation-like responses of a/R and adhesion energy is uniquely found in Lat-A induced cell detachment. Overall, our biophysical approaches have been proven as a highly quantitative platform for screening drug interactions with targeted cells.

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Chapter 1: Introduction

1.1 Background

Cell adhesion is mediated by the recognition of adhesion receptors on cell surface with underlying extracellular matrix proteins or biomaterials, which acts as a biophysical cue for mechanochemical transduction [Keselowsky et al., 2005]. Moreover, the intracellular domain of adhesion receptors such as integrin directly tethers to cytoskeleton inside the plasma membrane through accessory binding proteins for modulating cytoskeletal network and transmitting external signals [Miyamoto et al., 1995; Mostafavi-Pour et al., 2003]. In particular, these adhesion receptors form signaling complexes such as focal adhesion contact towards the recognition with immobilized ligands on extracellular matrix or biomaterial and modulate cell functions including differentiation, proliferation, morphogenesis, organogenesis and migration etc. Most importantly, cell adhesion alters the biochemical signaling fluxes involved in actin polymerization, cytoskeletal targeting and adhesion contact assembly [Pichard et al., 2001]. The disruption of normal adhesion or promotion of inappropriate temporal and spatial mechanism of adhesion will change regulatory signaling and subsequently disease progression [Tavares et al., 2005].

For adherent cell to sense external stimuli, cytoskeleton, an intracellular structural element plays a critical role in transmitting signal from cell surface, across the cytoplasm and into the nucleus, as well as in the triggering of specific biochemical responses [Ingber 1993; van Zundert et al., 2002; Wang et al., 1993]. To understand the influence of external stimuli on cellular functions, one has to reveal how cytoskeleton changes its organization and properties in response to external challenges surrounding

cells. Cytoskeleton is an interconnected structure including three major filaments: actin filaments, microtubules and intermediate filaments. Although their roles in biological functions have not been fully understood, several experimental studies in living cell *in vitro* have revealed that actin filament carries tensile force, stress fibers (bundling actin filaments) transmits tensile force and compression force, and microtubules transmit compression force [Ingber 1993; Hucker et al., 1999; Wang et al., 2001]. More importantly, these cytoskeleton elements are tightly coupled to the biochemical signaling cascades of most mammalian cells.

Extracellular matrix (ECM) composed of a variety of polysaccharides and proteins has been found as another important component in the signal transduction cascades of anchorage dependent cells. In turn, the mechanical forces within the cytoskeleton are fed backward from cytoplasm to the ECM via transmembrane integrin receptors and/or coreceptors that condense to form focal adhesion plaques [Wang et al., 1993; Mostafavi-Pour et al., 2003]. Thus the determination of the correlation between cytoskeleton properties and external stimuli has fundamental implications for understanding cellular responses governing regular physiological functions.

Receptors on cell membrane are essential components for mediating cell adhesion and transmitting external stimuli. For example, integrin receptors are alpha-beta heterodimers with a transmembrane segment, a short cytoplasmic domain and a large extracellular domain. The transmembrane receptors form significant linkage between extracellular matrix and cytoskeleton components. It is evident that integrin receptors play a very important role in cell adhesion, cell migration and other related biological functions. Although there are other adhesion receptors on cell surface such as cadherin, selectin and immunoglobulin receptors that can mediate cell-cell interactions, several

groups have shown that these classes of receptors have no affinity against ECM proteins that are critical for promoting cell migration [Calof and Lander 1991; Ludwig et al., 2004; Lemmon et al., 1992]. The activation of integrin receptor influences multiple intracellular signaling cascades, including tyrosine phosphorylation. Moreover, integrin mediates processes alter the activities of focal adhesion kinase (FAK), Rho GTPase, and protein kinase C α (PKC α), etc [Schleapfer et al., 1994; Mostafavi-Pour et al., 2003]. However, the intracellular signal transduction cascades that follows receptor mediated recognition at cell-ECM interface is not known. To date, several classes of integrin have demonstrated their important roles in the signal transduction of anchorage-dependent cells. The structural features of integrin suggest that the mechanical property of integrin-cytoskeleton linkage has led to the unique roles of integrin in cellular functions. Recently, a group has applied optical tweezers to induce external force on cell surface through ligand coated polystyrene bead. Vascular smooth muscle cells stressed via integrins have shown increased stiffening against the increase of external force, while this stiffening is significantly less for cells stressed via the transferrin receptor ($P < 0.001$) [Huang et al., 2001]. Moreover, global, cell-wide changes in cytoskeletal structure have been shown to regulate and drive key cellular responses upon the integrin-mediated adhesion [Alenghat et al., 2004].

Cellular responses induced by external stimuli through the mediation of cytoskeleton network dictate various physiological functions, such as organogenesis, cell migration, immunological cascades, etc [Chen et al., 1997; Harris et al., 1980; Thomas et al., 2002]. The study of a pathway of mechanochemical transductions poses significant challenge to experimental techniques *in vivo* because the complex metabolic networks underlying the cellular processes. To investigate cellular responses as a result

of external factors including chemicals and forces, advanced bioanalytical techniques must be designed and developed for the applications *in vitro*. For instance, micropipette aspiration, atomic force microscope, optical tweezers, and magnetic tweezers have been used to probe the mechanochemical of single cells [Evans and Yeung, 1989; Hochmuth, 1993; Mathur et al., 2001; Leckband 2000; Alenghat et al., 2000]. The transfer of external stimuli in the form of biochemical signals or mechanical force from ECM or biomaterial scaffold to intracellular structures is correlated with the strength of cell attachment to the substrate, as well as on geometry of the adhesion site. Therefore, ensuring appropriate adhesion of cells to the underlying substrate is a critical requirement for major *in vitro* applications of cells in engineered tissue equivalents and other cell therapeutics devices.

In addition, mechanochemical signal transduction plays an essential role in the functional maintenance of various tissues. The design of engineered tissue equivalents must consider the environmental influence on tissues *in vivo*. Cells likely have numerous mechanisms to sense and respond to external stimuli, but our focus is on the mechanistic connection of cytoskeleton with the activated adhesion receptor. Focal adhesion contacts, which are clusters of integrin receptors and associated with cytoskeletal proteins, provide a direct physical linkage between the extracellular matrix and the cytoskeleton, and paves the way for mechanochemical signaling. Overall, cell adhesion and mechanochemical signal transduction are tightly coupled with each other through the intricate interplay between receptor-ligand interaction, cytoskeleton transformation, gene expression, etc. The biophysical mechanisms involved in receptor-mediated process are not clear due to technique limitation and shortage of

advanced biophysical techniques. For example, how biological cells sense external stimuli, deform, adhere and convert them into biological responses.

With the recent development of new biophysical techniques, in depth study on cell deformation and adhesion energy become possible. The marriage between molecular biology techniques, and unique biophysical approaches based on confocal reflection interference contrast microscopy, phase contrast microscopy and confocal fluorescence microscopy facilitates the determination of biomechanical behavior, cytoskeleton structure and adhesion strength of cells in a highly quantitative manner. In this project, studies on the adhesion contact dynamics, deformation, morphology, cytoskeleton organization under different external stimulation are carried out at molecular and cellular levels.

1.2 Objective and Scope

The primary goal of this work is to study the morphology, cytoskeletal organization, adhesion energy, cell deformation and adhesion contact dynamics of HepG2 cells on ligand-modified substrates under external stimuli including GFP-actin expression, Hepatitis B virus, and cytoskeleton disruptive drugs. An understanding of HepG2 cells behavior as a model system will elucidate the underlying correlation between external stimuli and cellular functions. A novel biophysical probe known as confocal-reflection interference contrast microscopy (C-RICM) has been set up in our lab, which is used for probing key biophysical parameters of adherent cells including morphology, cytoskeleton organization and adhesion dynamics stimulated by GFP-actin expression, Hepatitis B virus, some drugs targeting cytoskeleton. In this project, our specific aims are listed as follows:

- To apply molecular biology techniques for transfecting cells with GFP-tagged cytoskeletal proteins and immunostaining actin filaments with FITC-phalloidin. These approaches allow one to observe change in cytoskeleton structures *in-situ* or *ex-situ* under external stimuli.
- To understand the biophysical mechanism of cell adhesion on model ECM towards cytoskeleton alteration (GFP-actin expression) by using C-RICM for elucidating the link between cytoskeleton integrity, receptor-mediated recognition and external stimuli.
- To investigate the effect of hepatitis B virus infection and/or GFP-actin expression on HepG2 cell adhesion dynamics on ECM model, it is useful for one to understand the change in liver cell morphology, cytoskeletal rearrangement and functions under HBV infection.
- To add cytoskeleton disruptive drugs to HepG2 cells for elucidating the global correlation between cell morphology, biomechanical properties, adhesion contact recession and cytoskeleton structures on a model ECM.

Our approach as mentioned above will enable researchers to gain a quantitative understanding of cellular behavior which will lead to further exploitations in tissue engineering, especially clinical application including artificial liver and self-repairing system for treatment of acute liver failure; enhance capabilities in evaluating and designing biomaterials and drugs at a lower cost through the modeling of mechanical signaling pathways.

1.3 Organization

This thesis is organized into seven chapters. The background, scopes and objectives of my present research has been described in the current chapter. In Chapter 2, we present a brief literature review from previous research about cell biophysics, receptor-mediated adhesion, common experimental methods, cytoskeleton structure and functions. Materials and experimental methods are covered in Chapter 3. The influence of GFP-actin expression, Hepatitis B viruses, and cytoskeleton disruptive drugs on the biophysical properties of HepG2 cells on a model ECM is presented in Chapter 4, 5, and 6, respectively. And finally, conclusions and future work are given in Chapter 7.

Chapter 2: Literature Review

2.1 Overview

Cell adhesion to other cells, extracellular matrix (ECM), and various substrates is fundamental in the designs of tissue engineering device and biotechnological processes [Athanasίου et al., 1999]. The attachment, subsequent spreading and adhesion of a cell on a ligand coated substratum are resulted from many different intermolecular forces. These biological forces are classified into nonspecific interaction and specific binding that are originated from physical forces and molecular recognition, respectively [Koller and Papoutsakis, 1995]. Nonspecific interactions include electrostatic force, hydrogen bonding, van der Waals force, hydrophobic force, and steric stabilization forces. Actually, specific binding is also brought about by countless nonspecific forces between specific functional sites on two complementary molecules. The distinction between specific binding and nonspecific interactions is stemmed from the molecular specificity and high affinity.

The recent progress in cell and molecular biology has paved the way for the study of mechanochemical transduction of anchorage-dependent cell. The new paradigm of biophysics has been developed along with the advances of instrumentation as suggested by several recent reviews on cell biophysics [Zhu et al., 2002], molecular biomechanics [Bao, 2002], cell morphology regulation and biochemical signaling [Goldmann, 2002], kinetics of cell spreading [Frisch and Thoumine, 2002], cell and molecular mechanics [Bao and Suresh, 2003], mechanochemical transduction [Huang et al., 2004], and adhesion-dependent cell mechanosensitivity [Bershadsky et al., 2003]. In this section, we will emphasize on recent progress on the understanding of cell adhesion molecules

and intracellular anchor proteins (integrins, selectins, cadherin, vinculins, catenins); focal adhesion contact and signal transduction; relation between adhesion, osmosis; cytoskeleton structure and function; and experimental techniques for measurement of cell adhesion.

2.2 Adhesion molecules and intracellular anchor proteins

Integrins, a major class of adhesion receptor of cell for ECM and cell-cell adhesions, are a family of heterodimeric membrane glycoprotein expressed on diverse cell types. Several studies indicate the formation of integrin-cytoskeleton focal adhesion complexes triggers subsequent cell signaling pathway. In order to exploit the influence of receptor-ligand interaction on the mechanism of focal contact formation, Athanasiou et al. (1999) seeded bovine articular chondrocytes on a substratum coated with either fibronectin or bovine serum albumin, on standard microscope slide to quantify the force required to displace attached cells utilizing a cytodetacher. Their results demonstrate that a larger force is required to detach cells from fibronectin coated substrate in comparison with the two other substrates and suggest that actin filament of cytoskeleton and perhaps focal adhesion contribute significantly to the adhesive strength of the cells. Does the intercellular dynamic of the cytoskeletal molecules enhance the affinity between the ECM and the extracellular domain of the cell surface receptor (Integrins)? By using soft micro-needles coated with Arg-gly-Asp (RGD) peptides, a sequence commonly found in most ECM proteins, Rahman et al. (2002) applied a localized ramp shear stress to the surface of a HeLa cell in order to determine the cell stiffness as well as the collective unbinding lifetime between cell surface receptor and RDG. These measurements demonstrate that both cell stiffness and the collective cell receptor-RGD unbinding lifetime increase with the increase of the

duration of the pre-shear cell-micro-needle contact and with the rate of shear applied to the cell membrane. These parameters are also highly dependent on the integrity of the network of actin filament. The results indicate that RGD-mediated initial recruitment of cytoskeletal proteins to the cytoplasmic domain of integrins directly enhances the interaction between the extracellular domain of integrins and the RGD sequences of ECM molecule.

Bierbaum (1998) applied mechanical stress to specific receptors on the cell surface and probe the reaction kinetics of trans-membrane receptors against the change of mechanical stress. An increase in tyrosine phosphorylation of a migration cell is observed as a result of applied shear stress on the beta (1)-subunits of the integrin family. On the other hand, mechanical stress applied to other trans-membrane molecules such as transferrin or low density lipoprotein receptors without connection to the cytoskeleton does not lead to the change mentioned above. Potard (1997) probed the cytoskeleton through integrins and E-cadherins in a confluent epithelial cell layer (MCF7). At high applied stress ($>10\text{dyn/cm}^2$), the stiffness (stress/strain) of the cytoskeleton coupled to integrins is greater than that of cytoskeleton coupled to E-cadherins. The stiffness response of cell after actin (micro filament) or microtubule (MT) disruption with cytochalasin D or colchicine is greater for integrins than other receptors. Berry et al. (2000) explored integrin dynamics of cells during tumor invasion and metastasis. Their experiments demonstrate a 1.40 ± 0.14 -fold increase in beta (1) integrin expression on MCF-7 cells following treatment with angiotensin II. These findings provide evidence of association between integrins and the angiotensin A II in the function of human breast cancer cells and suggest a novel research direction for future anti-metastatic therapeutics in the management of invasive human breast cancer.

If the cytoplasmic domain of the beta subunit of integrin is deleted using recombinant DNA techniques, the shortened integrins still bind to their ligands, but they no longer mediate robust adhesion, and fail to form focal adhesions. It seems that integrins must interact with the cytoskeleton to bind cells strongly to the ECM.

Extracellular environment surrounding cell apparently influences cell morphology, spreading, as well as cellular function. However, the exact mechanism is clearly unknown. Pichard et al. (2001) investigated the role of epidermal growth factor (EGF) and type IV collagen on cell adhesion by using flow cytometry, immunofluorescence, immuno-precipitation and immuno-blotting techniques, as well as colon adenocarcinoma cell line HT29-D4 as *in vitro* model. The result shows that HT29-D4 cell adhesion and spreading is regulated by EGF through EGF-dependent FAK phosphorylation on type IV collagen coated coverslip. The EGF stimulation also leads to integrin activation and their recruitment in numerous focal adhesion complexes. Also, FAK induces aggregation of actin-associated proteins in focal complexes leading to actin filaments reorganization and lamellipodia formation. Binding of specific integrin receptors on different biomaterial surfaces also triggers specified downstream, higher-order cellular activities. Keselowsky et al. (2005) plated osteoblasts on fibronectin-coated biomaterial surface that contains different chemical group such as CH₃, OH, COOH, and NH₂ to explore the influence of biomaterial surface on cell behavior. The experimental result indicates that surface chemistry regulates osteoblast differentiation and matrix mineralization independently from alterations in cell proliferation. Main cause is that upon adsorption to biomaterial surface, fibronectin undergoes structural transformation such as alternations in the central cell adhesion binding site and varies its functional activity for integrin binding.

Besides integrin, selectins are cell-surface carbohydrate-binding proteins that mediate a range of transient and Ca^{2+} -dependent cell-cell adhesion in circulation. There are at least three types of selectins including L-selectins found on white blood cells, P-selectins found on blood platelet, and E-selectins on endothelial cells. Both classes of selectin receptors are activated during inflammatory responses. E-selectins are commonly found on activated endothelial cells. Selectins play an important role in causing blood cells to reach endothelium, subsequently enabling the blood cells to migrate out of the bloodstream into various tissues. Geng et al. (2004) explored P-selectin cell adhesion molecule in inflammation, thrombosis, cancer growth and metastasis. P-selectin, which expresses on stimulated endothelial cells and activated platelets, not only mediates leukocyte rolling on stimulated endothelial cells and heterotypic aggregation of activated platelets onto leukocytes but also mediates heterotypic aggregation of activated platelets to cancer cells and adhesion of cancer cells to stimulated endothelial cells. Recent study demonstrates the importance of P-selectin-mediated cell adhesive interactions in the pathogenesis of inflammation, thrombosis, growth and metastasis of cancers using P-selectin knockout mice. Based on the current knowledge and important progress in the biomedical research of P-selectin biology, they provide a suitable target for therapeutic interventions developed through both experimental and bioinformatics approaches.

Dong et al. (2000) has investigated the mechanics of leukocyte (WBC) deformation and adhesion to endothelial cells (EC) using a novel *in vitro* side-view flow assay. HL-60 cell rolling that eventually leads to cell arrest on surface immobilized with P-selectin was as a model system for WBC-EC adhesion process. The group has found that changes in flow shear stress, cell deformability, or substrate ligand

strength result in significant changes in the characteristic adhesion binding time, cell-surface contact and cell rolling velocity. A 2-Dimensional model indicated that cell-substrate contact area under a high wall shear stress (20 dyn/cm^2) is nearly twice of that under a low stress (0.5 dyn/cm^2) due to shear flow-induced cell deformation. An increase in adhesion contact area results in more energy dissipation to both adhesion bonds and viscous cytoplasm, whereas the fluid energy that inputs to a cell decreases due to a flattened cell shape. Hinds et al. (2001) applied shear flow to investigate monocyte adhesion to the endothelium and proposed the hypothesis that (a) under the same flow conditions, insolubilized E-selectin on the model's surface may mediate adhesive interaction at higher wall shear stresses, compared to uncoated model, and (b) pulsatic flow may modify the adhesion profile obtained under steady flow. Experimental result shows surface E-selectin greatly enhances cell adhesion in all regions of flow at $Re = 100$ and 140 , compared to an uncoated model under the same flow conditions. In regions where the cells near the wall are abundant (taper and stenosis), adhesion to E-selectin is correlated with the reciprocal of local wall shear stress when flow is steady.

Cell-cell adhesion is mediated by specific cell-surface molecules. A family of Ca^{+2} -dependent adhesion proteins called cadherins, which hold cells together by a homophilic interaction between these transmembrane proteins on adjacent cells. For this interaction to be effective, the cytoplasmic anchor proteins called catenins are required. In order to characterize the structure, binding strength (unbinding force), and binding kinetics of a classical cadherin (vascular endothelial (VE)-cadherin), Baumgartner et al. (2000) probed cadherin interaction by atomic force microscopy. As the full-length external domain fused to Fc-portion of human IgG of cadherin, in

physiological buffer, the external domain of VE-cadherin dimers is approximately a rod-shaped molecule with a length of 20-nm that collapses and dissociates into monomers (V-shaped structures) in the absence of Ca^{+2} . Trans-interaction between cadherin dimers is a low-affinity reaction with a relatively low unbinding force of 35-55 pN at retract velocities of 200-4,000 nm/s. The higher order unbinding forces, which increase with interaction time, indicate the association of cadherins to form complexes with cumulative binding strength. These observations favor a model by which the inherently weak binding strength and affinity of cadherin trans-interaction requires clustering and cytoskeletal immobilization for amplification. Binding is regulated by low-affinity Ca^{+2} binding sites ($K_D = 1.15\text{mM}$) with high cooperativity (hill coefficient of 5.04). Local change of free extracellular Ca^{+2} in the narrow interaction space may be of physiological importance to facilitate rapid remodeling of intercellular adhesion and communication.

Attenborough et al. (2000) studied the cell-cell adhesion of erythrocytes. They tested red blood cells from three different species by using a new image analysis method to determine cell-cell adhesion with a high level of precision. Various literature sources have suggested that horse or rat erythrocytes adhere to form aggregates more readily than human red cells but this paper provides the first accurate quantification of this phenomenon. The effects of surface active agents such as glutaraldehyde, papain and fibronectin on cell-to-cell adhesion are studied. Glutaraldehyde reduces the extent of adhesion whereas both papain and fibronectin increases it. Furthermore, cadhesins are critical determinants of tissue organization, which support cell-cell interaction that including cell-cell cohesion, cell-cell discrimination, and cell-upon-cell locomotion. Radice et al. (1997) investigated the functions of N-cadherin in vivo using mice with

mutated N-cadherin. Mutant embryo is malformed with small and less cohesive somites, as well as partially disrupted epithelial organization in the somites compared with wild-type littermates. Simultaneously, dramatic dissociation of myocytes from each other due to mutated N-cadherin spell out the effect of cell-cell adhesion in the development of heart tube. The results indicate that cadhesins support the cell-cell adhesion necessary for cohesion of tissues and organs. In addition, cadherin is composed of ectodomains and cytoplasmic domain that form core cadherin-catenin complex with p120-catenin, α -catenin and β -catenin in cytoplasm of cell. Therefore, cadhesins is intimately associated with the actin cytoskeleton through the core complex to modulate and control actin dynamics. Kobiela et al. (2004) studied the correlation between α -catenin and actin polymerization using α -catenin-null keratinocytes compared with wild-type keratinocytes. Formin-1 is found to interact with α -catenin, localize in adherens junctions, as well as nucleate unbranched actin filaments. Moreover, disruption of the α -catenin-formin-1 interaction obstructs the assembly of radial actin filament and interferes intercellular adhesion. Most notably, a complex of α -catenin and formin-1 adding into α -catenin-null keratinocytes has the capability of rescuing formation of adherens junctions and associated actin cytoskeleton. This result shows that actin binding at the core cadherin-catenin complex contacts is a critical process, which involves cell signaling and the action of forming-1 that can support actin assembly in bundles. This implies that during cell-cell adhesion from nascent to mature stage, cadherins play a key role in actively participating in the progressive remodeling of the actin cytoskeleton that promote effective formation of cell-cell contacts and mechanically couple cells with one another into coherent sheets.

Several classes of attachment proteins in the cytoskeleton network also play critical roles in mechanochemical signal transduction. For instance, vinculin belongs to one class of intracellular anchor proteins, which is a relatively large molecule that contains multiple binding domains for other cytoskeleton proteins and focal adhesion sites as well as phosphorylation sites. It functions as one of several interacting proteins involved in anchoring F-actin to the membrane. Previous studies have revealed that Vinculin plays a key role in mechanical coupling of integrins to the cytoskeletal network as well as in the control of cytoskeleton elasticity, cell shape and motility. Alenghat (2000) used a magnetic tweezer to study the cell biophysics of a single vinculin-deficient cell. The application of the system of the wild-type F9 Vin (-/-) as mentioned above reveals significant differences in the stiffness of the transmembrane domains of integrin that is linked to the cytoskeleton. The measured mechanical properties vary widely among cell types and exhibit an approximately log-normal distribution. The median lateral bead displacement is two-fold higher in F9 Vin (-/-) cells than in wild-type cells, whereas the arithmetic mean displacement is only increased by 37%. It means that vinculin serves a larger role in mechanical integrity of cell.

Goldmann (2002) used vinculin-deficient F9 embryonic carcinoma cell to analyze the structure-function relationship of the focal adhesion protein vinculin. Atomic force microscope is applied to quantify the effects of different vinculin fragments (head and tail) on cell shape and cytoskeletal mechanics. This study reveals that the transfection of the head and/or tail domain of vinculin into vinculin-deficient cell is not sufficient to fully reverse the decrease in cell stiffness, spreading, and lamellipodia formation, compared to cells with expressed vinculin. In contrast, transfection of intact vinculin

into the cell completely restores normal cell mechanics and spreading regardless of the deletion of its tyrosine phosphorylation site. Constitutively active Rac also induces extension of lamellipodia when it is micro-injected into cells that expressed intact vinculin protein. Thus, vinculin contributes to the mechanical integrity of the cytoskeleton and shape control, by simultaneously binding multiple focal adhesion proteins and that this interaction is significantly enhanced when the relevant binding sites of the head and tail domains are maintained in close proximity within the same molecule. Vinculin, which is a highly conserved intracellular protein, play a crucial role in the maintenance and regulation of cell adhesion and migration. In the cytosol, vinculin adopts a default autoinhibited conformation. On recruitment to cell-cell and cell-matrix adherens-type junctions, vinculin becomes activated and mediates various protein-protein interactions that regulate the links between F-actin and the cadherin and integrin families of cell-adhesion molecules. Recently, Bakolitsa et al. (2004) described the crystal structure of the full-length vinculin molecule (1,066 amino acids), which shows a five-domain autoinhibited conformation where the carboxy-terminal tail domain is held pincer-like by the vinculin head, and ligand binding is regulated both sterically and allosterically. They have shown that conformational changes in the head, tail and proline-rich domains are linked structurally and thermodynamically, and propose a combinatorial pathway to activation that ensures that vinculin is activated only at sites of cell adhesion when two or more of its binding partners are brought into apposition.

2.3 Focal contact and signal transduction

Focal contacts are adhesion plaques through which cell attach to the underlying substrate through the interaction of extracellular matrix proteins, such as collagen or

fibronectin, and adhesion receptor called integrins, which are linked to the actin filaments via accessory proteins.

Fig. 2.1. A scheme summarizing known interactions between the various constituents of cell-matrix adhesions [adopted from Zamir and Geiger, 2001].

Many experiments have substantiated that a large number of specific proteins concentrate at focal contacts, these molecules are usually structural and cytoskeletal proteins but many are also signaling molecules including focal adhesion kinase (FAK), ERK/MAP kinase, MEKK1 (A mitogen-activated protein kinase (binds FAK)), etc. Upon the binding of the extracellular matrix proteins to integrins, intracellular protein kinases are activated leading to the activation of signaling pathways that can promote the survival, growth, proliferation, movement, gene expression, morphology of cell in response to the extracellular environment. Zamir and Geiger (2001) have reported that

focal adhesion formation requires more than 50 proteins. Most of these proteins with multiple domains can interact with different molecular partners, potentially forming a dense and heterogeneous protein network at the cytoplasmic side of the adhesion site. According to their presumed functions, these proteins are sorted as cytoskeleton proteins, tyrosine kinases, serin/threonine kinases, modulators of small GTPase, tyrosine phosphatases, other enzymes, and so on, besides cytoplasmic tail of receptors. The molecular and structural diversity of this focal contact is regulated by a wide variety of mechanisms, including competition, tyrosine phosphorylation, and conformational changes in component proteins, which can affect their reactivity. From Fig. 2.1, we can see molecular complexity and relation between one another.

Correlation between focal adhesion and signal transduction is rather sophisticated. Distinct mechanism between focal adhesion and signal transduction are not known. Many studies have been performing and obtained new understanding at the molecular level on this field. For instance, clustering of adhesion receptor integrins, organization of actin filaments, and aggregation of signaling molecules (adaptors and enzymes) enter into specific structures (such as focal adhesion, focal complex and fibrillar adhesion) and mediate adhesion-dependent signals. Mostafavi-Pour et al. (2003) focused on studying $\alpha_5\beta_1$ or $\alpha_5\beta_1$ integrin-fibronectin-signaling pathway. $\alpha_5\beta_1$ integrin receptors need proteoglycan coreceptor syndecan-4 for focal adhesion formation and migration but $\alpha_4\beta_1$ integrins do not. Furthermore, adhesion through $\alpha_5\beta_1$ integrin receptors leads to stronger protein kinase C α (PKC α) activation than that via $\alpha_4\beta_1$ integrin receptors. Inhibition of PKC α and transient expression of dominant-negative PKC α apparently influence focal adhesion formation and migration mediated by $\alpha_5\beta_1$ integrin. These results reveal that different integrin receptors can control focal adhesion formation and

cell migration through different signaling pathway. Danen et al. (2002) explored the formation of different types of cell matrix adhesion in cell that bind to fibronectin through $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrin receptor by applying GD25 (β_1 -deficient fibroblast cells), GE11 (β_1 -deficient epithelioid cells) and transfection technique. Experimental results demonstrate that upon β_1 expression in GD 25 and GE11, RhoA-GTP levels are significantly increased by $\alpha_5\beta_1$ integrins instead of $\alpha_v\beta_3$ integrins but high levels of RhoA activation occurs at later stages of cell spreading, which are associated with a translocation of focal adhesions to peripheral cell protrusions, recruitment of tension into fibrillar adhesions, and fibronectin fibrillogenesis. Activated RhoA is able to rescue impaired fibronectin fibrillogenesis to form fibril adhesions in GD β_3 and GE β_3 . Notably, apart from the central cell-binding domain of fibronectin for $\alpha_5\beta_1$ integrin to adhere, other regions (HepII domain) of fibronectin are needed for development of $\alpha_5\beta_1$ integrin mediated focal adhesion. This implies that the fibronectin-binding integrin $\alpha_5\beta_1$ and $\alpha_v\beta_3$ differentially regulate RhoA activation, organization of cell matrix adhesions, and fibronectin fibrillogenesis.

2.4 Adhesion and temperature, osmosis

Cell adhesion is a rather complex process, strongly influenced by temperature and osmosis. Sagvolden et al. (1999) used atomic force microscope to measure the adhesion force of cervical carcinoma cells in tissue culture. The forces were studied as a function of time and temperature for cells cultured on hydrophilic and hydrophobic polystyrene substrates with pre-adsorbed proteins. They found the cells attached faster and stronger at 37 °C than at 23 °C and better on hydrophilic than on hydrophobic substrates. Wan and Liu (2001) reported the detail of the contact mechanics modeling

of an adherent model cell. A thin-walled capsule, modeled as an incompressible liquid droplet contained in a thin flexible membrane, is allowed to adhere onto a rigid substrate. The contact mechanics model is formulated, based on linear elasticity, to portray quantitatively the relationship between osmotic inflation, contact area, contact angle, membrane stretching and adhesion strength. The predicted results shed light on the fundamental mechanisms of adhesion contact mechanics in a typical cell culture process of anchorage-dependent cells. The lipid bilayer is a main component of a cell membrane, but the effect of its physiochemical properties on the contact mechanics and adhesion energy of cells or model liposome has not been systematically studied. Chan et al. (2002) applied confocal reflectance interference contrast microscope, phase contrast microscope and contact mechanics modeling to probe the interfacial adhesion contacts of phospholipid vesicles on fused silica substrates during gel to liquid crystalline transition of the bilayer. It is shown that the contact area, contact angle, and adhesion energy of adhering liposome increase during the thermotropic transition. The trend of adhesion energy against temperature is indicated by the complex interplay of the elastic modulus of the membrane wall and deformation of the liposome. In the presence of osmotic stress, the magnitude of adhesion is solely dictated by the elastic modulus and is dependent on vesicle size. Overall, the study provides direct evidence that the adhesion strength of model membrane vesicle can be tuned by the change of temperature. Liu et al. (2002) studied the adhesion of a cellular entity liquid-filled microcapsule adhered to a flat glass substrate in response to an osmosis change. A sensitive microscope visualization instrument has been developed to measure the cell-substrate contact area and inflated capsule volume. A theoretical model is developed to quantitatively correlate the adhesion energy to the contact area and osmotic inflation of cell volume. The results show that the contact area increases with increasing adhesion

energy, while it shrinks in dimension as cell inflation is enlarged. This observed phenomenon is consistent with the theoretical prediction. This work demonstrates the possibility of obtaining quantitative interfacial adhesion energy by using the present technique and represents the first step in extending this approach to study more complicated system such as cell-substrate interaction.

2.5 Cytoskeleton Structure and Function

Cytoskeleton is an interconnected structure of various cross-linked and interlinked filamentous biopolymers that have three major types such as intermediate filaments, microtubule filaments and actin filaments. They extent from the cell nucleus to the cell membrane [Amos and Amos, 1991]. Their usefulness to the cell depends on a large number of accessory proteins that link the filaments to other components, as well as to each other.

These filaments consist of subunits by a large number of specific interaction and noncovalent bonds. Actin filaments are built up from actin molecules and are the contractile elements of the cell, playing an important role that determines the shape of the cell's surface and is necessary for the whole-cell locomotion. Intermediate filaments constitute a large family of proteins, providing mechanical strength and resistance to shear stress. Microtubules are made of polymers of tubulin heterodimers, determining the positions of membrane-enclosed organelles and directing intracellular transport [Alberts et al., 2001].

Cytoskeleton network are dynamic and adaptable. The dynamic character of filaments is controlled by an ATP bound to each monomer. The state of the ATP determines the stability of the filament. Free subunit typically holds an ATP molecule

and binds tightly to growing filaments. After attachment, the ATP is broken and the subunit subtly changes shape. The new form, with ADP bound, is not stable in the filament and dissociates more easily. One of the unusual consequences of these behaviors is called treadmilling. Stossel (1989), Theriot and Mitchison (1991) investigated the alternations in actin filament assembly, serving, and crosslinking. They found these phenomena occurred in local regions of the leading edge of migrating cell, and thus come up with the concept of actin polymerization. Kolega et al. (1991) studied the role of solvation-contraction coupling in regulating stress fiber dynamic in nonmuscle cells. The result showed that sol-gel transformations of the actin lattice act as a dragging force for cell extension during locomotion

2.5.1 Actin Filament function

A complex reorganization of actin filaments plays a critical role in cell spreading which is correlated with changes in important cell functions including DNA synthesis, motility, and differentiation. Bhadriraju et al. (2002) studied extracellular matrix- and cytoskeleton-dependent changes in cell shape and stiffness. Their result demonstrates the relationship between cell stiffness and spreading on hepatocytes. Normal actin and myosin function is required for hepatocyte spreading and DNA synthesis and showed opposite effects on cell stiffness. Cell spreading is accompanied by changes in the structure and composition of the cytoskeleton, which in turn accommodate and facilitate the change in cell shape. To understand the role of cytoskeleton in cell adhesion, Mooney *et al.* (1995) measured the changes in cytoskeletal filament assembly that occur following cell attachment to ECM. Their result shows that cell adhesion to ECM induces changes in the assembly of microfilaments and microtubules. However, large-scale alterations in cell form do not appear to be driven directly by these polymerization

events. Rather, microfilaments and microtubules appear to function as a single, structurally integrated unit which changes its form in response to changes in cell-ECM binding.

Actin expression can regulate cellular contractile activity. Hinz et al. (2001) proofed the point. Alpha-smooth muscle actin exists in fibroblasts. They used several models and methods to test the possible correlation between the alpha-smooth muscle actin and contractile activity of fibroblastic cells. They concluded that an increases alpha-smooth muscle actin expression could be sufficient to enhance fibroblast contractile activity.

2.5.2 Effect of cell adhesion molecules on cytoskeleton

Integrins are transmembrane cell adhesion proteins that act as matrix receptors and tie the matrix to the cell's cytoskeleton. After the binding of a typical integrin to a ligand of the ECM, the cytoplasmic tail of its α subunit binds to several intracellular anchor proteins, including talin, α -actinin, vinculin and filamin. These anchor proteins can bind directly to actin or to other anchor proteins such as vinculin, thereby linking the integrin to actin filaments in the cortex [Alberts et al, 2001]. Integrin mediated cell adhesion results in the transmission of signals that induce cytoskeletal reorganizations and lead to changes in cell behavior.

The cytoskeletal reorganizations are regulated by transient activation and deactivation of Rho GTPases. Kulkarni et al. (2002) previously identified mu-calpain as an enzyme that is activated by signaling across β_1 and β_3 integrins. Their results indicate that the calpain cleaves RhoA and generates a form that inhibits integrin-induced stress fiber assembly and cell spreading. Integrins regulate cell adhesion and motility through

tyrosine kinases, but initiation of this process is poorly understood. Oberegfell et al. (2002) found out that Src associates constitutively with integrin $\alpha\text{IIb}\beta\text{3}$ in platelets. Platelet adhesion to fibrinogen causes a rapid increase in $\alpha\text{IIb}\beta\text{3}$ -associated Src activity, and active Src localizes to filopodia and cell edges. Their studies establish that platelet spreading on fibrinogen requires sequential activation of Src and Syk in proximity to $\alpha\text{IIb}\beta\text{3}$, thus providing a paradigm for initiation of integrin signaling to the actin cytoskeleton.

Most cadherins function as transmembrane adhesion proteins that indirectly link to the actin filament of cells. This arrangement occurs in the adhesion junctions. The highly conserved cytoplasmic tail of these cadherins interacts indirectly with actin filaments by means of a group of intracellular anchor proteins called catenins. This interaction is essential for effective cell-cell adhesion because of the functional relationship between cadherin receptors and actin filament organization, Braga et al. (1997) investigated whether members of the Rho family of small GTPases are necessary for cadherin adhesion. Their study results provide new insight into the role of the small GTPases in the cadherin-dependent cell-cell contact formation and the remodeling of actin filaments in epithelial cells.

2.5.3 Cytoskeleton labeling techniques

Cytoskeleton has been demonstrated to be a system for mechanochemical signal transduction [Stamenovic et al., 2002]. To study the influence of external stimuli on cell behavior, the behavior of cytoskeleton under different experimental conditions must be first understood. These changes of cytoskeletal filaments in a cell can be studied by using immunostaining technique [Rotsch et al., 2000]. In cultured cell, the

three primary cytoskeletal components can be visualized utilizing fluorescent microscopy or confocal fluorescence microscopy. But fluorescent labels have certain limitations, for instant, filament dynamics in real time may not be studied using this method.

The use of antibodies to localize proteins within cells requires that the cells be chemically fixed and rendered permeable to the antibody molecules. In general, one aims to achieve optimal structural preservation combined with intense antibody labeling. But the properties of many antibodies do not allow us this luxury. The stronger is fixation, the better structural preservation can be gotten. But this leads to weaker antibody labeling. So formaldehyde was selected to fix cell. Permeabilization of the cell membrane is achieved with triton to make antibody conjugated with fluorescent enter cytoplasm and bind cytoskeleton.

Green fluorescent protein (GFP) owes the widespread application in life sciences to its remarkable properties. It is a fluorescent protein enabling the fusion of its cDNA to either any other codon sequences to visualize the expressed fusion protein. Among its application are the intracellular localization of proteins and the investigation of the organization, regulation and dynamics of the cytoskeleton for living cells [Schmitz et al., 2001]. GFP expression in mammalian cells requires appropriate vectors that may be transfected into mammalian cells by a variety of techniques, including those using calcium phosphate [Chen and Okayama, 1988], DEAE-dextran [Rosenthal, 1987], various liposome-based transfection reagents [Samborook et al., 1989], and electroporation [Ausubel et al., 1994]. In most cases, GFP expression may be detected by fluorescence microscope, confocal microscope, or fluorometer assays 24-72 hours post-transfection.

GFP labeling technique has competitive advantages over immunostaining technique. GFP tag eliminates the need for fixation, cell permeabilization, and antibody incubation steps normally required when using antibodies tagged with chemical fluorophores. The most importance is to offer us to investigate cytoskeleton dynamics in real time.

2.6 Experimental techniques in cell biophysics

Various cellular functions are closely related to the intrinsic mechanical properties of individual cells. Over the years, various experimental techniques such as micropipette aspiration, atomic force microscope, confocal microscope, optical tweezers, and shear flow are developed to directly quantify the biophysical properties of a single cell.

In order to study the biophysical properties of single cell, the experimental techniques must provide the capability of probing biomechanical parameters at the cellular level. The dimensions of cells are in the order of 10 μm and thus, the magnitude of cellular deformation will be in the order of 1 μm or so. Forces required to deform soft cells such as red blood cells or white blood cells are in the order of 10-100 pN while those exerted on stiff cells such as endothelial cells or chondrocytes are in the order of 1nN. The diameter of micropipettes is the order of 1-10 μm and the applied forces range from 10pN to 1nN. As a result, the stresses or pressures acting on a cell are in the unit of either $\text{pN}/\mu\text{m}^2$ or $1 \text{ nN}/\mu\text{m}^2$ [Hochmuth, 2000].

2.6.1 Micropipette aspiration technique

It provides a versatile method for determining cellular properties such as the elastic modulus (stiffness), viscosity, and time constants for recovery from deformation [Evans and Yeung, 1989; Hochmuth et al., 1993; Sato et al., 1990; Theret et al., 1988].

Fig. 2.2. Setup for micropipette technique [adapted from Jones et al., 1999].

The experimental system (Fig. 2.2) was initially used to induce a tare pressure (0.01 kPa) for 60 s for drawing the chondrocytes against the micropipette and to define a reference state for measuring the deformation of cell. A series of 8-10 step increases in suction pressure were then applied to the cell which allowed to equilibrate at each pressure for 60 s. The length of cell projection sucked into the micropipette was measured at the end of each pressure. Maximum pressures between 0.05 and 1 kPa were applied. Jones et al. (1999) applied the technique to study chondrocytes. With this technique, real-time measurements of pressure and deformation can be made. In

conjunction with theoretical modeling, this technique was used to determine the intrinsic mechanical and volumetric properties of a single cell. In one study, no difference was found between the Young's moduli of normal (0.65 ± 0.63 kPa, $n = 44$) and osteoarthritic human cartilage cells (0.67 ± 0.86 kPa, $n = 69$). On the other hand, a significant difference in cell volume was observed immediately and 600 s after complete aspiration of the cell into the micropipette. The magnitude of this volume change between normal ($11 \pm 11\%$, $n = 40$) and osteoarthritic ($20 \pm 11\%$, $n = 41$) chondrocytes was significantly different at both time points. The results strongly indicate that normal human chondrocytes behaved as a viscoelastic solid and chondrocytes from osteoarthritic cartilage may have altered volume regulation capabilities in response to mechanical deformation.

Sato *et al.* (1990) applied the micropipette aspiration technique to study the viscoelastic properties of cultured porcine aortic endothelial cell. They describe the viscoelastic behavior of endothelial cells cultured under no-flow conditions. Using cytoskeletal disrupting agents, they investigated the influence of two different cytoskeletal elements, the actin microfilaments and the microtubules, on the viscoelastic properties of cells. The cells were divided into four groups: (1) cells detached by trypsin (control (T)); (2) mechanically detached cells (control (M)); (3) cells treated by cytochalasin B (CB); and (4) cells treated by colchicine. The value of k_I in the Kelvin model for control (T) cells (760 dyne/cm^2) was slightly lower than that for control (M) cells (920 dyne/cm^2). The value for CB-treated (110 dyne/cm^2) and colchicine-treated (280 dyne/cm^2) cells were significantly lower, approximately 1/3 and 1/10 of the control (M) value. The results showed the microfilaments were playing a very important role in the process of viscoelastic deformation. Tran-Son-Tay *et al.* (1991)

investigated time-dependent recovery of passive neutrophils after large deformation using micropipette technique. Under a suction pressure of 5000 dyn/cm^2 and the micropipette diameter of $4 \text{ }\mu\text{m}$, a neutrophil was aspirated into a pipette and deformed into an elongated “sausage” shape. The cell was held within the pipette for at least a few seconds and then rapidly expelled, and its spherical shape eventually recovered in 1 minute. This recovery process was analyzed according to a Newtonian, liquid-drop model with cortical tension. The theoretical model gave a good fit to the experimental data for a ratio of membrane cortical tension to cytoplasmic viscosity of $1.7 \times 10^{-5} \text{ cm/s}$. When the cell was held in the pipette for only a short time interval of 5 s or less, and then expelled, the cell undergoes an initial, rapid elastic rebound. This result suggests that the cells behave as a Maxwell viscoelastic liquid rather than a Newtonian liquid with constant cortical tension.

Hochmuth et al. (1993) studied the viscosity of passive human neutrophils undergoing small deformations. The diameter of the pipette was $7.5 \text{ }\mu\text{m}$. A neutrophil was held in the pipette under small aspiration pressure for at least seven seconds, the cell was rapidly expelled and allowed to recover its original, spherical shape. The recovery took around 15 s. An analysis of the recovery process by treating the cell as a simple Newtonian liquid drop with a constant cortical tension gave a value of $3.3 \times 10^{-5} \text{ cm/s}$ for the ratio of the cortical tension to cytoplasmic viscosity. This value was about twice as large as the value published in 1991 with the same model from studied of large deformation of neutrophils.

Shao et al. (1996) used the micropipette aspiration technique to measure piconewton forces of adhesion and tether formation from neutrophil membrane. They found that the minimum force required to form a membrane tether from neutrophil is 45 pN,

and the length of the microvilli on the surface of neutrophil was inferred. In 1999, they studied mechanical anchoring strength of L-selectin, β_2 integrins and CD45 to neutrophil cytoskeleton and membrane. They found that it took 1-2 s to extract an L-selectin at a force range of 25-45 pN, 1-4 s to extract a β_2 integrin at a force range of 60-130 pN, and 1-11 s to extract a CD45 at a force range of 35-85 pN. In his review on micropipette aspiration of living cells, Hochmuth (2000) mentioned that neutrophil has a cortical tension of about 30 pN/ μm and a viscosity on the order of 100 Pa s, chondrocytes and endothelial cells has an elastic modulus of the order of 500 pN/ μm^2 (0.5 kPa).

2.6.2 Atomic force microscope (AFM)

AFM is another remarkable biophysical instrument that can measure forces down to pico-newton regime and can resolve force changes caused by the displacement of its probe by a fraction of a nanometer. Furthermore, these measurements can be carried under water, allowing the study of biological material under conditions that resemble those in vivo. The AFM functions like a miniature phonograph. A sharp tip, mounted on a cantilever, interacts with the sample, causing minute deflections that can be calibrated as a force. As the AFM probe scans across biological surfaces, it characterizes adsorbed lysozyme, images cell topology, and purple membranes, followed the action of enzymes and changes in the local elasticity of cells, and has been used in several other application. AFM can also be used to determine the mechanical properties of the different cell types.

To accurately determine the elastic modulus and the relative contribution of viscous losses, Mathur et al. (2001) examined the effect of probe velocity, indentation

depth, and the assumed shape of the probe on the calculated elastic modulus. The results of study showed that cardiac cells were the stiffest (100.3 ± 10.7 kPa), the skeletal cells were intermediate (24.7 ± 3.5 kPa), and the endothelial cells were the softest with a range of elastic module (1.4 ± 0.1 kPa) depending on the location of the cell surface tested. Cardiac and skeletal muscle exhibited nonlinear elastic behavior. These passive mechanical properties are generally consistent with the biological function of these different cell types. Mathur et al. (2000) applied AFM technique loading forces 0.3-0.5 nN and scanning velocity $0.25 \mu\text{m}$ to measure the elastic modulus of human umbilical vein endothelial cells. They obtained the elastic modulus of 7 kPa over the nucleus, 3 kPa over the cell body in proximity to the nucleus and 1 kPa over the cell body near the edge respectively.

Radmacher et al. (1996) studied the viscoelastic properties of human platelets with the atomic force microscope. They applied a load of 3nN on the platelet. The probe was engaged to the surface at a scan velocity of $70 \mu\text{m/s}$. The elastic modulus was determined as 1.5-4 kPa over the nucleus and 100 kPa over the cell body near edge.

AFM is used for imaging and elasticity measurements. It possesses very soft cantilevers with certain spring constants as determined by the thermal noise method and certain shape. Cantilever has tip semi-angles of 35° . To allow convenient positioning of the tip above the cells, the AFM is put on top of an inverted optical microscope. Petri dishes are mounted in a custom-built clamp that is magnetically attached to the microscope table. The table could be heated from below with resistors. The AFM is covered with a gas-tight PMMA box that could be flooded with CO_2 . It is able to maintain optimum environmental conditions for the cells throughout the experiment ($5\% \text{CO}_2$, 37°C).

Fig. 2.3. Schematic of the indentation of a sample or cell with a conical and blunt cone probe [adapted from Mathur et al., 2001].

2.6.3 Optical tweezers

It is also an important technique for us to study cell biophysics. It can measure the inhibition of adhesion [Leckband, 2000], the forces that control protein interactions [Bongrand, 1999], ligand-receptor interaction [Lambert, 2002], investigate the dynamics of ligand-induced Rac1-dependent anchoring of cadherins to the actin cytoskeleton [Thoumine et al., 2000], mechanical properties of neuronal growth cone membranes [Dai and Sheetz, 1995]. Henon et al. (1999) first used optical tweezers to measure the elastic coefficients of the red blood cell membrane. They reported simultaneous forces and deformations exerted on a red blood cell, from which they

inferred the elastic shear modulus of the membrane. They found the same result of shear modulus ($2.5 \pm 0.4 \mu\text{N/m}$) for both discotic and nearly spherical swollen cells. This value was smaller than the one deduced from micropipettes experiments. However, the methods did not operate in the same deformation regime and were not expected to lead to the same result. The cell was seized and deformed by means of two small silica beads bound to the membrane surface. The beads were trapped in split optical tweezers and used like handles to pull on the membrane with two opposite forces, which were determined by a preliminary calibration. The following scheme of the optical tweezer was set up by Henon et al. (1999). They used a upright optical microscope to trap the RBCs and simultaneously to observe them. The $1.064 \mu\text{m}$ Nd:YAG laser beam has a maximum emission power $P_{\text{max}} = 605\text{mW}$. The beam is reflected by a dichroic mirror inside the microscope and focused at the observation point through an oil immersion objective.

Fig. 2.4. Scheme of the optical tweezers experimental setup [adapted from Henon et al, 1999].

Qian et al. (2002) used three optical traps to measure the inhibition of adhesion of lectins to the surface of erythrocytes. The near-infrared (852 nm) beams were used to trap the cells and the visible (632 nm) beam was used to trap the polystyrene microspheres. Sleep et al. (1999) applied optical tweezers to study the elasticity of the red cell membranes. The apparatus was based on an inverted microscope. Polarizing prisms were used to split the laser beam to give two independently movable single-beam gradient traps, the stiffness of the two traps was equalized with a $\lambda/2$ plate. A 63x, infinity-corrected, 1.4 N.A. objective was used for these studies. In the experiments, one of the traps was left stationary, and the position of the bead in this trap was monitored by focusing an image onto a photodiode quadrant detector. The theory of Parker and Winlove for elastic deformation of axisymmetric shells allows them to determine the function BH^2 for the spherical saponin-permeabilized ghost membranes (where B is the bending modulus and H the shear modulus); taking the literature value of 2×10^{-19} Nm for B , H then emerges as 2×10^{-6} Nm⁻¹. Li et al. (2002) studied membrane tether formation from outer hair cells with optical tweezers. Their optical tweezers setup composes of a continuous wave, tunable (650-1100nm) Titanium-Sapphire laser pumped by a 5-W solid state, frequency doubled Nd: YVO₄ laser. The laser beam was expanded 5 times by a beam expander to fill the back aperture of a 100 × microscope objective with a numerical aperture of 1.3, thereby achieving a high convergence angle required for strong trapping. That found a great force was required for tether formations from OHC lateral wall (499 ± 152 pN) than from OHC basal end (142 ± 49 pN). The apparent plasma membrane stiffness, estimated under the static conditions by measuring tether force at different tether length, was 3.71 pN/μm for OHC lateral wall and 4.57 pN/μm for OHC basal end. The effective membrane

viscosity was measured by pulling tethers at different rates while continuously recording the tether force, and estimated in the range of 2.39 to 5.25 pN·s/μm.

2.6.4 Magnetic tweezers

The principle of the magnetic tweezers technique is to arrange a small “tug-of-war” between the electromagnet and the cell. In Fig. 2.5, the magnet is mounted on a micromanipulator secured on a light microscope, with the entire working area heated to 37 °C and cold water circulating through the brass housing of the magnet to prevent overheating of the wire as high current passes through it. A microscopic view (inset) of the area on the cover-slip shows that beads (4.5μm diameter) bound to the surfaces of cultured cells relative to the position of the tip of the magnet (M) [Alenghat et al., 2000]. Alenghat et al. (2000) have constructed a magnetic tweezers which applied tensional forces (10 pN to greater than 1 nN) to transmembrane receptors via bound ligand-coated microbeads while optically measuring lateral bead displacements within individual cells. The examination of wild-type F9 embryonic carcinoma cells and cells from a vinculin knockout mouse F9 Vin (-/-) with magnetic tweezers revealed significant differences in the stiffness of the transmembrane integrin linkages to the cytoskeleton than previously reported using related techniques that measured average mechanical properties of entire cell. The measured mechanical properties varied widely among cells, exhibiting an approximately log-normal distribution. The median lateral bead displacement was 2-fold larger in F9 Vin (-/-) cells compared to wild-type cells whereas the arithmetic mean displacement only increased by 37%. They conclude that vinculin serves a greater role in maintaining cell integrity than previously reported and that the magnetic tweezers may be useful for probing the molecular basis of mechanochemical transduction within single cells.

Fig. 2.5. Diagram of the magnetic tweezers [adapted from Alenghat et al., 2000].

2.6.5 Other Biophysical Techniques

In addition to those aforementioned biophysical techniques for probing single cell, various experimental techniques for probing mechanochemical transduction at the multi-cell/tissue level are available. For example, cell stretching device as shown in Fig. 2.5 consists of a set of 6 Teflon rods embedded in a polycarbonate sheet. A 6-well culture dish with a flexible substrate (Bio-Flex plate, Flexcell Intl Corp.) is placed in an aluminum collar, loaded over the Teflon rods, and clamped into place with a wing nut/carriage blot assembly. The degree of strain is controlled by clamping the plate down to specific distances, with the distance controlled by aluminum spacers between the polycarbonate sheet and the aluminum collar. Putnam et al. (1998) applied the

technique to study the effects of externally applied strain to microtubule assembly. In the study, the assembly of microtubules was analyzed in rat aortic smooth muscle cells cultured on silicon rubber substrates exposed to step increases in applied strain. Cytoskeletal and total cellular protein fractions were extracted from the cells following application of the external strain, and tubulin levels were quantified with competitive ELISA and western blotting using bovine brain tubulin as a standard. In the first set of experiments, smooth muscle cells were subjected to a step-increase in strain and the distribution of tubulin between monomeric, polymeric, and total cellular pools was followed with time. Microtubule mass increased rapidly following application of the strain, with a statistically significant increase ($P < 0.05$) in microtubule mass from 373 ± 32 pg/cell ($t = 0$) to 514 ± 30 pg/cell ($t = 15$ minutes). In parallel, the amount of soluble tubulin was decreased by fivefold. The microtubule mass decreased after 1 hour to a value of 437 ± 24 pg/cell. In the second set of experiments, smooth muscle cells were subjected to increased doses of externally applied strain using a custom-built strain device. Monomeric, polymeric, and total tubulin fractions were extracted after 15 minutes of applied strain and quantified in the earlier experiments. Microtubule mass increased with increasing strain while total cellular tubulin levels remained essentially constant at all strain levels. These findings are consistent with a thermodynamic model which predicts that microtubule assembly is promoted as a cell is stretched and compressive loads on the microtubules are relieved. Furthermore, these data suggest microtubules are a potential target for translating changes in externally applied mechanical stimuli to alterations in cellular phenotype.

Fig. 2.6. Schematic of the cell stretching device by Flexcell [Adapted from Putman et al., 1998].

A similar cell stretching device is shown in Fig. 2.7, demonstrating its major components. A laser displacement transducer (LDT), mounted on a three-axis manipulator, measures the dynamic displacement of the membrane, allowing for calculation of the peak membrane strain. The brain slice cultures are grown in custom-built wells on a silicone membrane that is deformed by an applied vacuum as is depicted in the exploded view illustration by Morrison et al [Morrison et al., 2000].

Fig. 2.7. Schematic of a cell stretch device [adapted from Morrison et al, 2000].

Fig. 2.8. Schematic of a cell stress unit used to measure $[Ca^{2+}]_i$ in bovine aortic EC mono- layers subjected to cyclic strain [adapted from Rosales et al., 1997].

Rosales et al. [1997] used a Flexercell strain unit (Flexcell Corp., McKeesport, PA, U.S.A.), as shown in Fig.2.8, to study the effect of cyclic strain on ion channels. In this setup (similar to those shown in the previous figures), cells are grown on culture plates with flexible silastic bottoms coated with collagen type I (Flex I plate; Flexcell) which can be deformed to a known percentage elongation by applying a precise vacuum level. Release of the vacuum returns the plate bottoms to their original conformation. Vacuum is regulated by solenoid valves that are controlled by a computer program. Thus the magnitude, duration and frequency of the applied force can be varied. Force analysis of the strain that was exerted on the flex plate (attained by various vacuum levels) was determined mathematically, by finite element analysis, and empirically by measuring the distance between (radial strain), or the diameter (axial strain) of, concentric circles marked on the membrane. The strain is biaxial, but very little change is observed in the axial direction, and the force on the attached cells is predominantly radial. A conceptually similar device is also shown in Fig. 2.9. To apply pulsating fluid flow, a schematic of the flow chamber is shown in Fig. 2.10. A summary of the commonly used techniques is provided in Fig. 2.11.

Fig. 2.9. Schematic diagram of device to apply homogeneous and uniform biaxial strain to monolayer human vascular smooth muscle cell culture. The dish is secured by a locking collar. The circular elastic membrane in each dish is deformed by a platen as the platen assembly is displaced sinusoidally by a speed-controlled motor-driven cam [adapted from Cheng et al., 1997].

Fig. 2.10. Diagram of apparatus to apply pulsating fluid flow (PFF). *A*: fluid culture medium. *B*: parallel-plate flow chamber. *C*: glass slide with cell monolayer. *D*: polycarbonate plate with two slits through which medium enters and exits channel. *E*: revolving pump. *F*: 37°C incubator. *G*: water reservoir. *H*: atmosphere of 5% CO₂ in air. *I*: flow probe [adapted from Sterck et al., 1998].

Fig. 2.11. A schematic representation of biophysical techniques for studying mechanotransduction. *A*: cells are cultured on an elastic membrane that is pushed upward (arrow) statically or by pulsation. *B*: cells cultured on an elastic membrane are pulled downward (down arrow) by negative pressure. *C*: cells are cultured on a Gelfoam sponge, one end of which is glued to bottom of a Petri dish while the other end is attached to a metal bar. A magnetic force generated through a computer-controlled solenoid (arrow) acts on the metal bar to apply strain to organotypic cell cultures on the sponge. *D*: a parallel-plate flow channel applies shear stress to cells cultured as a monolayer. *E*: a micropipette touches apical membrane of a single cell and distorts cellular membrane. *F*: after ligand-coated micro-beads attached to surface receptors are magnetized in 1 direction, a second, weaker magnetic field oriented at 90° "twists" the beads with a controlled shear stress [adapted from Liu et al., 1999].

2.7 Summary

In this section, we present a brief review of the mechanochemical transduction of cell adhesion: mechanisms, experiments, cytoskeleton structure and function, cytoskeleton labeling techniques, focal adhesion contact and biological functions. One of the major topics is concerning about the effect of external stimuli on cell adhesion dynamics. However, Cell adhesion mechanism is still unclear. Cell adhesion strength can not be assessed directly and effectively due to shortage of proper techniques and advanced instrument. For this study, it should focus on the use of novel biophysical techniques for studying cell adhesion dynamics and cytoskeleton rearrangement under the influence of external stimuli.

Chapter 3: Experimental Materials and Methods

3.1 Materials

CLONfectin reagent and p-enhanced green florescent protein (pEGFP)-actin plasmid were obtained from Clontech Inc. (Palo Alto, CA). Gibco Dulbecco's Minimal Essential Medium (DMEM) and anti-mycotic were purchased from Invitrogen Corporation. HBV replicative genome (genotype A) was constructed by GeneLab of SBS in NTU. Effectene Transfection Reagent and 60 mm dishes were obtained from QIAGEN and Nunc, respectively. Phosphate buffer saline, Dulbecco Modified Eagle Medium (DMEM), L-glutamine, collagen, streptomycin, penicillin, essential amino acids, fetal bovine serum (FBS), methanol, trypsin-EDTA solution, mouse anti-vinculin, Cytochalasin D, Latrunculin A, and Doxorubicin were obtained from Sigma-Aldrich Chemical Inc. (USA). HepG2 cells were a gift from Johns Hopkins Singapore. Fibronectin was obtained from Chemicon International Inc. or Sigma-Aldrich Chemical Inc. (USA)

3.2 Substrate preparations

To prepare collagen and fibronectin gel-coated substrate, 400 μ l stock solution of collagen (1mg/ml in 0.012 N HCl, 400 μ l) was neutralized before use by adding 50 μ l of 0.1 N NaOH and 50 μ l of 10X PBS (final concentration of 0.8 mg/ml). Fibronectin stock solution (1 mg/ml, 50 μ l) and 12.5 μ l of PBS were added to produce a solution with fibronectin concentration of 0.8 μ g/ μ l. Glass coverslip was cleaned with the solution mixture of 30% 1 N NaOH and 70% methanol in an ultra-sonic bath for 20

minutes then soaked in pure methanol solution for 15 minutes, autoclaved and sterilized under UV light for 30 minutes. A drop of neutralized collagen solution was placed on the coverslip and spread out evenly with the help of a cell scraper. After 24 hours of collagen or fibronectin incubation at 4 °C, the glass coverslips were then washed with 1X PBS for three times and dried in air before use.

3.3 Cell Culture and Transfection

3.3.1 Cell culture

HepG2 cells were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin (called complete medium), at 37 °C under 5 % CO₂ and 100% humidity.

3.3.2 Cell transfection with pEGFP-actin plasmid

Transiently transfected HepG2 cells were established with pEGFP-actin by using CLONfectin delivery system (Clontech Inc., USA). CLONfectin reagent was liposome based system and promoted delivery of plasmid DNA into mammalian cells. P EGFP-actin plasmid encoded a fusion protein containing both green fluorescent protein and actin. Generally, 2 µg of pEGFP-actin plasmid and 8 µg of CLONfectin (1 µg/µl in HBS) solution were incubated for 20 minutes in 200 µl serum free DMEM medium and then diluted with 1.8 ml serum free medium. This solution was added to cells at 70% confluence in a 35 mm tissue plate. After 4 hours of incubation at 37 °C, the entire medium was removed from the cell culture flask. The transfected cells were washed with PBS following by 2 ml of fresh complete DMEM medium. After 72 hours of

transfection, HepG2 cells were detached by trypsin-EDTA solution, re-suspended in complete DMEM medium (Sigma-Aldrich Chemical Inc., USA) and were placed on collagen and fibronectin coated coverslips for our biophysical measurements.

Stable EGFP-actin expressing clones were obtained by using G418 (800 μ g/ml; Geneticin, Clontech) supplemented media. Clone expressing EGFP-actin was expanded for further analysis.

3.3.3 Cell transfection with HBV replicative genome and/or pEGFP-actin plasmid

HepG2 cells (ATCC, USA) were cultured in Gibco Dulbecco's Minimal Essential Medium (Invitrogen Inc., USA), supplemented with 10% fetal bovine serum (Invitrogen Inc., USA), 1% anti-mycotic (Invitrogen Inc., USA) under 37 °C and 5% CO₂. Effectene transfection reagent (QIAGEN Inc., Germany) was the expression system for GFP-actin and HBV in HepG2 cells. 6 x 10⁵ HepG2 cells were seeded and cultured on a 60mm dish (Nunc Inc., USA) under 37 °C and 5% CO₂ for twenty four hours before transfection. After adherent HepG2 cells reach 70% confluency, the cells were transfected with 2 μ g of pEGFP-actin plasmid construct (BD Clontech, USA) or/and 2 μ g replicative HBV genome (rHBV; genotype A) constructed as described previously [Chen et al., 2000]. Specifically, the replicative HBV genome was constructed by cloning a linear form of viral genome into mammalian expression vector pcDNA3.1. The linear genome contains the viral promoter at its 5' end and the region for termination of transcription at its 3' end. The ability of this genome in producing a replicative virus was assessed by the amount of HBsAg particles produced in the cell culture medium two days after its transfection into HepG2 cells, using ImX semi-

quantitative measurement (Abbott Laboratories, USA). In brief, the plasmid constructs are mixed with 16 μ l of enhancer followed by 60 μ l of Effectene transfection reagent. Then the resulted transfection complexes were added to the cells. After two hours of transfection, the medium was removed and rinsed twice with 1X PBS, before addition of fresh medium. Transfected cells were maintained at 37°C and 5% CO₂ for 48 hours. Transfected cells are detached by trypsinization, pelleted and resuspended in fresh medium before further biophysical studies.

3.4 Vinexin and HBX Interaction

The proline rich region of HBX was cloned into pGEX-5X-1 (Amersham Biosciences) as a fusion protein of Glutathione-S-Transferase (GST) with the addition of 6x His at the C-Terminal. The construct was then expressed in BL21 cells and purified by Glutathione Sepharose 4B (Amersham Biosciences). It was then incubated with array membrane (Panomics SH3 Domain Array II). The anti-His antibodies (from kit) were used to detect for the binding of positive interactors. Chemiluminescence was then performed using ECL (Amersham Biosciences).

3.5 Immunostaining

After HepG2 cells seeding on collagen coated substrate at 2 hours in incubator at 5 % CO₂ and 37 °C, Cyto-D or Lat-A or Dox at a concentration of 5 μ M or 10 μ M was added. 5 and 150 minutes after treatment with various drugs, the cells were removed from incubator for labeling the actin filaments with FITC-phalloidin. HepG2 cells washed three times with cold PBS were fixed with 3.5% formaldehyde in PBS at room temperature for 20 minutes. The cells were permeabilized with 1% triton X-100 in PBS

for 4 minutes at room temperature following by a wash with 1X PBS. For the localization and structure of actin filaments, the fixed and permeabilized HepG2 cells were incubated with FITC-phalloidin in 5% BSA for 30 minutes, and subsequently mounted with 3:1 glycerol and PBS to prevent bleaching before performing the fluorescence microscopy.

Each of the three types of HepG2 cells (HBV-replicating, transfected with pcDNA3.1 or normal cells) was seeded separately on collagen-coated coverslip for 20 or 50 minutes and washed with pre-warmed PBS and fixed with 1ml 3% paraformaldehyde / PBS, followed by another wash with PBS. They were then permeabilized with 0.2% Triton-X100/ PBS, and blocked with 10% FBS/0.1% Triton-X100/ PBS followed by a wash with PBS. Cells were incubated with mouse anti-vinculin (Sigma, USA) at 37°C for 2 hrs. After washing with 0.1% Triton-X100/PBS, cells were incubated with anti-mouse secondary antibody for 1hr at room temperature. The coverslip was then mounted with mounting.

3.6 Atomic Force Microscopy

Collagen or fibronectin coated coverslip were examined with Thermomicroscope Atomic Force Microscope (Veeco Instruments, USA). All measurements were carried out in contact mode with dry samples. A cantilever with a spring constant of 0.03 N/m and a scan rate of 1 Hz were used in all experiments. Each AFM topographic image was representative of at least three samples and was highly reproducible under the same procedures of sample preparation. The root-mean-square roughness of each image was determined with the AFM software.

3.7 Fluorescence Imaging

HepG2 cells transfected with the pEGFP-actin plasmid were cultured on fibronectin or collagen coated coverglass and placed on a temperature and carbon dioxide-controlled chamber at 37°C. The images of HepG2 cells were collected by using confocal fluorescence microscopy with a 63X oil-immersion objective, an Argon-ion laser (Carl Zeiss), as well as 1024 x 1024 pixels (8 bits, 256 colour levels) at 1X zoom corresponding to a pixel resolution of 0.15 μm with the slow scan speed. Excitation was set at 488nm and emission was collected between 505 and 530nm. Detector gain and pinhole aperture were automatically adjusted. The method of image of immunostained HepG2 cells is the same as mentioned above.

3.8 Phase Contrast Microscopy

Pascal 5 confocal microscope system (Carl Zeiss, Germany) equipped with a 63X oil-immersion objective and a transmitted light analyzer (Carl Zeiss, Germany) was used for imaging the adherent cells. Immediately following the seeding of cells on fibronectin or collagen or fibronectin coated coverglass, a series of phase contrast images were taken for probing the initial two-dimensional spreading kinetics of adherent cells at different time. An image analysis software, ZSMS (Carl Zeiss, Germany), was used for measuring the mid-plane diameter of adhering cells. Student's *t*-test was applied to determine the statistical significance in the comparison between GFP-actin expressing cells and normal cells in Chapter 4 & normal cells after adding drug at 5 μM and 10 μM in Chapter 6.

3.9 Confocal Reflection Interference Contrast Microscopy (C-RICM)

3.9.1 Setup

In our lab, we have developed confocal reflection interference contrast microscopy (C-RICM) by implementing the optical trains in RICM into a confocal microscope. We apply the technique same as that of RICM. The difference of our C-RICM with RICM is that we are using the confocal platform. For C-RICM we used an Axiovert 100M inverted microscope (Zeiss, Oberkochen, Germany) with a Zeiss Antiflex-Neofluar 63 X /1.25 oil immersion objective. In conventional RICM, illumination was carried with a mercury lamp (Osram, Berlin-Munich, Germany).

3.9.2 Principle of C-RICM

C-RICM technique is built on the technological platform of confocal microscopy. The general theory of RICM in the formation of interference fringes at cell-substrate interface is also applicable to our C-RICM technique. C-RICM images are formed by the interference of light reflected from the surface of the glass substratum (I_1) with light reflected from the cell surface (I_2), as shown in Fig. 3.1A. The distance $h(x)$ between substratum and cell surface leads to the formation of visible interference fringes which decreases with increasing angle α of illumination. For the setup used, the maximum detectable distance of cell-substrate separation is $\sim 4 \mu\text{m}$. The schematic (Fig. 3.1B) of image of an adhering cell reveals that the adhesion area of a cell appears dark, because of a phase jump of light reflected at the buffer-cell interface. The adhesion area appears as dark zone on the C-RICM image due to destructive interference between light from

the substrate and apposing cell surface. The contact line L circumscribes the adhesion area, where the cell forms close contact with the substratum. Outside of the area, the increasing distance between cell and substratum gives rise to a series of interference fringes.

Fig. 3.1. (A) Principle of Reflection Interference Contrast Microscopy [adopted from Kloboucek et al., 1999]. (B) The schematic of RICM image of an adhering cell [adopted from Simson et al., 1998].

The cell contour of adhering cells was examined in terms of the spacing $h(x)$ between cell membrane and substratum, in the proximity of their contact zone, using C-RICM. The principle of image formation (Simson et al., 1998; Kloboucek et al., 1999) is illustrated in Fig. 3.1A. Based on basic interference theory of optics, light reflected from the substratum (I_1) interferes with light reflected from the ventral cell surface (I_2), giving rise to the following intensity distribution of the image:

$$I(x) = I_1 + I_2 + 2\sqrt{I_1 I_2} \cdot F[\alpha, h(x)] \cdot \cos(2kh(x) \cdot (1 - G(\alpha)) + \delta) \quad (1)$$

Both F and G are functions of the angle α of the illumination aperture, and $k = 2\pi\eta/\lambda$ denotes the wave vector of the illuminating light in the buffer. The characteristic length $\lambda = \sqrt{\kappa/\gamma}$ embedded in this differential equation separates regions of the cell contour dominated by lateral tension γ from regions near the contact line that are dominated by the membrane bending modulus κ (Fig. 3.2). Assuming that the refractive index of the cell membrane is higher than the index of the surrounding buffer, light reflected on the buffer-cell interface experiences a phase jump of $\delta = \pi$. Therefore, the contact area of an adhering cell appears dark in C-RICM. Around the adhesion area, interference produces a specific fringe pattern (Fig. 3.1B) that depends on the shape of the cell contour $h(x)$.

Fig. 3.2. Theoretical height profile of adhering soft shells near the contact area [adopted from Simson et al., 1998].

The contour $h(x)$ perpendicular to the contact line is governed by two boundary conditions, the balance of tensions and the equilibrium of bending moments (Fig. 3.2). The characteristic length λ separates two regimes. One is a straight regime determined by a slope $\tan \alpha$. Another is a curved regime characterized by a contact curvature R_c .

For distance $x < \lambda$ the cell contact is dominated by the bending energy and is therefore curved. For distance $x > \lambda$ the contour is dominated by the lateral tension of the composite membrane, and approached a straight line. Extrapolation of the line to the substratum defines both λ and the contact angle α . Considering the following boundary conditions: $h(x) = 0$ within the contact area and $h(x) =$ for x large and position (Fig. 3.2), R_c and α can be determined most accurately by application of the following equations for the contour $h(x)$ (Bruinsma 1996; Alberdorfer et al., 1997).

$$h(x) = \alpha \cdot x - \alpha \cdot \lambda \left[1 - \exp\left\{-\frac{x}{\lambda}\right\} \right] \quad \text{for } x > 0, \quad (2)$$

$$h(x) = 0 \quad \text{for } x \leq 0 \quad (3)$$

and $R_c = \frac{\lambda}{\alpha} \quad (4)$

λ is equal to the distance between the contact line and the intersection of the tangent $\vec{t}$ with the surface of the substratum (Fig. 3.2). In most of our experimental measurement, cells don't form detectable cohesive zone.

The main difference between C-RICM and RICM is the replacement of the RICM optics with the confocal scanning mechanisms. In general, the laser beam passes through a high-NA objective and becomes focused at the cell-substrate interface. The light reflected from the cell-substrate interface passing through the same high-NA objective is projected onto the variable pinhole diaphragm. The focused laser beam inside the specimen and the pinhole are situated at optically conjugate points (confocal imaging). The main advantage of confocal imaging mechanism is that essentially no other light than that coming from a particular focal plane of interest can pass the narrow pinhole and be detected by a the photomultiplier tube (PMT). Most importantly, scattered light coming from different focal planes is eliminated by the pinhole in front

of the PMT. The smaller the pinhole, the less stray light from the out-of-focus areas will reach the PMT detector. Therefore the resulted image is sharper than that from optical microscopy. In order to obtain the overall image under the entire field of view, it is necessary to scan the cell-substrate interface in a point-by-point, line-to-line raster with the aid of an XY light deflection system. PMT converts the optical signal into electric signals. This allows the image of the object plane to be generated and stored in the computer within a second.

3.9.3 Method for Using C-RICM

In this study, the system was based on a laser scanning confocal microscope (Pascal 5, Carl Zeiss, Germany) equipped with one 80/20-filter set. Moreover, the laser scanning confocal microscope was integrated with a temperature/CO₂ control chamber (Carl Zeiss, Germany). The illumination source was a He-Ne laser with a maximum power of 1mW and excitation wavelength of 488nm. 63X (Neofluar, N.A.: 1.25) oil immersion objective was used in this study. The optical system carried the excitation light to the cell-substrate interface through the microscope objective and collected the direct reflected light simultaneously from the cell and glass surfaces. The pinhole for the scanning module was set at 69μm. The reflected light was eventually passed through the filter set and directed to a photo-multiplier tube. The constructive or destructive interference of reflected light from the two opposing surfaces resulted in the formation of fringes propagating from the interfacial region of strong contact zone to the peripheral region of the cell. Strong contact zone of the adhering liposome appears as dark region on the C-RICM image. Immediately following the seeding of cells, a series of C-RICM images was taken to follow the evolution of adhesion contact for adherent cells at different time. ZSMS software (Carl Zeiss, Germany) was used for all

image analysis. The contact area was determined with a drawing tool of the software. The error bar on cell deformation was originated from the standard derivation of at least three sets of experimental data under each condition. Student's t -test was applied to determine the statistical significance in the comparison between GFP-actin expressing cells and normal cell& drug at 5 μ M and 10 μ M in Chapter 6.

3.10 Data Analysis

The details of the contact mechanics model have been reported previously [Wan and Liu, 2001]. Briefly, the equilibrium geometry of a water-filled cell adhering on non-deformable substrate is modeled as a truncated sphere with a mid-plane radius R (Fig. 3.3). Degree of deformation, $\sin\theta = (a/R) = \alpha$ is an experimentally measurable parameter where a is the contact zone radius and θ is the contact angle. R and a is measured by C-RICM and phase contrast microscopy, respectively.

Fig. 3.3. The contact mechanics model of adherent cell. R , a and θ correspond to mid-plane radius, contact zone radius and contact angle, respectively.

The cell wall is under a uniform equi-biaxial stress, $\sigma = C\epsilon$. C is equivalent to $Eh/(1-\nu)$ in a linear system under small strain where E , h and ν is the elastic modulus, membrane thickness and Poisson's ratio, respectively. Only stretching model with negligible bending and rigidity is considered in the model. In a related work, our group has considered the effect of bending rigidity and pointed out the truncated sphere model worked just as good as other theories that are based on the bending rigidity [Fang et al., 2002]. The average biaxial strain, ϵ , is directly calculated from experimental data including R and α as follows:

$$\epsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{1/2}}{4/R^2 - \alpha^2} - 1 \right] \quad (1)$$

In the absence of external force, the cell spontaneously adjusts its distance of approach towards the substrate until an equilibrium is achieved. It was shown earlier that the adhesion energy, W , is

$$W = (1 - \cos\theta) C\epsilon + C\epsilon^2 \quad (2)$$

Based on the experimental measurements of the mid-plane diameter R (phase contrast microscope) and the radius of contact zone, a (C-RICM), W can be found by Eqs.(1) and (2). Elastic modulus E of HepG2 cell is taken as 2000 N/m² according to the experimental results obtained from micropipette aspiration and AFM indentation [Mathur et al., 2000].

Chapter 4: GFP-Actin Expression Modulates the Adhesion Dynamics of HepG2 Cells on Model Extracellular Matrix

4.1 Introduction

Cell adhesion to extracellular matrix (ECM) plays a vital role in regulating key physiological processes including organogenesis, inflammation and endothelialization, etc [Hynes 1992]. In addition, the control of cell adhesion on biomaterial is critical for the design of cell therapy device, engineered tissue equivalents, bio-artificial organs [Yarmush et al., 1992]. In general, the adhesion of anchorage-dependent cell is mediated by the recognition of receptor on cell surface with the ligand on biomaterials or extracellular matrix (ECM). Upon the ligand-receptor binding between cell and biomaterials, mechanochemical signal transduction is subsequently triggered by the intracellular domains of bound receptor which is coupled to the cytoskeleton resulting in focal adhesion kinase (FAK) phosphorylation, gene expression and cytoskeleton remodeling. It has been shown that fibroblast migration on biomaterial surface is driven by the mechanochemical signal transduction and the speed of migrating cell is dependent on the type of ECM proteins on the biomaterials [Sheetz et al., 1998]. In particular, integrin receptor mediates the connection between cytoskeleton and ECM. Although there are other adhesion receptors on cell surface such as cadherin and immunoglobulin receptors that can mediate cell-cell interaction, these receptors do not have a direct role in cell adhesion and migration on ECM [Calof and Lande, 1991; Lemmon et al., 1992].

Each class of integrin receptor recognizes specific ligand on ECM. For example, integrin $\alpha_5\beta_1$ recognizes an Arg-Gly-Asp (RGD) motif within fibronectin, forms a prototypic receptor-ligand complex, induces fibrogenesis and remodels extracellular matrix [Hynes and Yamada, 1982; Cukierman et al., 2001]. Moreover, integrin $\alpha_2\beta_1$ is the major adhesion receptor that recognizes the α_2 subunit von Willebrand factor A-type domain on collagen [Tuckwell et al., 2000]. The binding of integrin receptor to model ECM composed of collagen or fibronectin is accompanied by a series of structural and compositional transformations in the cytoskeleton, which eventually facilitates the change of cell morphology [Stossel 1993]. Several types of cells including hepatocytes, fibroblast, smooth muscle cells and endothelial cells display unique phenotypes upon adhesion on model ECM. Upon integrin-mediated adhesion on ECM proteins, cells like fibroblasts and smooth muscle cells actively extend lamellipodium or filopodia and encounter new extracellular matrix environment. As a result, cell spreading and motility lead to the generation of mechanical forces, which are believed to be originated from the coupling between the actin polymerization and active membrane transport [Pollard and Borisy, 2003].

The process as mentioned above facilitates cells to perceive the biochemical and mechanical cues in the external environment through the dynamics of actin network within cytoskeleton after integrin-ECM interactions [Welch et al., 1997]. Thus the “real time” probing of cytoskeletal organization of adherent cells would provide a vital link between cell-ECM interaction and subsequent cellular responses. To visualize cytoskeleton in living cells, microinjection of fluorescently labeled actin or actin specific antibodies into the cytoplasm has been conventionally applied [Wang 1984; Bhadriraju and Hansen, 2002]. In addition, green fluorescence protein (GFP)

technology has emerged as a superior approach of biological labeling through the expression of recombinant GFP-proteins in living cells compared with immunostaining. By transferring ligated DNA vector into cytoplasm, GFP tagged proteins can be visualized in living cells under conventional fluorescence microscopy. In order to apply GFP technology to study mechanochemical signal transduction and cell adhesion on biomaterials, the effect of GFP-actin expression on receptor-mediated adhesion must be elucidated.

While cell adhesion mechanics is known to be correlated with the spatial organization of cytoskeleton, the molecular and biophysical linkage between receptor-ligand binding, cytoskeleton remodeling and the overall adhesion phenotypes is currently unknown. For example, extensive spreading of smooth muscle cells adhering on biomaterials is accompanied by the formation of actin stress fibers in cytoplasm [Karnik et al., 2003]. In this study, we aim to elucidate the effect of GFP-actin expression on the biophysics of integrin mediated adhesion for HepG2 cells on model ECM proteins supported on glass substrate. Our results would provide a benchmark for applying GFP technology in cell adhesion investigations. HepG2 cell line is chosen as our model system because it carries most major phenotypes of hepatocytes that are important for biological function regenerations in bio-artificial liver. Confocal reflection interference contrast microscopy (C-RICM), phase contrast microscopy, fluorescence microscopy are applied to simultaneously probe the adhesion contact dynamics and cytoskeleton structure of GFP-actin expressing HepG2 cells on collagen- and fibronectin-coated substrate. Using the two ECM proteins as mentioned above provides a way of tuning ligand-receptor interactions because each ligand recognizes a unique class of integrin on HepG2 cells. The results first show that adhesion contact

evolution, cell deformation, and adhesion energy of GFP-actin expressing HepG2 cells are significantly altered for HepG2 cell seeding on either fibronectin- or collagen-coated substrates compared to that of intact HepG2 cells. At the same time, the degree of modulation in the biophysical parameters is dependent on the type of immobilized ligand. Furthermore, the coupling between cytoskeleton remodeling and adhesion mechanism is revealed.

4.2 Results and Discussions

We presented here a new experimental approach based on the GFP-actin expression to elucidate the role of cytoskeleton composition on the adhesion contact dynamics of HepG2 cells on model ECMs. The Integrin receptors on HepG2 cell are a family of heterodimeric transmembrane proteins (with α and β subunits) and play a critical role in several cellular processes including tissue morphogenesis, organogenesis, tumor metastasis [Hynes 2002]. It is known that integrin $\alpha_5\beta_1$ specifically recognizes fibronectin on ECM and triggers ECM remodeling [Cukierman et al., 2001]. Also, integrin $\alpha_2\beta_1$ is responsible for the adhesion of cell to collagen and is closely associated with intracellular signal transduction [Tuckwell and Humphries, 1996]. Eventually, integrin receptors transmit the biochemical signal generated from the molecular recognition to cytoskeleton through RACK1 kinase activation and focal adhesion formation [Cox et al., 2003]. It has been shown that the overexpression of RACK1 dampens cell migration and increase cell adhesion [Buensuceso 2001]. As a result, the mechanochemical transduction of adherent cells to ECM proteins is highly intricate for detailed biophysical characterizations.

The adsorption of ECM proteins on model substrates leads to the formation of molecularly structured film. In turn, nanoscale topology of biomaterial surfaces is known to directly tune key phenotypes of certain anchorage dependent cells. Fig. 4.1A shows an AFM topographic image ($2\ \mu\text{m} \times 2\ \mu\text{m}$) of a representative region on collagen-coated coverglass. The result indicates that collagen forms fibrillar structures upon physical adsorption on plain coverglass. The root-mean-square (RMS) roughness of the collagen-coated substrate averaged from three different samples (at least five regions on each) is $2.5 \pm 0.5\ \text{nm}$. The RMS roughness of pure glass coverslip as a control is around $0.18 \pm 0.05\ \text{nm}$. Fig. 4.1B shows an AFM topographic image for a typical region of fibronectin-coated coverglass. The result indicates the thicker fiber with thickness of around $200\ \text{nm}$ is formed by fibronectin adsorption. Moreover, the averaged RMS roughness of fibronectin-coated coverglass is around $5.4 \pm 1.50\ \text{nm}$ and is 2.2 times higher than that of collagen-coated glass. The surface coverage of collagen and fibronectin on coverglass is determined as 55 % and 46 %, respectively. The detection of fibrillar structures upon ECM adsorption on gold film and glass has been reported by other groups [Kato and Mrksich, 2004]. Moreover, it has been shown that the root-mean-square (RMS) roughness of biomaterial surface is directly correlated with the extent of cell attachment [Hallab et al., 2001]. It has also been proved that increased surface roughness results in greater tissue adhesion [Bundy et al., 1991; Hallab et al., 2001]. In this study, the increase of surface roughness is caused by the macromolecular aggregation of ECM proteins on glass coverslip. The differences in the RMS roughness and fiber thickness between the collagen and fibronectin coated coverglass are likely caused by the different physical and biochemical properties of the two proteins, e.g., pKa [Shibutani et al., 2000]. Therefore, to determine the RMS

roughness of collagen or fibronectin not only displays topology of adsorbed protein on coverslip but also assists to explore cell attachment.

Fig. 4.1. AFM topographic image of a typical region ($2\ \mu\text{m} \times 2\ \mu\text{m}$) on collagen-coated coverslip (A). AFM topographic image of a typical region ($2\ \mu\text{m} \times 2\ \mu\text{m}$) on fibronectin-coated coverslip (B).

Cell-spreading is a main phenotype of anchorage-dependent cells and is triggered by receptor-mediated signal transduction. Intact and transfected HepG2 cell are cultured on glass coverslip coated with collagen or fibronectin (with the same solution concentration of $0.8\ \mu\text{g}/\mu\text{l}$) in order to study the effect of the GFP-actin expression in the regulation of cell geometry on model ECMs. Fig. 4.2A and 4.2B show the phase contrast image of intact HepG2 cells adhering on fibronectin- and collagen-coated coverslip, respectively, during the first two hours of cell seeding. Intact HepG2 cells extensively spread on fibronectin-coated coverslip during initial cell seeding. For example, the two cells (labeled with arrows on Fig. 4.2A) change from spherical to disc-like shape. On the other hand, intact HepG2 cells slightly spread and remain

spherical in shape on collagen-coated coverslip after two hour of cell seeding (arrows on Fig. 4.2B). Fig. 4.2C and 4.2D show the phase contrast image of GFP-actin expressing HepG2 cells on fibronectin- and collagen-coated coverslip, respectively. Interestingly, the morphology change of GFP-actin expressing HepG2 cells is less significant compared with that of intact HepG2 cells on fibronectin-coated coverslip. More specifically, GFP-actin expressing cells spread less on fibronectin-coated coverslip compared with intact HepG2 cells as shown by the rather round geometry towards 2 hours of cell seeding (arrows on Fig. 4.2C). Similarly, the transfection of HepG2 cells with GFP-actin reduces the extent of cell-spreading on collagen-coated coverslip. It is possible that GFP-actin transfection causes HepG2 cell to synthesize GFP-actin, which dampens cytoskeleton reorganization through the interaction between actin and GFP-actin. In fact, intact HepG2 cell and GFP-actin expressing HepG2 cell-spreading are driven by intracellular processes such as actin filament growth and are modulated by external cues including ligand density and surface roughness.

The change of cell morphology mediated by collagen and fibronectin can be quantitated through the determination of percentage change of two-dimensional (2-D) spread area during initial cell seeding (Fig. 4.3). Each error bar represents the standard deviation for at least fifty cells under each condition. On fibronectin-coated coverslip, the average percentage change of 2-D spread area for intact HepG2 cells and GFP-expressing HepG2 cells is 37.25% and 18.96%, respectively, after 2h of cell seeding (Student's *t*-test: $p < 0.001$). The percentage change of 2-D spread area of HepG2 cells on collagen-coated coverslip is reduced by three-folds from 22.09 % to 7.35 % towards the transfection with GFP-actin (Student's *t*-test: $p < 0.001$). It is shown that fibronectin is more effective than collagen in promoting HepG2 cell-spreading. Also, the results

indicate that GFP-actin expression restrains the 2-D spreading of HepG2 cells on both ECM coated surfaces. Interestingly, the rate and extent of spreading for intact HepG2 cells on fibronectin are higher than those on collagen.

Fig.4.2. Phase contrast images of a typical population of intact HepG2 cells on fibronectin (a) and collagen-coated coverslip (b) and that of GFP-actin expressing cells on fibronectin (c) and collagen-coated coverslip (d) The scale bar represents a length of 20 μm .

It is widely recognized that cell-spreading is caused by a complex re-organization of the cytoskeleton following receptor-ligand interactions at cell-substrate interface [Zutter et al., 1999]. Thus the result implies that the morphology change of intact HepG2 cell is highly dependent on the nature of molecular recognition between cell and ECM. In comparison with the intact cells, GFP-actin expressing HepG2 cells do not significantly increase in their 2-dimensional spread areas and remains rather compact in shape. The rounded morphology resulted from GFP-actin expression is

likely associated with low DNA synthesis rate [Hansen et al., 1994]. Moreover, the extent of HepG2 cell-spreading is less than that of fibroblast [Sheetz et al., 1998]. This trend is likely caused by the lacks of selective ECM protein binding to the integrin on the lamellipodia and the subsequent tension generated through cytoskeleton extension towards cell periphery [Sheetz et al., 1998]. In addition, HepG2 cell and fibroblast are found in liver and connective tissue, respectively. HepG2 cells aggregate onto the microstructures of surface but fibroblasts elongate in the microchannel as shown by optical microscope [Lecterc et al.]. It is evident that the orientation and alignment of HepG2 cell and fibroblast is driven by the biological functions of their tissues [Curtis and Riehle, 2001]. The lower percentage changes in 2D spread area for GFP-actin expressing HepG2 cells on both ECMs further indicates that the substitution of actin with its GFP variant perturbs the cytoskeleton responses towards integrin mediated adhesion. It has been shown that smooth muscle cells with GFP-actin expression spreads more on soft collagen-coated gel compared to normal cells [Engler et al., 2004]. The lack of enhancement in cell-spreading upon GFP-actin expression observed herein is stemmed from the following factors: (1) adherent HepG2 cells maintain round geometry; (2) the total time of cell seeding in the previous study is two times longer than that in our study; (3) the effect of GFP-actin expression previously observed is only obvious at a particular collagen density on the soft gel. It is possible that the less spreading of GFP-actin expression cells is caused by the injury induced by the lipid-based transfection. From our recent data of long-term cell culture, it is clearly shown that the transfected cells extensively spread on fibronectin- or collagen-coated substrate after 20 hours of cell seeding (Fig 4.4). As a result, it is unlikely that the cells were injured by the gene transfection.

Fig. 4.3. The average percentage change in the two-dimensional spread area for a population of HepG2 cells during the first two hours of cell seeding on collagen and fibronectin (Student's *t*-test: $p<0.001$).

Fig. 4.4. Phase contrast images of transfected HepG2 on collagen-coated coverslip (a) and fibronectin-coated coverslip (b) at different time. Scale bar = 10 μ m.

Conventionally, extensive cell-spreading on biomaterial surfaces has been used as a benchmark of successful cell adhesion. On the other hand, the extent of cell-spreading has no direct correlation with the adhesion contact formation during initial cell seeding [Yin et al., 2003]. In order to reveal the actual mechanism of mechano-signal transduction, both cell-spreading and adhesion contact dynamics must be simultaneously measured. In parallel with the phase contrast imaging, confocal-reflection interference contrast microscopy (C-RICM) reveals the adhesion contact evolution of HepG2 cells against GFP-actin expression and different ECM proteins. Fig. 4.5 shows a series of C-RICM images of a typical intact HepG2 cell adhering on fibronectin-coated coverslip from 6 to 124 minutes after cell seeding. From C-RICM image, strong adhesion contact between cell membrane and solid substrate is represented by dark region due to the destructive interference of the two reflected laser beams [Fang et al., 2002]. The result shows that significant adhesion contact of intact HepG2 cell with an area of $104\ \mu\text{m}^2$ (half of the steady-state value) emerges on the fibronectin gel-coated substrate at 6 minutes after cell seeding. Also, the adhesion contact area rapidly reaches the steady-state level of around $215\ \mu\text{m}^2$ at 46 minutes after cell seeding. Along the way, the shape of the adhesion contact zone is remodeled from round to elliptical.

The effect of GFP-actin expression on the adhesion contact evolution of HepG2 cells needs to be elucidated for determining the link between cytoskeleton properties and mechanochemical signal transduction. Figure 4.6 shows a series of C-RICM images of a GFP-actin expressing HepG2 cell on fibronectin-coated coverslip from 7 to 126 minutes after cell seeding. It is demonstrated that the adhesion contact of the GFP-actin expressing cell is significantly smaller than that of intact cell (Fig. 4.3) at the

initial period of cell seeding on fibronectin-coated substrate. For example, the adhesion contact area of the transfected cell is around 2.4 % ($5 \mu\text{m}^2$) of the steady-state value at 7 minutes after cell seeding. The adhesion contact area gradually grows from 5 to $200 \mu\text{m}^2$ against the increase of seeding time from 7 to 86 minutes. Beyond 86 minutes of cell seeding, the adhesion contact area remains constant. Moreover, negligible remodeling in the shape of adhesion contact of the transfected cell is detected.

Fig.4.5. A series of C-RICM images of a typical HepG2 cell (intact) within a cell population adhering on fibronectin gel-coated substrate from 6 min to 125 min. The scale bar represents $5 \mu\text{m}$.

It is known that both collagen and fibronectin recognize different types of integrin receptors. Different pair of ligand and receptor may modulate the adhesion contact evolution of HepG2 cells. Fig. 4.7 shows a series of C-RICM images for a typical intact HepG2 cell within a cell population adhering on collagen-coated substrate from 8 to 123 minutes after cell seeding. During the initial stage of cell seeding (till

eight minutes), the adhesion contact area is $70 \mu\text{m}^2$. The adhesion contact area eventually reaches a steady-state level of $210 \mu\text{m}^2$ at around 60 minutes after cell seeding. Fig. 4.8 shows a series of C-RICM images of a typical GFP-actin expressing HepG2 cell on collagen-coated coverslip from 8 to 123 minutes after cell seeding. The result shows that negligible adhesion contact after 8 minutes of cell seeding. Later on, a small and dark region representing an adhesion zone with an area of $50 \mu\text{m}^2$ emerges at 18 minutes post cell seeding. The cell adhesion contact of the transfected cell gradually grows in size against time until it reaches a steady-state level of around $200 \mu\text{m}^2$ after 100 minutes of cell seeding.

Fig.4.6. A series of C-RICM images of a typical transfected HepG2 cell with a cell population adhering on fibronectin gel-coated substrate from 8 min to 126 min after cell seeding. The scale bar represents $6 \mu\text{m}$.

Fig. 4.7. A series of C-RICM images of a typical HepG2 cell (intact) with a cell population adhering on collagen-coated substrate from 8 min to 125 min after cell seeding.

Fig. 4.8. A series of C-RICM images of a typical transfected HepG2 cell with a cell population adhering on collagen gel-coated substrate from 8 min to 123 min after cell seeding.

Our results show that GFP-actin expressing HepG2 cell delays in the adhesion contact evolution in comparison with intact HepG2 on ECMs. It is likely that GFP-actin transfection influences the signaling from the intracellular domain of the β integrin, which not only promotes cell-spreading but also activates signaling through focal adhesion kinase [Hall 1998; Ilic et al., 1997]. Also, GFP-actin which is similar to TMR-actin (tetramethylrhodamine-modified actin) perturbs the actin filament and results in more fragile and shorter filaments compared to unlabeled actin [Kudryashov et al., 2004]. In addition, an integrin clustering in combination with receptor ligation is locally detected, and then key features of the prototypical anchorage response appear at the site: focal adhesion kinase assembles with the induction of phosphorylation, and local cytoskeletal nucleation happens at the nascent focal adhesions [Miyamoto et al., 1995]. The cluster of integrin receptors into focal adhesion complexes is possibly diminished by GFP-actin transfection which leads to the decrease of adhesion contact area.

At the same time, our C-RICM results reveal that HepG2 cells not only attached to fibronectin-coated substrate of higher RMS roughness earlier but also form large dark region compared with that on collagen surface of lower RMS roughness. The dark region formation (defined as contact zone) after cell seeding on fibronectin- or collagen-coated substrates is closely associated with the total number and density of adhesion bonds between immobilized fibronectin or collagen molecules and cell adhesion receptors. The finding may have significant biological implication, especially related to the receptor-ligand interaction between cell and substrate. It could explain why cells attain higher adhesion energy by receptor clustering and by coupling to the intracellular domain of receptors to actin filaments through focal adhesion complexes.

The strength of adhesion energy and tethering force exerted by a focal adhesion domain increases dramatically with the diameter of the adhesion domain.

Based on our data in C-RICM and phase contrast microscopy, an experimental parameter known as the degree of deformation (a/R) that quantifies the geometry of adherent cells can be determined. Fig. 4.9A shows the average a/R of intact and GFP-actin expressing HepG2 cells adhering on fibronectin-coated coverslip against the time of cell seeding. Each error bar represents the standard deviation for the data collected from at least sixty cells on at least four samples. The result indicates that a/R of intact HepG2 cell on fibronectin substrate rapidly increases from 0 to 20 minutes post cell seeding compared with that of GFP-actin expressing HepG2 cell. Specifically, the initial rate of increase of a/R against time (initial cell deformation rate; Table 4.1) for intact HepG2 and GFP-expressing HepG2 on fibronectin-coated coverslip is 0.083 min^{-1} and 0.025 min^{-1} , respectively. By comparing the two sets of data with paired Student's t -test, it is shown that the effect of GFP-actin transfection on the data is not arisen from random events ($p < 0.0001$). The extent of cell deformation in terms of a/R eventually reaches a steady state after 60 and 80 minutes of cell seeding for intact and GFP-actin expressing cells, respectively, on fibronectin-coated coverslip. At a steady state, average a/R for intact and GFP-actin expressing cells reaches 0.87 and 0.84, respectively. The result basically shows that the kinetics of cell deformation of GFP-actin expressing HepG2 cells on fibronectin is significantly dampened compared to intact cells.

Table 4.1: Summary of rate of adhesion contact evolution

GFP-actin Expression	Substrate	Initial cell Deformation rate (min ⁻¹)
Yes	Collagen	0.024
No	Collagen	0.042
Yes	Fibronectin	0.025
No	Fibronectin	0.083

Fig. 4.9A. Cell deformation (defined as a/R according to our contact mechanics model) against time, averaged over a population of intact and transfect HepG2 cells on fibronectin substrates.

Fig.4.9B. Degree of cell deformation against the time of cell seeding on collagen-coated glass.

Fig. 4.9B shows that average a/R against time for intact and GFP-actin expressing HepG2 cells seeded on collagen gel substrates. Each error bar represents the standard deviation for the data collected from at least sixty cells on at least four collagen-coated samples. Moreover, the initial cell deformation rate for intact HepG2 and GFP-expressing HepG2 on collagen-coated coverslip is 0.042 min^{-1} and 0.024 min^{-1} , respectively (Student's t -test: $p < 0.01$). The averaged a/R eventually reaches a steady state after 100 and 104 minutes of cell seeding for intact and GFP-actin expressing cells, respectively, on collagen-coated coverslip. At steady state, average a/R for intact and GFP-actin expressing cells adhering on collagen-coated coverslip reaches 0.78 and 0.63, respectively. The results further support the role of GFP-actin expression in reducing the rate of cell deformation on model ECM proteins. On both collagen and

fibronectin, GFP-actin expression directly moderates the initial cell deformation rate. This result is supported by the role of GFP-actin expression in inducing the round-up and detachment of adherent dictyostelium discoideum cells [Aizawa et al., 1997]. Comparing between the results on collagen and fibronectin substrates, intact HepG2 cells achieve a higher average a/R at steady state and initial rate of a/R vs. time on fibronectin. In contrast, the initial cell deformation rate and steady state a/R for GFP-actin expressing cells on fibronectin is the same and higher, respectively, compared to that on collagen. The trend as mentioned above demonstrates the intricate interplay between GFP-actin expression and the nature of ECM proteins that dictates the ultimate biomechanical responses of adherent cells. Our results are supported by the faster adhesion of H10 cells on fibronectin-coated substrates compared to that on collagen-coated substrates [de Gennes et al., 2003].

With the experimentally determined a/R and R , the adhesion energy between cell and model ECM is calculated from Eqn. 2. Fig. 4.10A shows the average adhesion energy against time for intact and GFP-actin expressing HepG2 cells after seeding on fibronectin-coated coverslip. Each error bar represents the standard deviation for the data collected from at least sixty cells on at least four fibronectin-coated samples. For both cell types, the average adhesion energy spans four orders of magnitude. During the initial stage of cell seeding, the average adhesion energy of intact HepG2 cells seeding on fibronectin is higher than that of GFP-actin expressing HepG2 cells. The time for the adhesion energy of intact and GFP-actin expressing cells to reach steady state is 40 minutes and 70 minutes, respectively. The steady-state level of adhesion energy for both cell types on fibronectin-coated coverslip is around $1 \times 10^{-7} \text{ J/m}^2$. Fig. 4.10B shows the average adhesion energy against time for a population of intact and

GFP-actin expressing HepG2 cells after seeding on collagen gel substrates. In collagen-mediated adhesion, the adhesion energy spans three orders of magnitude. The result also indicates that the initial rate of increase in adhesion energy against time for infected HepG2 cell is significantly lower than that of intact HepG2 cell. The time for the adhesion energy of intact and GFP-actin expressing cells to reach steady state is 70 minutes and 90 minutes, respectively, on collagen-coated substrate. The steady-state level of adhesion energy for intact and GFP-actin expressing cells on collagen-coated coverslip is $6 \times 10^{-8} \text{ J/m}^2$ and $1 \times 10^{-8} \text{ J/m}^2$, respectively. On either collagen- or fibronectin-coated coverslip, intact HepG2 cells achieve higher adhesion energy than transfected cells. In a recent study, it is shown that cofilin, actin-regulating protein, facilitates the remodeling of actin monomers into functional bundles. [Aizawa et al., 1997]. Therefore, the physical origin of the reduction in adhesion strength of HepG2 cells is probably correlated to the impaired interaction between GFP-actin and cofilin. Based on the similar coverage of fibronectin and collagen on the coverslips (as shown in our AFM data), the apparent affinity of integrin $\alpha_5\beta_1$ -fibronectin for both intact and GFP-actin expressing HepG2 cells is higher than that of integrin $\alpha_2\beta_1$ -collagen interaction. Although the transfected cells do not extensively flatten on the ECMs, our C-RICM result shows that strong adhesion contacts are developed after 22h of cell seeding on ECMs (Fig. 4.11). As long as the cells are strongly attached to the substrates, they are not undergoing anokis induced by cell detachment during cell culture on model ECMs.

Fig. 4.10A. The average adhesion energy for a population of intact and transfect HepG2 cells against time after seeding on fibronectin substrates.

Fig. 4.10B. The average adhesion energy for a population of infected HepG2 cells against time after seeding on collagen substrates.

Fig. 4.11. Phase contrast image (a) and C-RICM image (b) of transfected HepG2 cell on collagen-coated substrate at 22 hours at 37°C.

Fig. 4.12A. The ln-ln plot of radius of adhesion contact against the time of cell seeding on fibronectin-coated glass.

Fig. 4.12B. The ln-ln plot of radius of adhesion contact against the time of cell seeding on collagen-coated glass.

Fig. 4.12A shows the ln-ln plot of radius of adhesion contact zone and time for intact and GFP-actin expressing HepG2 cells on fibronectin-coated coverslip. By determining the slope of the ln-ln plot, the coefficient n of the kinetics of adhesion contact evolution ($R \propto t^n$) is determined for each type of cell on ECM-coated substrates. On fibronectin-coated substrate, n for HepG2 cells is increased from 0.35 to 0.50 upon GFP-actin expression. It is shown that the cell adhesion kinetics moves from intermediate to slow reaction rate on the sticky cell surface [de Gennes et al., 2003]. Fig. 4.12B shows the ln-ln plot of radius of adhesion contact zone and time for intact and GFP-actin expressing HepG2 cells on collagen-coated coverslip. On collagen-coated substrate, n for HepG2 cells is increased from 0.23 to 0.35 upon GFP-actin expression. The result indicates that the adhesion of intact cell is driven by the rapid

reaction between the stickers or receptor on cell surface and the ligand on the model substrate [de Gennes et al., 2003]. Interestingly, GFP-actin expression tunes the adhesion kinetics to an intermediate regime between fast and slow reactions on the sticky cell surfaces. The result strongly suggested that the change of adhesion kinetics induced by GFP-actin expression is dependent on the type of ECM proteins

We also observed and explored live cells on a model substrate during long-term cell culture. Fig. 4.13 shows a series of phase contrast images of GFP-actin expressing HepG2 cells adhering on collagen-coated coverglass against the time of cell seeding at 37 °C. The result indicates that the cells remain round in shape during the first 30 minutes of seeding on collagen-coated substrate. Lamellipodium distributed at the peripheral cell membrane progressively increases over time. During this dynamic process, there is a major role for the cytoskeleton, which drives the protrusive events via polymerization of actin in the lamellipodium, following by actomyosin contractility [Zimmerman et al., 2004]. Furthermore, there is no significant change of cell morphology as shown by the round geometry of the adherent cells towards seven hours of cell seeding. In parallel with phase contrast microscopy, C-RICM is used to probe the adhesion contact area that appears as dark region in the image. Fig. 4.14 shows a series of C-RICM images for a typical group of GFP-actin HepG2 cells on collagen-coated substrate during initial cell seeding. The result indicates that strong adhesion contact evolves at around 30 minutes after cell seeding. The adhesion contact zone of HepG2 cells on collagen-coated substrate apparently extends from 30 minutes to 3 hours. From 3 hours to seven hours after cell seeding, the adhesion contact area of cells remains constant. Interestingly, the adhesion contact of all cells concurrently evolves against

time. The result implies that the cell population is homogeneous towards collagen-mediated adhesion.

Fig. 4.13. A series of phase contrast images GFP-actin HepG2 cells seeding on collagen-coated substrate. Scar bar = 10 μm .

Fig. 4.14. A series of C-RICM images for GFP-actin expressing HepG2 cells seeding on collagen-coated substrate.

It is known that different forms of integrin receptor on cell surface recognize distinct ligands on ECM or biomaterial surface. Integrin receptor affinity and signaling in responses to extracellular ligand binding affect intracellular actin polymerization and recruit distinct focal adhesion protein to change cell morphology and adhesion contact [Miyamoto et al., 1995]. Fig. 4.15 shows a series of phase contrast images for GFP-actin expressing HepG2 cells during long-term cell culture on fibronectin-coated substrate at 37 °C. During the first 30 minutes of cell seeding, the adherent cells remain round in shape on fibronectin. Moreover, the boundary of cell membrane laterally extends on the fibronectin-coated substrates as shown by the significant portraying of lamellipodia beyond 1.5 hours of cell seeding. Later on, the cell morphology is progressively changed until the cells extensively spread after 7.5 hours of cell seeding. Also, the extent of cell spreading on fibronectin is significantly higher than on collagen-coated substrate. The result is closely associated with cellular cytoskeleton. It is a complex dynamical network that constantly remodels as cells spread and move. Fig. 4.16 shows a series of C-RICM images for a typical group of GFP-actin expressing HepG2 cells on fibronectin-coated substrate during initial cell seeding. The result indicates that significant adhesion contact is formed at 30 minutes after cell seeding. From 30 minutes to 3.5 hours, the area of adhesion contact is significantly increased until it reaches a steady-state level. After 3.5 hours of cell seeding, the adhesion contact area doesn't change against the time of seeding. In comparison with C-RICM images for GFP-actin expressing HepG2 cells seeding on collagen-coated substrate, the adhesion contact area for GFP-actin expressing HepG2 cells seeding on fibronectin-coated substrate is much more distinctly enhanced. Because $\alpha_5\beta_1$ integrin not only mediated cell attachment to fibronectin but also play an essential role in the assembly of a fibronectin matrix. This role includes direct binding to fibronectin and modulating a

distinct binding event of receptor-ligand interaction [Wu et al., 1993]. In addition, It is possible that the intracellular domain of $\alpha_5\beta_1$ integrin connects fibronectin to the actin cytoskeleton and trigger intracellular signaling pathways including focal adhesion kinase, integrin linked kinase, extracellular signal-regulated kinase leading to intracellular kinase cascade [Kuphal et al., 2005].

Fig. 4.15. A series of phase contrast images for GFP-actin expressing HepG2 cells seeding on fibronectin-coated substrate.

Fig. 4.16. A series of C-RICM images for GFP-actin expressing HepG2 cell seeding on fibronectin substrate.

By combining the data of phase contrast microscopy and C-RICM, the degree of deformation a/R which quantifies the exact geometry of adherent cells can be determined. Fig. 4.17 shows a/R of GFP-actin expressing HepG2 cells adhering on collagen- or fibronectin-coated substrate against time of cell seeding. Each error bar represents the standard deviation for the data collected from at least sixty cells on at least four collagen- or fibronectin-coated samples. During the initial stage of cell seeding, the rate of increase of a/R against time is 1.8 and 1.1 hr^{-1} on fibronectin- and collagen-coated surface, respectively (Student's t -test: $p < 0.0012$). Beyond two hours of cell seeding, a/R reaches a steady-state level of 0.94 and 0.78 on fibronectin- and collagen-coated surface, respectively. The result basically indicates that the initial rate and extent of cell deformation on fibronectin-coated surface are significantly higher

than those on collagen-coated surface. To assess the effect of ligand-receptor bond on the adhesion energy of HepG2 cells during the cell attachment, contact mechanics model that has been successful constructed is useful to measure adhesion energy [Liu et al., 2002]. Fig. 4.18 shows the averaged adhesion energy of GFP-actin expressing HepG2 cells against time of seeding on fibronectin- and collagen-coated surfaces. The result indicates that adhesion energy spans two orders of magnitude during long-term cell culture on both ligand-coated surfaces. On fibronectin surface, the initial rate of increase of adhesion energy is significant higher than that on collagen-coated surface. Interestingly, the enhanced cell deformation and adhesion energy of HepG2 cells are accompanied with the higher degree cell spreading. The trend as observed herein is likely related to the intricate signal transduction cascades of cell adhesion induced between integrin variants and different ligands on the model substrate.

In comparison with GFP-actin expressing HepG2 seeding on collagen- and fibronectin-coated substrates for 2 hours culture, the degree of cell deformation of GFP-actin expressing HepG2 for 24 hours culture obvious increases. Moreover, adhesion energy of GFP-actin expressing HepG2 seeding on collagen- and fibronectin-coated substrate rises and keeps constant, respectively. Therefore, the results demonstrates that the interactions between extracellular matrix proteins and cell surface receptors provide physical support for cell adhesion but the contractile cytoskeleton to plasma membrane tethered with integrin receptor still needs to be proved through experiment.

Fig. 4.17. Degree of cell deformation a/R of GFP-actin expressing HepG2 cell on fibronectin- and collagen-coated surface.

Fig. 4.18. The averaged adhesion energy for a population of GFP-actin expressing HepG2 cells against time after seeding on collagen and fibronectin substrates.

To reveal the role of GFP-actin expression in mechanotransduction, the spatial organization of cytoskeleton during ECM induced adhesion needs to be determined. Fig. 4.19A shows a series of fluorescence images of a typical GFP-actin expressing HepG2 cell adhering on fibronectin-coated coverslip from 8 to 128 minutes after cell seeding. Moreover, the phase contrast images for the same cell are also included in the figure. At the early stage of cell adhesion (8 minutes), the fluorescence image indicates that F-actin concentrates at the peripheral region of the cell membrane. As cell spreading and adhesion proceed, the spatial organization of actin is transformed from an elliptical to circular pattern. Actin remodeling significantly leads to cell morphology change. It implies that the mechanistic connection between cytoskeleton and adhesion receptor causes redistribution of tensile force. Moreover, cell-spreading on fibronectin is synergized by the localization of actin filaments at the lamellipodia. The immunostaining of HepG2 cells with phalloidin shows that the actin filaments concentrate around the cell periphery as seen in GFP-actin expressing cells, as shown Fig. 4.19C (a). The result supports that the GFP-actin expression does not apparently affect the cell phenotypes. It is shown that integrin-mediated adhesion mainly causes the localization of actin filaments in the cytoplasmic cortex. Fig. 4.19B shows a series of fluorescence images for a typical GFP-actin expressing HepG2 cell on collagen-coated coverslip during cell seeding. At eight minutes after cell seeding, actins form a donut-like structure that diffuses inward from the edge to the inner part of the adherent cell. As cell seeding time increases from 8 to 47 minutes, the thickness of the donut-like structure is progressively reduced. Beyond 87 minutes of cell seeding, the actin molecules mostly concentrate at the peripheral of the cell. The results demonstrate that actin polymerization of GFP-actin expressing HepG2 cell is restrained from influencing cell-spreading, regardless of cell seeding on fibronectin or collagen substrate.

Fig. 4.19A. A series of GFP-actin images of typical HepG2 cell adhering on fibronectin gel-coated substrate from 7 min to 122 min after cell seeding. Error bar represents 5 μm .

Fig. 4.20A shows a series of fluorescence images of GFP-actin expressing HepG2 cells adhering on fibronectin-coated coverglass from 12 minutes to 22 hours after cell seeding. Moreover, the phase contrast images for the same cells are also included in the figure. At the early stage of cell adhesion (12 minutes), the fluorescence image indicates that actin concentrates at the peripheral region of the cell membrane. As cell spreading and adhesion proceed, the spatial organization of actin is transformed from a round to more extended shape. The geometric transformation of actin network coincides

with the morphology development of the adherent cell as shown by the phase contrast images (in Fig. 4.20A). Specifically, cell-spreading on fibronectin is characterized by the localization of actin filaments at the lamellipodia. Also, actin filament reorganization process occurs not only at the peripheral of cell membrane but also in the cellular interior. Fig. 4.20B shows a series of fluorescence images for GFP-actin expressing HepG2 cells on collagen-coated coverslip during cell seeding. At 12 minutes after cell seeding, actins form a donut-like structure around the edge of the adherent cell. As cell-seeding time increases from 12 minutes to 22 hours, the thickness of the donut-like structure is progressively reduced but the donut-like structure is increased in size. Beyond 22 minutes of cell seeding, the actin molecules mostly concentrate at the peripheral of the cell. From the fluorescence images of Fig 4.20A and 4.20B, we can see that GFP-actin protein expression is apparently enhanced due to the interaction of integrin and ligand.

It is noticed that transfected HepG2 cells adhered to collagen substrates spread to a lesser extent. On both fibronectin and collagen coating, transfected HepG2 cells do not form stress fibers. Instead, the actin filaments localize in the cytoplasmic cortex. Our results agree well with the data reported by Kusano *et al.* in P29 cells adhering on ECM proteins. In detail, it is shown that integrin-mediated adhesion only causes the localization of actin filaments in the cytoplasmic cortex. Kusano *et al.* have pointed out that stress fiber formation in various cell types requires the dual receptor system with integrin and cell surface proteoglycan (latter is not present on HepG2 cells) [Kusano *et al.*, 2000]. We also determined that normal HepG2 cells have not developed stress fibers after seeding on rigid glass substrate for 22h (in Fig. 4.19C (b)). Furthermore, actin filament of GFP-actin expressing HepG2 cell mainly distributes at cell peripheral

after seeding on ECMs model for 22h in comparison with that of normal HepG2 (in Fig. 4.19C (b)). The result verifies that GFP-actin attenuates actin filament polymerization. On the bottom line, the main objective of this study is to correlate the overall cytoskeletal reorganization with the spreading and adhesion kinetics of transfected HepG2 cells upon integrin mediated signaling on ECM proteins. Therefore, the other intricate mechanisms such as focal contact formation, kinase activation involved in mechanotransduction are not focused herein [Tang et al., 1999].

Fig. 4.19B. A series of GFP-actin images of typical HepG2 cell adhering on collagen gel-coated substrate from 8 min to 128 min after cell seeding. Error bar represents 5 μm .

Fig. 4.19C. Immunostaining of normal HepG2 cell on collagen-coated substrate at 50 minutes (a) and on rigid glass substrate at 22 hours (b).

Fig.4.20A. A series of GFP-actin images for HepG2 cell seeding on fibronectin substrate.

Fig. 4.20B. A series of GFP-actin images for HepG2 cell seeding on collagen substrate.

4.3 Conclusion

In summary, current investigation aims to probe the cell adhesion contact dynamics, cell spreading and cytoskeleton structure on model ECM proteins with confocal reflection interference contrast microscope (C-RICM), fluorescence microscopy, phase contrast microscopy in conjunction with GFP-protein expression technology. Generally, it is demonstrated that the initial rate of cell deformation of intact HepG2 cell seeded on fibronectin- or collagen-coated glass are higher than that of transfected HepG2 cells. Similarly, the rate of adhesion energy increase against time during initial cell seeding is higher for intact HepG2 cells. Moreover, GFP-actin expression increases and maintains the average adhesion energy of the cells on

collagen- and fibronectin-coated glass, respectively, at steady state compared with the non-transfected samples. Our research results are different from previous studies of immunostaining as GFP-actin fusion proteins are successfully integrated into actin filaments. Therefore our study leads to the simultaneous observation of cell spreading, cytoskeleton dynamics and adhesion contact dynamics. Regardless of HepG2 cell on fibronectin or collagen gel substrate, actin filaments are concentrated in the cytoplasmic cortex. Importantly, this study provides a benchmark in the application of GFP-actin expression for studying the cell-biomaterials interaction in real time but we also find that GFP-actin transfection has certain disadvantage which attenuates actin filament polymerization. The exact biophysical responses of GFP-actin expressing cells towards receptor-mediated adhesion on engineered biomaterials can be used to deduce the actual properties of non-transfected or intact cells.

Chapter 5: Effect of Hepatitis B Virus Infection on the Adhesion Contact Dynamics of HepG2 Cells on ECM

5.1 Background

Hepatitis B virus (HBV) is a partially double-stranded circular DNA virus belonging to the hepadnaviridea family. HBV infection is associated with the development of major hepatic diseases such as acute liver diseases, chronic liver disease, liver cirrhosis, and hepatocellular carcinoma [Castilla et al., 1991; Foo et al., 2002]. There are three stages involved in the HBV infection of host cell including virus attachment, fusion and entrance. It is known that HBV attachment is mediated by the specific recognition between the viral surface proteins and membrane-bound receptors of hepatocyte [Lonberg-Holm and Philipson, 1981; Le Seyec et al., 1999]. Later on, the fusion of virus with cell membrane and subsequent viral genome intracellular release lead to viral replication cycle [Debra et al., 2001].

The HBV genome contains four genes: *pol*, *env*, *pre-core* and *X* that encodes the viral DNA-polymerase, envelope protein (HBsAg), core protein (HBcAg) and protein X (HBxAg), respectively [Ganem 1996; Ganem and Varmus, 1987]. During its replication, HBV makes use of components of host cell to synthesize the viral proteins as mentioned above and such an interference may affect the phenotypic characteristics and biological functions of infected host cell. For example, the phosphoprotein HBcAg is found to bind to the C-terminal region of actin-binding protein, which may modulate the function of membrane-bound receptor and interfere with the normal signaling pathways of adherent hepatocytes [Juang et al., 2000]. Therefore it is generally

expected that the interaction between HBcAg and actin binding protein contributes to the morphological changes of hepatocytes through the alteration of the cytoskeleton organization. Moreover, HBxAg has been shown to enhance the transcription, translation and secretion of matrix metalloproteinase-9 and metalloproteinase-3 [Chung et al., 2002; Yu et al., 2005]. These two proteinases as mentioned above reduce adhesion and enhance migration of several types of cells [Lee et al., 2005].

Cell adhesion to extracellular matrix (ECM) is achieved through the interaction between receptors on cell surface and ligand on ECM. The communication between cell and ECM is mainly mediated by integrin receptor which binds to ECM molecules with its extracellular domain and interacts with cytoskeletal proteins through its intracellular domain. HBV infection is known to disrupt the signal transduction cascades triggered by the adhesion receptors and to modulate cell adhesion and motility [Yeh 2000]. For instance, HBxAg breaks the molecular linkage between the actin filament and cadherin complex and weakens the intercellular adhesion in a Src-dependent manner [Laza-Pezzi et al., 2001]. Moreover, HBxAg reduces the expression of α_1 and α_5 subunits of integrin, impairs cell adhesion on fibronectin substrate and promotes cell migration [Laza-Pezzi et al., 2001]. However, little is known on the effect of HBV infection on the mechanics and dynamics of host cell adhesion on ECM.

In our current work, we aim to understand the biophysical cascades of cell adhesion underlying the intricate interplay between HBV infection, ligand-receptor interaction and cytoskeleton remodeling. Along the way, we apply our unique biophysical approaches including confocal reflection interference contrast microscopy (C-RICM), phase contrast microscopy and confocal fluorescence microscopy to measure the contact mechanics, adhesion dynamics and cytoskeleton rearrangement of

HepG2 cells challenged with HBV during the initial stage of cell seeding on collagen or fibronectin substrates. Four cell types have been established for our investigation including: 1. HepG2 cells infected with HBV; 2. HepG2 cells with GFP-actin expression; 3. HepG2 cells expressing GFP-actin and infected with HBV; 4. normal HepG2 cells (control experiment). The use of GFP-actin expressing HepG2 cells enables the real time monitoring on the dynamic response of cytoskeleton under HBV infection with confocal fluorescence microscope. The temporal trends of degree of cell deformation, cytoskeleton organization and adhesion energy of the adherent cells are found to be perturbed by HBV infection.

5.2 Results

Fibronectin and collagen are common ECM proteins which influence cell morphology, survival and proliferation. In this study, fibronectin and collagen are coated on glass coverslip to form a layer of molecular film on the substrate. The nanoscale topography of biomimetic surface has been shown to influence the morphology and function of attached cells [Wilkinson et al., 2002]. Thus it is essential to probe the nanotopology of the ECM protein coated surfaces. Fig. 5.1 shows an AFM topographic image of a representative region on fibronectin-coated glass coverslip (A) and collagen-coated glass coverslip (B). The result indicates that fibronectin substrate appears to be rough, contains some rather large agglomerate with irregular shapes, and has a RMS roughness of 2.9 nm. In contrast, collagen substrate seems to be smooth and forms finer grain-like structures evenly distributing on glass coverslip. The root mean square (RMS) roughness of 0.7 nm is for collagen-coated substrate, which is apparently smaller than that for fibronectin substrate. Similar results have been found on the fibronectin or collagen films formed from physical adsorption on the self assembled

monolayer supported on gold substrate [Kato and Mrksich 2004]. The observed differences between the two surfaces coated with collagen and fibronectin possibly result from the different biochemical properties of the two ECM proteins [Shibutani et al., 2000].

Fig. 5.1. AFM topographic images of a typical region ($2\mu\text{m} \times 2\mu\text{m}$) on fibronectin-coated coverslip (A) and on collagen-coated coverslip (B).

Cell attachment and spreading is mediated by the interaction between the adhesion receptors on cell surface and biological ligands on ECM. For instance, different variants of integrin receptor mediate the spreading of most anchorage-dependent cells on fibronectin- or collagen-modified substrates [Koivisto et al., 1997; Feng et al., 2005]. Four types of cell including HepG2 cells transfected with replicative HBV (rHBV) genome, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV, and normal HepG2 cells as a control are utilized in

this study. Fig. 5.2 shows the phase contrast images of the four types of cell on the collagen (A) and fibronectin (B) coated glass coverslip at 5 and 150 minutes after cell seeding. During the early stage (time < 10 minutes) of cell seeding, the four groups of cells readily attach to collagen or fibronectin substrate and adopt a round shape. After 150 minutes of cell seeding, HepG2 cells transfected with rHBV tends to spread significantly on collagen-coated surface in comparison with the normal HepG2 cells. Also, normal HepG2 cells and HepG2 cells transfected with rHBV form significant numbers of lamellipodium at the cell peripheral on collagen-coated substrate. Interestingly, the extent of cell-spreading of GFP-actin expressing HepG2 cells on collagen-coated substrate is impaired by HBV transfection (Fig. 5.2A). In general, both normal HepG2 cells and GFP-actin expressing HepG2 cells more extensively spread after 150 minutes of seeding on fibronectin-coated substrate than on collagen-coated substrate (Fig. 5.2B). In resemblance to the result on the collagen-coated substrate, HBV infection increases the extent of cell-spreading of normal HepG2 cells on fibronectin-coated substrate. On the other hand, HBV infection has no apparent effect on the spreading of GFP-actin expressing cells on fibronectin-coated substrate as shown by the similar cell morphology change of GFP-actin expressing HepG2 cells and GFP-actin expressing HepG2 cells transfected with rHBV plasmid.

Percentage change of 2-dimensional (2-D) spread area is a critical biophysical parameter for quantifying cell morphological change during cell seeding. According to spread areas of HepG2 cells seeded on collagen and fibronectin substrate at 5 and 150 minutes, the percentage change of 2-D spread area is calculated. The average percentage change of 2-D spread area of normal HepG2 cells, GFP-actin expressing HepG2 cells, HepG2 cells transfected with rHBV, and GFP-actin expressing HepG2

cells transfected with rHBV on fibronectin- and collagen-coated substrates is listed in Table 5.1. It is obvious that HBV infection promotes normal HepG2 cell-spreading on both fibronectin- and collagen-coated substrate. Generally, the degree of cell-spreading of all cell types on collagen is reduced compared with that on fibronectin-coated substrate. The result indicates that HBV infection enhances normal HepG2 cells spreading but dampens GFP-actin expressing HepG2 cell-spreading on both collagen- and fibronectin-coated substrates. It is possible that an amount of synthesized HBV particles and secretion of HBV particles promote actin polymerization of normal HepG2 cell, which generates tensile force propelling cell-spreading. In contrast, HBV infection influences GFP-actin expression and actin polymerization. Specifically, GFP-actin is like TMR-actin (tetramethylrhodamine-modified actin), which perturbs the actin polymerization [Kudryashow et al., 2004]. The response of GFP-actin transfected HepG2 cell to HBV infection becomes more complex.

Table 5.1. Summary of percentage change of 2D spread area

Cell	Substrate	Percentage change of 2D spread area
Normal HepG2 cells	Fibronectin	117%
GFP-actin expressing HepG2 cells	Fibronectin	174%
HepG2 cells transfected with rHBV	Fibronectin	216%
GFP-actin expressing HepG2 cells with rHBV	Fibronectin	150%
Normal HepG2 cells	Collagen	73%
GFP-actin expressing HepG2 cells	Collagen	137%
HepG2 cells transfected with rHBV	Collagen	98%
GFP-actin expressing HepG2 cells with rHBV	Collagen	25%

Fig. 5.2. The phase contrast images of GFP-actin expressing HepG2 cells infected with HBV, GFP-actin expressing HepG2 cells, HepG2 cells infected with HBV and normal HepG2 cells on collagen (A) and fibronectin (B) coated coverslip at 5 and 150 min after cell seeding.

The kinetics of cell adhesion is correlated with receptor-mediated signal transduction cascades such as the receptor expressions, ligand affinity, focal adhesion formation and cytoskeleton remodeling, etc [Gauet et al., 2003; Palecek et al., 1997; Garcia et al., 1998]. To measure the intrinsic kinetics of cell adhesion, both cell-spreading and adhesion contact development must be measured simultaneously. In parallel with the phase contrast microscopy, C-RICM probes the adhesion contact development of HepG2 cells and GFP-actin expressing HepG2 cells in response to HBV infection on different EMC proteins. Fig. 5.3 shows a series of C-RICM images of a typical HepG2 cell transfected with rHBV (A), GFP-actin expressing HepG2 cell (B), and GFP-actin expressing HepG2 cell transfected with rHBV (C) and normal HepG2 cell (D) against time of cell seeding (from 10 to 150 minutes) on collagen-coated substrate. Generally, the adhesion contact zone of the four cell types progressively increases in size against time until it reaches a steady state. At 20 minutes after the seeding of HBV transfected HepG2 cell on collagen, small dark patches indicating strong adhesion contact with a total area of around $48\mu\text{m}^2$ (9% of the steady-state value) emerges. The adhesion contact area slowly reaches a steady state of $530\mu\text{m}^2$ (100 % of the steady-state value) at around 130 minutes of cell seeding. Simultaneously, the two-dimensional spread area (phase contrast image) of the HBV infected cell increases from 217 to $633\mu\text{m}^2$ during the same period. In contrast, the normal HepG2 cell seeding on collagen displays larger adhesion contact zone of $173\mu\text{m}^2$ (59% of the steady-state value) at 20 minutes after cell seeding compared to the HBV infected cell. Interestingly, the time for the adhesion contact zone of the normal HepG2 cell to reach steady state of around $271\mu\text{m}^2$ (93% of the steady-state value) is around 70 minutes which is 46 % shorter than that of HBV infected cell. In parallel, the insignificant change of the two-dimensional spread area of the normal HepG2 cell

(from 220 To 235 μm^2) during the same period compared with the HBV infected cell leads to the smaller steady adhesion contact area.

It is interesting to shed light on the intricate interplay between cytoskeleton alteration and HBV infection during HepG2 cell adhesion. First of all, it is shown that the GFP-actin expressing HepG2 cell takes about 20 minutes to develop small patches of strong adhesion contact with an area of 15 μm^2 (3% of the steady-state value) on collagen-coated substrate (Fig. 5.3B). The cell contact area reaches a steady state of about 424 μm^2 (100 % of the steady-state value) at 70 minutes after cell seeding. The two-dimensional spread area (phase contrast image) of the GFP-actin expressing cell increases from 324 to 465 μm^2 during 150 minutes of cell seeding. The shape of adhesion contact zone changes from round to asymmetric over time. The apparent effect of HBV infection on the GFP-actin expressing HepG2 cells is the increase of lag time for forming initial adhesion contact (Fig.5.3C). Specifically, the extent of adhesion contact development for GFP-actin expressing cells (33% of steady state value) at 35 minutes is significantly higher than that for GFP-actin expressing cell with HBV infection (12% of steady state value) at 35 minutes after cell seeding. From 0 to 150 minutes of cell seeding, the 2D spread area and adhesion contact area of GFP-actin expressing cell with HBV infection is increased from 370 to 589 μm^2 and 0 to 573 μm^2 (99% of the steady-state value), respectively. Also, HBV infection significantly increases the time for the adhesion contact area of GFP-actin expressing cell in reaching steady state from 70 to 110 minutes.

Fig. 5.3. A series of C-RICM images of a typical HBV infected HepG2 cell (A), GFP-actin expressing HepG2 cell (B), HBV infected and GFP-actin expressing HepG2 cell (C), and normal HepG2 cell (D) adhering on collagen-coated coverslip from 5 to 150min at 37°C.

Fig. 5.4. A series of C-RICM images of a typical HBV infected HepG2 cell (A), GFP-actin expressing HepG2 cell (B), HBV infected and GFP-actin expressing HepG2 cell (C), and normal HepG2 cell (D) adhering on fibronectin-coated coverslip from 5 to 150min at 37°C.

The change of ligand-receptor interaction may directly tune the biophysical responses of adherent cells. Fig. 5.4 shows a series of C-RICM images of a typical HepG2 cell transfected with rHBV (A), GFP-actin expressing HepG2 cell (B), GFP-actin expressing HepG2 cell transfected with rHBV (C), and normal HepG2 cell (D) adhering on fibronectin-coated substrate from 5 to 150 minutes after cell seeding. The result indicates that the lag time for the emergence of adhesion contact of HBV infected HepG2 cells is 25 minutes longer than that of normal HepG2 cells (Figure 5.4A and 5.4C) on fibronectin-coated surface. Also, the adhesion contact area of HBV infected HepG2 cell and normal HepG2 cell reaches steady state level of $1230 \mu\text{m}^2$ and $465 \mu\text{m}^2$ at 130 minutes and 90 minutes, respectively, after cell seeding. Interestingly, HBV infection doesn't moderate the lag time of adhesion contact emergence (around 10 minutes for both cells) for GFP-actin expressing cell on fibronectin-coated substrate. Moreover, GFP-actin expressing HepG2 cells with or without HBV infection take around 110 minutes to reach steady state of adhesion contact development.

The focal adhesion formation plays a critical role in cell adhesion by serving as structural links between the cytoskeleton and ECM. The confocal fluorescence images of normal HepG2 cells, HepG2 cells transfected with rHBV and HepG2 cells transfected with the empty plasmid stained with vinculin at 20 minutes after cell seeding on collagen substrate are shown in Fig. 5.5(A). The significant expression of vinculin was detected in a typical group of normal HepG2 cells (top panel) while vinculin was barely detected in the group of HBV-replicating cells (bottom panel). Specifically, dot-like structures of vinculin which have a diameter ranging from 0.5 to $1.5 \mu\text{m}$ emerged at the lamellipodium of normal HepG2 cells (Fig. 5.5(A): single-cell image in top panel). The dot-like structures composed of vinculin are known as focal

complexes which served as precursor of focal adhesion [Geiger et al., 2001]. In contrast, only diffusive vinculin instead of clusters was detected in the cytoplasm of HepG2 cells transfected by HBV (single-cell image in bottom panel). As a control, HepG2 cells transfected by empty vector also demonstrated the expression of similar focal complexes found in normal HepG2 cells (single-cell image in middle panel). The result strongly supported that HBV replication delayed the formation focal complexes during initial cell seeding. Most importantly, the delay of focal complex evolution coincides with the reduction of initial cell deformation rate and adhesion energy during the first twenty minutes of cell seeding (Fig 5.11 and Fig 5.13). Towards 50 minutes of adhesion, the dot-like focal adhesion complexes in normal HepG2 cells and cells transfected by empty vector were transformed to dense patches at cell periphery (Fig. 5.5(B)). The elongated and oval vinculin containing structure had an averaged length of 4 μm and was generally known as focal adhesion (zoom-in view in all panels). Interestingly, vinculin patches also emerged at cell periphery of HBV-replicating HepG2 cells after 50 minutes of cell seeding. At the same time, all cell types demonstrated high level of exogenous vinculin in the cytosol. The formation of focal adhesion in HBV replicating HepG2 cells is likely caused by the integrin-collagen recognition and the subsequent cytoskeletal reorganization.

Cellular cytoskeleton not only provides structural integrity to cells, but also serves in the trafficking of proteins and the secretion of infecting virus. To investigate the role of HBV infection on the spatial organization of cytoskeleton during cell adhesion, GFP-actin vector is expressed in HepG2 cells (with or without rHBV expression). Fig. 5.6 shows a series of confocal fluorescence images of a typical GFP-actin expressing HepG2 cell adhering on collagen-coated substrate from 5 to 150 minutes after cell

seeding. Phase contrast images for the same cell at 5 and 150 minutes after cell seeding are also included in the same figure. Our results indicate that actin microfilament is initially concentrated at the peripheral region of the cell membrane (5 minutes). From 5 to 70 minutes, the accumulation of actin molecules leads to the formation smooth contour around the peripheral of the cell. After 90 minutes, the pattern of actin microfilament is transformed from elliptical to wave-like in shape. From 90 minutes onward, the wave-like structure continues to grow in number and size. Also, the trend of actin pattern evolution follows the extension of membrane, which leads to the formation of lamellipodium as shown in the phase contrast image. The result indicates that the unidirectional polymerization of actin provides the major driving force for membrane extension. Fig. 5.7 shows a series of confocal fluorescence images of a typical HepG2 cell adhering on fibronectin-coated substrate at different time. From 5 to 35 minutes, actin filaments accumulate and distribute at the peripheral of cell membrane. Simultaneously, boundary of actin filaments keeps smooth and round in shape. Following time, actin filaments become wave-like shape and extend towards outside. This reveals that actin polymerization and actin cytoskeleton tension is enhanced leading to cell spreading. Compared with Fig. 5.6, GFP-actin expressing HepG2 cell seeding on fibronectin forms wave-liked actin cytoskeleton is apparently earlier than on collagen. Moreover, C-RICM images in Fig 5.4 and Fig 5.3 have shown that GFP-actin expressing HepG2 cell attaches earlier to fibronectin coated substrate compared with GFP-actin expressing HepG2 to collagen substrate. In addition, fibronectin contains two binding sites for integrin to recognize and bind. Integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ receptors have also been reported to mediate cell adherence to collagen and they are the primary collagen-binding integrins [Emsley et al., 1997; Calderwood et al., 1995; Rich et al., 1997; Heino, 2000]. Collagen has only one binding site per molecule

against integrin. Therefore, it is evident that the recognition of fibronectin by integrin $\alpha_5\beta_1$ receptor on HepG2 cell surface leads to quicker mechanochemical transduction into intracellular compartments in comparison with that triggered by collagen.

Fig. 5.5(A) The confocal fluorescence images of normal HepG2 cells, HepG2 cells transfected with rHBV and HepG2 cells transfected with the empty plasmid stained with vinculin at 20 minutes after cell seeding on collagen substrate. The scale bar on left panels is 25 μm . The scale bar on the right panel is 10 μm .

Fig. 5.5(B) The confocal fluorescence images of normal HepG2 cells, HepG2 cells transfected with rHBV and HepG2 cells transfected with the empty plasmid stained with vinculin at 50 minutes after cell seeding on collagen substrate. The scale bar on left panels is 25 µm. The scale bar on the right panel is 10 µm.

Fig. 5.6. A series of GFP-actin images of a typical HepG2 cell adhering on collagen-coated substrate from 5min to 150 after cell seeding.

Fig. 5.7. A series of GFP-actin images of a typical HepG2 cell (normal) adhering on fibronectin-coated substrate from 5 min to 150 after cell seeding.

To probe whether HBV infection influences cytoskeleton rearrangement so as to change cellular morphology, replicative HBV genome was effectively introduced into GFP-actin expressing HepG2 cells using transfection technique. Fig. 5.8 shows a series of confocal fluorescence images of a typical GFP-actin expressing HepG2 cell transfected with rHBV on collagen-coated coverslip at different times after cell seeding. At 5 minutes after cell seeding, numerous wave-like structures with high actin concentration emerge at the peripheral region of the cell membrane. Actually, the role of the actin cytoskeleton is significant in endocytosis and phagocytosis in a variety of cell types. A number of studies demonstrated that the actin cytoskeleton can be manipulated by microbial pathogens [Frischknecht and Way, 2001]. Upon HBV attachment within minutes, the formation of wave-like structure is possibly caused by HBV stimulation rapidly which causes actin polymerization or reorganization. The HBV infection makes GFP-actin expressing HepG2 cell transfected with HBV form the formation of lamellipodium. Our result indicates that HBV infection accelerates the formation of lamellipodium for GFP-actin expressing cells (the lag-time of wave-like structure emergence is reduced by 85 minutes compared with GFP-actin expressing cell seeding on collagen substrate). The phenomena expressed during the early stage of HBV infection rapidly elicits major reorganization of cellular cytoskeleton, essentially the polymerization of actin filaments which generates tension to propel cell membrane projections and achieves HBV entrance into HepG2 cell as shown in phase contrast image of the cell at 5 minutes after seeding. From 5 to 70 minutes, the spatial organization of actin microfilament continuously extends outwards with a concurrent increase in size of bubble-like structure. During cell-spreading, GFP-actin in HepG2 cell transfected with HBV infection is not enriched at the edge of wave-like structure compared to its interior. Simultaneously, it is shown that progeny HBV particles

synthesized in intracellular space employ actin cytoskeleton to facilitate their spreading. In addition, expressed HBx is able to activate Rac1 small GTP-binding protein, which is a key signaling modulator of actin polymerization process [Kalra and Kumar, 2004]. The fact that HBV induced actin polymerization is remarkably similar to that seen in *Shigella* [Fernandez and Sansonetti, 2003]. Interestingly, the bubble-like structure gradually reduces in size beyond 70 minutes of cell seeding. As the bubble-like structure inflates, a cortical actin filament assembles underneath the membrane. The result is consistent with that of blebbing cells [Chattas et al., 2005]. The formation of wave-like structure is thought to be initiated by rupture of the plasma membrane from the underlying cytoskeleton, following by inflation of the detached membrane by intracellular fluid flow [Cunningham 1995]. Fig. 5.9 shows a series of confocal fluorescence images of a typical GFP-actin expressing HepG2 cell transfected with rHBV on fibronectin-coated coverslip from 5 to 150 minutes after cell seeding. From 5 to 20 minutes, there are small wave-like structures generated around actin cytoskeleton. At 35 minutes, small wave-like structures change into bubble-like structure. It is likely that a large amount of HBV particles attach to cell membrane leading to actin filament polymerization and extension. Also, the intracellular fluid flow causes the inflation of detached membrane to form the bubble-like structure. Furthermore, bubble-like structure grows in number and size progressively and concentrates at two ends of actin cytoskeleton over time. Although the structure is reduced at 130 minutes, actin filament structure still extends outwards. In short, HBV infection severely influences temporal and spatial cytoskeleton rearrangement resulting in cellular morphological change along with cytoskeletal structure. Similar trend has been found on fibronectin-coated substrate.

Fig. 5.8. A series of GFP-actin images of a typical HepG2 cell infected with HBV adhering on collagen-coated substrate from 5min to 150 after cell seeding.

Fig. 5.9. A series of GFP-actin images of a typical HepG2 cell infected with HBV adhering on fibronectin-coated substrate from 5 min to 150 after cell seeding.

As part of our ongoing effort to characterize HBV-host interactions, the proline rich region of HBX was expressed in *E. coli* and purified as a GST fusion protein with an additional His tag. The purified GST-HBV fusion protein was then used to incubate with SH3 array membrane containing various SH3-containing cellular proteins (Panomics). Fig. 5.10 shows that both vinexin β and c-src interacted with the praline rich region of HBX. The interaction with c-src has been previously reported [Klein and Schneider, 1997]. Our data revealed for the first time the interaction between vinexin β and HBX. Vinexin is an adaptor protein that is involved in the process of actin polymerization [Kioka et al., 2002]. Therefore the interaction of HBX with vinexin following HBV replication could significantly reduce vinculin self-assembly necessary for focal complex formation.

Fig. 5.10. Proline rich region of HBX interacts with SH3-containing cellular proteins. The interaction between purified wild type GST-HBX and SH3 array was detected by anti-His antibody. In the upper row, Spots 9 and 10 corresponds to vinexin SH3 domain while spots 11 and 12 contained c-Src. Negative controls provided by manufacturer were included in spots 13 to 18 in the upper row (Spots 13 and 14 is empty GST while spots 15 to 18 are not spotted).

We can quantify the degree of the cell deformation induced by receptor-ligand interaction and explore the influence of HBV infection and GFP-actin expression on the geometric transformation of adherent cells. Fig. 5.11 shows the averaged degree of deformation a/R of HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV, and normal HepG2 cell on fibronectin-coated substrate during cell adhesion. The results indicate that averaged a/R of normal HepG2 cells on fibronectin-coated substrate rapidly increases from 0 to 20 minutes. By determining the initial slope of a/R vs. time plot, an initial cell deformation rate of 0.027 min^{-1} is determined for normal HepG2 cells. Interestingly, the cell deformation rate is reduced by 22 % to 0.021 min^{-1} towards GFP-actin expression, HBV infection or combined GFP-actin expression/HBV infection. Averaged a/R of normal HepG2 cells eventually reaches a steady-state after 50 minutes of cell seeding. Furthermore, HBV infection and/or GFP-actin expression increase the time for approaching steady-state by 70 minutes during the fibronectin mediated adhesion. At a steady-state, averaged a/R for HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV and normal HepG2 cell is 0.85, 0.85, 0.85 and 0.80, respectively.

It is critical to determine the combined effect of HBV infection and receptor-ligand interaction on the biomechanical response of adherent cells. Fig. 5.12 shows the averaged a/R of HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV and normal HepG2 cell on collagen substrate during cell adhesion. The result indicates that either HBV infection or/and GFP-actin transfection increases the lag-time for the detection of significant cell deformation from 5 minutes (for normal HepG2 cells) to 10 minutes (for

the three types of transfected cells). The initial cell deformation rate of HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells and normal HepG2 cells is 0.013, 0.013, and 0.019 min⁻¹. The lowest initial rate of cell deformation of 0.0056 min⁻¹ is found on GFP-actin expressing HepG2 cells transfected with rHBV. The average a/R of normal HepG2 cells reaches a steady state at 70 minutes prior to that of HepG2 cells transfected with rHBV and GFP-actin expressing HepG2 cells (120 minutes after seeding) on collagen substrate. Nevertheless, average a/R of GFP-actin expressing HepG2 cells transfected with rHBV has not reached steady state during the 150 minutes of cell seeding.

Fig. 5.11. The average a/R of HepG2 cells infected with HBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells infected with HBV, and normal HepG2 cell on fibronectin substrate against the time of cell seeding.

Fig. 5.12. The average a/R of HepG2 cells infected with HBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells infected with HBV, and normal HepG2 cell on collagen substrate against the time of cell seeding.

Contact mechanics model of adherent cell has been successfully used to determined adhesion energy of HepG2 cells [Yin et al., 2003; Feng et al 2005]. With the biophysical approaches used in this study, both the adhesion contact area and spreading area are probed experimentally. Our biophysical approaches provide an effective mean in elucidating the effect of HBV infection and cytoskeleton remodeling on the adhesion energy of HepG2 cells. Fig. 5.13 shows the averaged adhesion energy of HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV and normal HepG2 cell against time after cell seeding on fibronectin-coated coverglass. Generally, the averaged adhesion energy for all four types of cells spans five orders of magnitude during the course of cell seeding. After 5 minutes of cell seeding, the averaged adhesion energy of GFP

expressing HepG2 cells infected with HBV is lowest among the four types of cell. The time for the adhesion energy of normal HepG2 cells to reach steady state is 50 minutes. On the other hand, the other three cell types take 90 minutes to reach steady state after cell seeding on fibronectin-coated substrate. Interestingly, the steady-state level of adhesion energy for all four-cell types on fibronectin is around $1 \times 10^{-7} \text{ J/m}^2$.

The change of immobilized ligand may tune adhesion strength of HBV infected cell. Fig. 5.14 shows the averaged adhesion energy of a population of HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV and normal HepG2 cell against time of cell seeding on collagen-coated substrate. In collagen mediated adhesion, the averaged adhesion energy of HepG2 cells under the combined influences of HBV infection and GFP-actin expression spans six orders of magnitude. The results indicate that normal HepG2 cells rapidly achieved averaged adhesion energy of $2.6 \times 10^{-11} \text{ J/m}^2$ at 10 minutes after seeding on collagen-coated substrate. In contrast, GFP-actin expressing HepG2 cells infected with HBV take 30 minutes more to yield the same adhesion energy of normal HepG2 cells at 10 minutes. From 5 to 80 minutes after cell seeding, both HBV infected HepG2 cells and GFP-actin expressing cells attain significantly lower adhesion energy compared to normal HepG2 cells. Moreover, normal HepG2 cells require 70 minutes to approach steady state in adhesion energy. GFP-actin expressing HepG2 cells transfected with rHBV take longest time of about 150 minutes in reaching a steady state of adhesion energy compared with the other three cell types. At steady state, the adhesion energy of all four types of cells approaches the same level of $1.2 \times 10^{-7} \text{ J/m}^2$. The results demonstrate that adhesion energy of cell on ECMs depends on the number and density of bonds of receptor-ligand.

Fig. 5.13. The average adhesion energy of a population of four type cells against time after cell seeding on fibronectin-coated coverslip.

Fig. 5.14. The average adhesion energy of a population of four type cells against time after cell seeding on collagen-coated coverslip.

Fig. 5.15 (A). The $\ln$ - $\ln$ plot of radius of adhesion contact zone against time for the four different type cells on collagen-coated coverslip.

Fig. 5.15 (B) The $\ln$ - $\ln$ plot of radius of adhesion contact zone against time for the four different type cells on fibronectin-coated coverslip.

Several biophysical models have been proposed to simulate the growth of adhesion patch at membrane-substrate interface [de Gennes et al., 2003]. For the established growth law of adhesion patch, $R \sim t^n$, R and t represent radius of adhesion contact zone and time, respectively. Fig. 5.15 shows the ln-ln plot of radius of adhesion contact zone against time for HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, GFP-actin expressing HepG2 cells transfected with rHBV and normal HepG2 cell on fibronectin (A) or collagen (B) coated substrate. Each curve represents the best fit of the data with non-linear least square routine. The slope of the fitted line is the coefficient n . On fibronectin-coated substrate, n of normal HepG2 cell, HepG2 cell transfected with rHBV, GFP-actin expressing HepG2 cell, and GFP-actin expressing HepG2 cell transfected with rHBV is 0.36, 0.68, 0.50, and 0.55, respectively. Also, n of normal HepG2 cell, HepG2 cell transfected with rHBV, GFP-actin expressing HepG2 cell, and GFP-actin expressing HepG2 cell transfected with rHBV is 0.45, 0.58, 0.65 and 0.74, respectively, on collagen-coated substrate. Our results clearly demonstrate the adhesion contact kinetics is dependent on several factors including HBV infection, GFP-actin expression, and immobilized ligand.

5.3 Discussion

It is noticed that hepatocyte cell lines including HepG2 and Huh-7 cells are refractory to HBV infection [Paran et al., 2001]. In this study, the effect of HBV infection on cellular responses upon adhesion has been explored with our recently developed cell-based HBV replication system [Chen et al., 2000]. Briefly, a linearized form of HBV genome is constructed in the mammalian expression vector pcDNA3.1. The linear HBV genome contains the viral promoter at its 5' end and the region for termination of transcription at its 3' end. The ability of this genome in behaving as a

replicative virus after its transfection in HepG2 cells is then measured by the amount of HBsAg particles secreted into cell culture medium two days after its transfection [Onn et al., 2002]. The results suggest that the replicative genome delivered through transfection was able to produce significant amount of HBV particles which in turn infect other HepG2 cells [Toh et al., 2005]. In addition, HepG2 cells infected with HBV form long and thin filopodia which extend out from the cells [Fang et al., 2006]. The filopodia seem to search for a target in the extracellular matrix or surfaces of other cells for adhesion and also provide the driving force for cells to migrate. This implies that actin cytoskeleton projecting out from the HBV infected cell is driven by the dynamic actin reorganization process. This result suggests that our investigation of HBV infection of HepG2 cells is effectively carried out with our cell-based replication system, and therefore the transfection is chosen as the standard technique in this study. Based on the same rationale, results of our investigations are reported and interpreted in the context of HBV infection.

Cell-spreading on model ECM depends not only on the affinity of receptor-ligand interaction but also on cytoskeleton rearrangement [Nicolas et al., 2004]. For instance, HepG2 cells transfected with rHBV spread more efficiently than normal HepG2 cells on collagen-coated substrates although the adhesion of all four types of cells is mediated by the interaction between $\alpha_1\beta_1$ & $\alpha_2\beta_1$ integrin on HepG2 membrane and the collagen-coated substrate. The interaction mediates cell spreading has been proven by Koivisto et al. [Koivisto et al., 1999]. Moreover, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins belong to members of the integrin and are two of the best-known collagen receptor [Nicolas et al., 2004]. Also, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins on HepG2 cell membrane have been verified by Yang et al [Yang et al., 2003]. In our experiment, HepG2 cells are seeded on collagen-

coated substrate, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins on HepG2 cell recognize and bind collagen, which result in HepG2 cell adhering to the surface and spreading on collagen substrate. It means that upon the binding of the extracellular matrix proteins to integrins, intracellular protein kinases are activated leading to the activation of signaling pathways. This has been confirmed by the increase of percentage change of 2-D spread area for normal HepG2 cells from 73% to 98% upon HBV infection. It is possible that HBV infection affects the signal transduction cascades of receptor mediated adhesion of HepG2 cells on collagen. The result is likely correlated with HBV induced gene expression in HepG2 cells. In particular, HBX protein resulted from HBV infection stimulates nuclear factor κB , which has been shown to induce fibronectin gene expression at the promoter level [Norton et al, 2004]. The process leads to the synthesis and deposition of fibronectin. This in turn should contribute to the uplifted synthesis and accumulation of fibronectin on the substrate and the enhanced spreading of HBV infected HepG2 cell. Interestingly, HBV infection leads to the reduction in the degree of spreading of GFP-actin expressing cell on collagen-coated substrate as shown in Fig. 5.2A. The result is likely caused by the intricate interplay between HBV induced gene expression and the alteration of cytoskeleton induced by GFP-actin substitution. In addition, cells also decrease the expression of β_1 integrin subunits after HBV infection [Laza-Pezzi et al., 2001]. The reduction of the extent of spreading for GFP-actin expressing cells transfected by rHBV on collagen is attributed to HBV infection which apparently impairs the affinity between integrin with β_1 subunits and collagen. On the other hand, GFP-actin expressing HepG2 cell with HBV infection possibly has no/less synthesis and deposition of fibronectin on collagen-coated substrate after seeding during short time.

In contrast, the change of cell morphology for the four cell types on fibronectin is more apparent than that on collagen. The distinction of the cell-spreading behavior induced by HBV infection and/or GFP-actin expression becomes more blur. The trend as mentioned above is likely caused by the change of the nature of ligand-receptor interaction. In particular, it has been shown that integrin $\alpha_5\beta_1$ and fibronectin form a prototypic integrin-ligand pair, which mediates fibronectin fibril formation and governs extracellular matrix assembly that is vital to cell function *in vivo* [Hynes and Yamada, 1982]. Apart from the RDG sequence present in fibronectin, there is a set of residues referred to as the synergy site in fibronectin which also contributes to the high-affinity recognition by integrin $\alpha_5\beta_1$ [Takagi 2004; Cukierman et al., 2001]. Those factors as mentioned above help to elucidate why HBV infection promotes the extensive spreading of HepG2 cells and GFP-actin expressing HepG2 cells on fibronectin-coated substrate.

The adhesion of HepG2 cells on a model ECM or biomimetic scaffold is important in maintaining the resulted cellular functions. The formation of adhesion contact for HepG2 cells transfected with rHBV, GFP-actin expressing HepG2 cells, and GFP-actin expressing HepG2 cells transfected with rHBV on collagen substrate are delayed compared with that of normal HepG2 cell. The organization of the eukaryotic cell functions relies on the assembly and maintenance of a filamentous protein network called the cytoskeleton. This network extends throughout the cytosol and is connected to the cell plasma membrane. The cytoskeleton is composed of three different types of fibers: microtubules, intermediate filaments, and microfilaments [Carraway and Carraway, 1989] and form a complex dynamic structure, which responds to intracellular and extracellular stimuli. Microfilaments, which are important for cell movement, are

composed of polymerized actin. Actin polymerization occurs beneath the cell surface and creates various tridimensional structures, depending on the nature of the associated actin-binding proteins [Bretscher 1991], transmembrane cadherins and a complex formed of catenin, plakoglobin, and vinculin [Geiger and Ginsberg, 1991]. Bundles of actin filaments are linked to these complexes of attachment proteins through α -actinin. Because some of their protein components are kinases, focal contacts also participate in cell signaling during cell adhesion [Ridley and Hall, 1994].

Integrin-mediated cell adhesion triggers the formation of focal adhesion complex through the association with actin cytoskeleton and subsequent clustering. In detail, focal adhesion sites compose of cytoskeletal proteins such as vinculin, talin, and α -actinin, and signaling molecules, including FAK, Src, and paxillin [Wehrle-Haller and Imhof, 2002]. The focal adhesion formation is playing a critical role in the cell adhesion by serving as structural links between the cytoskeleton and ECM. In our study, the confocal fluorescence images of normal HepG2 cells, HepG2 cells transfected with rHBV and HepG2 cells transfected with the empty plasmid stained with vinculin at 20 and 50 minutes after cell seeding on collagen are shown in Fig. 5.5 (A) and 5.5 (B). The result not only display the lag time of the formation focal complexes of HepG2 cells transfected with rHBV during initial cell seeding but also support the transient effect of HBV replication on the adhesion energy and degree of deformation during the initial time of cell seeding on collagen substrate. In particular, the formation of focal complexes serves as a traction base for cell movement, anchor point at the cell-cell connection, and as a cement during morphogenesis [Cohen et al., 2004]. Our results were likely caused by the interaction of HBxAg with the C-terminal region of vinexin β containing one SH3 domain, which is an adaptor protein that is involved in the process

of actin polymerization [Kioka et al., 2002]. Moreover, the expression of vinexin β was found to enhance the formation of focal adhesion and cell spreading in 3T3 fibroblasts [Kioka et al., 1999]. Therefore the disruption of vinexin following HBV replication could significantly reduce vinculin self-assembly necessary for focal complex formation. The formation of focal adhesion in HBV replicating HepG2 cells is likely caused by the integrin-collagen recognition and the subsequent cytoskeletal reorganization.

In HBVPathDB database, HBV infection-related molecular interaction network contains 20 types of molecules including of focal adhesion kinases (FAK). These molecules involve in a series of physiological and biochemical reactions after HBV infects host cell [Zhang et al., 2005]. Thus the impairment of adhesion contact formation upon HBV infection is likely linked to the altered activity of FAKs that are induced by ligand binding to various adhesion receptors. Moreover, HBX expression induces the activation of FAK, which in turn triggers SRC and HBV DNA replication [Bouchard et al., 2001]. In turn, FAKs are known to target paxillin and serve as substrates for Src family kinases [Calalb et al., 1995]. These kinases contain phosphotyrosine in growing BALB/c3T3 cells but little or no phosphotyrosine is found in cells detached by trypsinization [Andre and Becker-Andre, 1993]. The tyrosine-phosphorylated state is regained within minutes when the cells are replated and anchored onto fibronectin or collagen substrates. Our results strongly suggest that HBV infection and GFP-actin expression could dampen the affinity of integrin-ligand interaction by affecting the activity of FAKs. From a broader perspective, our results may have provided the direct evidence on the link between HBV infection and the biophysical behavior of HepG2 cells which are a liver cancer model.

Cytoskeleton indirectly tethers to intracellular domain of adhesion receptor via accessory proteins in order to increase adhesive interaction through the stimulation of the mechanical force [Cohen et al., 2004]. In order to gain a better understanding of the organization dynamics of the actin cytoskeleton in HepG2 cell in response to HBV infection, we have visualized the actin organization in live HepG2 cells. The structural dynamics of cytoskeleton of GFP-actin expressing HepG2 cell on collagen demonstrates the significant delay of lamellipodium evolution. The actin cytoskeleton of GFP-actin expressing HepG2 cell changes from round to asymmetric shape through the formation of small lamellipodium towards the later stage of cell seeding. In contrast, HBV infection rapidly promotes GFP-actin expressing HepG2 cells to develop lamullipodium around cell edge via actin cytoskeleton reorganization. Also, bubble-like structures of actin microfilament distributed at the periphery of cell are closely associated with the secretion process of progeny HBV particles from HepG2 cells, which is similar to pancreatic acinar cell secretion [Muallem et al., 1995].

In addition to the small GTP-binding proteins of the Rho subfamily [Hall 1994], adaptor proteins generally consist of two or more protein-protein interacting modules. For example, the SH2 and SH3 containing Grb2 protein play a key role in the regulation of the complex organization of polymerized actin [Tari and Lopez-Berestein, 2001]. One of such adaptor proteins is vinexin β which is composed of three SH3 domains [Kioka et al., 2002]. From the Panomics SH3 Array II result, HBX-proteins which are formed during HBV infection are found to directly interact with vinexin β . Recent evidence has indicated the diverse regulatory roles of vinexin β , from cytoskeleton reorganization via its binding to vinculin, to growth factor induced signal transduction via its binding to Sos [Mandai et al., 1999; Akamatsu et al., 1999]. The molecular

interplay of intracellular proteins as mentioned above further supports our observed modulation of adhesion contact dynamics and cytoskeleton remodeling following HBV infection.

In this study, the degree of cell deformation reflects the biophysical characteristics of cell population adhering on the ligand-coated substrate. Our results show that the kinetics of cell deformation is dependent on HBV infection, cytoskeleton structure, focal adhesion formation and receptor-ligand interaction. The initial rate of cell deformation of HepG2 cell is reduced by the same magnitude immediately after HBV infection and/or GFP-actin expression on fibronectin-coated substrate. Interestingly, the same parameter is minimized by combined GFP-actin expression and HBV infection towards collagen-coated substrate. The result strongly implies that the biophysical responses of cells are dynamically governed by both ligand affinity and cellular properties. Cell adhesion to ECM or biomaterials is a dynamic process. The complex dynamical response of the adhesion energy between cell and ECM or biomaterial has been measured [Feng et al., 2005; Tan et al., 2005]. Our result indicates that HBV infection or/and GFP-actin expression reduce the initial adhesion energy compared with that of normal HepH2 cell, regardless to the seeding on collagen or fibronectin substrates. HBV infection obviously delays cell adhesion to collagen- or fibronectin-coated substrate. It is expected that HBV infection may influence integrin-mediated cell adhesion to collagen or fibronectin substrate through the reduction of β_1 integrin subunit expression and cytoskeleton rearrangement [Laza-Pezzi et al., 2001]. Also, GFP-actin expressing HepG2 cells display the delay of cell deformation and adhesion energy to collagen or fibronectin substrate. It is possible that GFP-actin expression influence the interaction of receptor and ligand. Moreover, confocal

fluorescence microscope images of GFP-actin expressing HepG2 cells and GFP-actin expressing HepG2 cells transfected with rHBV show reorganization of actin microfilaments. Morphological alteration on GFP-actin expressing HepG2 cells is obviously attributed to HBV infection.

Adhesion contact area of cell is one of critical biophysical parameters. Accumulation of the experimental data has driven the development of a number of mathematical models [Zhu et al., 2002]. The construction and application of these models including kinetic, thermodynamic, and mechanical models enable the analysis of cellular processes by highly quantitative approaches. Kinetic model of cell adhesion as a process driven by the reaction between receptor and ligand molecules has been developed during the last two decades [Bell 1978]. It has been shown that adhesion contact area and the bond distribution of receptor-ligand pair are modulated during the receptor mediated signaling cascades [Grakoui et al., 1999]. The growth law of adhesion patch on a planar substrate has been shown as $R \sim t^n$ [de Gennes et al 2003]. The magnitude of coefficient n is correlated to the rate of ligand-receptor interaction between cell and substrate. On collagen-coated substrate, our experimental results imply that the cell adhesion kinetics moves from intermediate reaction rate (in normal HepG2 cells) to slow reaction rates on the sticky cell surface upon HBV infection or/and GFP-actin expression. On fibronectin-coated substrate, the cell adhesion kinetics of normal HepG2 cells fits to the model intermediate reaction rate on the sticky cell surface. GFP-actin expression in the presence or absence HBV infection leads to the emergence of slow reaction rate between HepG2 cells and fibronectin. Interestingly, HBV infection in normal HepG2 cells leads to the most significant change of adhesion kinetics from a sticky cell with fast binding kinetics to that with slow binding kinetics

on fibronectin [de Gennes et al., 2003]. Overall, we demonstrate that the kinetics of ligand-receptor binding on cell surface is governed by intricate interplay between HBV infection, cytoskeleton alteration, ligand property, etc.

5.4 Conclusion

In this study, we have successfully combined biophysical techniques based on confocal microscopy with transfection technique to investigate the effect of HBV infection on the adhesion contact dynamics of HepG2 cell on model ECMs. Our result indicates that HBV infection affects the reorganization of actin microfilament and the interaction of HBX with vinexin β leading to alteration of cell morphology. Intricate interplay between HBV infection, cell deformation, adhesion dynamics and adhesion contact kinetics has been revealed herein. Our results indicate that HBV infection adversely affects cell spreading and adhesion processes of normal HepG2 cells on collagen and fibronectin substrates. Also, during early stage of cell seeding, only exogenous vinculin is detected in cells infected by HBV while vinculin-associated focal complexes were found. In addition, the adhesion energy of such HBV-infected cells is smaller than that of normal HepG2 cells. Finally, the trend of adhesion kinetic coefficient n indicates that the adhesion kinetics is transformed from intermediate reaction rate on the stick cell surfaces of normal HepG2 cells to other reaction regimes upon HBV infection or/and GFP-actin expression. Our results have provided the direct biophysical and molecular evidence on the link with HBV infection, focal adhesion formation and cytoskeleton remodeling.

Chapter 6: Effect of Cytoskeleton Disruption on the Adhesion Recession Dynamics of HepG2 cells from Extracellular Matrix

6.1 Introduction

The integrity of cytoskeleton plays pivotal roles in the regulation of physiological functions including cell adhesion, proliferation, differentiation, immune response, and migration [Giancotti and Ruoslahti, 1999]. Upon the receptor-ligand recognition on cell surface, actin filaments directly linked to the intracellular domain of adhesion receptor participate in mechanochemical signal transduction of most anchorage-dependent cells [Martin et al., 2002]. Several studies have shown that common drugs including latrunculin A (Lat-A), cytochalasin D (Cyto-D) and doxorubicin (Dox) lead to significant modification in the organization of cytoskeletal component, e.g., actin microfilaments of adherent cells [Mundy et al., 2002; Nemeth et al., 2004; Colombo et al., 1990].

Lat-A is an inhibitor of actin polymerization through the specific binding with actin monomers [Spector et al., 1983]. Lat-A has been shown to cause the depolymerization of F-actin and reduction of number of actin microfilament. The exact binding site on actin for Lat-A has not been identified, yet some studies indicate that Lat-A specifically binds to a region within or adjacent to the nucleotide-bind clef of actin [Aysough et al., 1997; Belmont et al., 1999]. Generally, Lat-A dictates the intricate dynamics of actin polymerization/depolymerization through the modulation of the gene expressions of actin and possibly other attachment proteins [Spector et al.,

1989]. For instance, Lat-A inhibits the binding between thymosin β_4 , an actin-binding protein, and actin by reducing the flexibility of the adenine nucleotide-binding cleft and by prohibiting nucleotide exchange between the two proteins [Yarmola et al., 2000]. Moreover, depolymerization of the actin filaments induced by Lat-A triggers yeast to up-regulate ACT1 expression, which encodes actin [Allen 2000].

Similar to Lat-A, Cyto-D has been shown to influence the kinetics of actin polymerization and the aggregation of actin within the cytosol. Cyto-D is fungal alkaloid that binds to the plus end of actin monomers, prevents further addition of G-actin, and disrupts the cytoskeletal microfilaments [Flanagan and Lin, 1980; Neweth et al., 2004]. However, Lat-A and Cyto-D induce actin-depolymerization with distinct mechanisms. Generally, latrunculin-A sequesters monomeric actin, while Cyto-D binds the barbed ends of actin filaments. The addition of Cyto-D to living cells not only obstructs cell locomotion and cytokinesis activity [Ohmori et al., 1992] but also regulates gene expression [Lavagnino and Arnoczky, 2005]. For example, Cyto-D exerts pressure on the microfilaments of HeLa cells and quiescent WI-38 cells and results in the increase of the concentrations of the c-fos protooncogene and c-fos mRNAs [Zambetti et al., 1991]. The study strongly indicates that the cytoskeleton network is linked to the cell nucleus, which selectively adjusts gene expression in response to biochemical factors. The mechanism of Cyto-D induced disruption on cells is influenced by several experimental factors including Cyto-D concentration [Isowa and Liu, 2001; Wakatsuki et al., 2000]. For example, there is a threshold of Cyto-D concentration for effective actin depolymerization.

Dox used in cancer chemotherapy is an anthracycline antibiotics produced by the fungus *Streptomyces peucetius*. It can intercalate between DNA base pairs, chelate

metal ion, or generate free radical resulting in DNA damage. It inhibits DNA topoisomerase II, which directly regulates DNA transcription [Haskell 1990]. Moreover, proteasome is a specific translocator of Dox from the cytoplasm to the nucleus [Kiyomiya et al., 2001]. The nuclear transport of Dox-proteasome complex is regulated by an ATP-dependent nuclear pore-mediated mechanism. Also, Dox enhances the transduction of recombinant adeno-associated virus serotype 2 in gene therapy [Tang et al., 2005]. Most importantly, Dox negatively affects actin polymerization as demonstrated by the inhibition of filament growth and reduction of mass and size of actin filament at steady-state in an ion-dependent manner [Colombo et al., 1984; Colombo et al., 1990; Colombo et al., 1988].

Until now, relatively little is known on the correlation between the cytoskeleton integrity and the biophysical responses for cell on model ECMs under the influence of the cytoskeleton disruptive agents. In this study, we seed HepG2 cells on collagen-coated substrate until they reach steady-state in cell spreading and adhesion. Then confocal reflection interference microscopy (C-RICM) in conjunction with phase contrast microscopy and confocal fluorescence microscopy is applied to investigate the adhesion recession dynamics of HepG2 cell under the influence of Lat-A or Cyto-D or Dox. In general, it is demonstrated that cell morphology, the kinetics of adhesion contact recession, and change of adhesion energy are dependent on the drug types and concentrations. Interestingly, only Lat-A induces oscillation in the degree of deformation and adhesion energy at different concentrations. Furthermore, clear evidence on the role of actin polymerization in the regulation of the biophysical signaling cascades of adherent cell is demonstrated herein.

6.2 Results and discussions

Cell-spreading on ECM or biomaterial depends on the interaction between cell receptor and immobilized ligands. Generally, receptor is closely associated with cytoskeleton, which transmits traction and contraction forces generated from cell to the ECM or biomaterial [Wakatsuki et al., 2000]. In addition, cell attaching to extracellular matrix through integrin spreads on the matrix, eliciting cellular signals needed for survival. Phase contrast microscopy is first applied to probe the degree of cell spreading upon receptor-mediated adhesion on ligand-coated surface under the influence of the three drugs. It is important to access the morphology of HepG2 cells before the drug disruption assay. Fig. 6.1 shows the phase contrast images of normal HepG2 cells on collagen-coated substrate at 5 and 120 minutes after cell seeding. The result indicates that normal HepG2 cells readily attach to collagen-coated substrate and most HepG2 cells retain their round shapes after seeding for 5 minutes. As time lapses, HepG2 cells progressively spread and form small lamellipodium evenly distributing at the periphery of cell membrane as shown in the image of 120 minutes. In the lamellipodium, actin nucleation and polymerization of HepG2 cells drive its assembly at the peripheral cell membrane as shown in Fig 6.15. At the cell membrane, protein complexes such as Arp2/3, WASP/Scar family, Ena/VASP family enhance actin nucleation and polymerization [Mullins et al., 1998; Ku et al., 1999]. Therefore, actin assembly powers HepG2 cell edge extension. In addition, the spreading of HepG2 cell is quite different from that of fibroblast whose actin nucleation and depolymerization proteins drives assembly at the front and disassembly at the rear, leading to cellular motility [Pantaloni et al., 2001. Giannone et al., 2004. Wolgemuth 2005]. Moreover, the degree of cell-spreading of normal HepG2 is not significant compared to other cell

types like fibroblast and smooth muscle cells as shown by other studies [Pollard and Borisy, 2003]. Actually, the process of cell-spreading on collagen-coated substrate involves integrin-collagen recognition [Camper et al., 1997], signal transduction [Seller and Hart, 2000], as well as reorganization of cytoskeleton [Muller et al., 2005]. Also, the spreading of HepG2 cells on collagen substrate causes tension-dependent changes in cell shape that is closely linked to gene expression, growth regulation, and survival [Chen et al., 1997]. Basically, cell-spreading is dependent on the regulation of actin filament, focal contact dynamics, and force generation.

Fig. 6.1. Phase contrast images of HepG2 cells on collagen substrate at 5 and 120min after cell seeding.

In order to investigate the influence of Cyto-D, Lat-A and Dox on cell morphology and cell-spreading, they are separately added to adherent HepG2 cells around 120 min after seeding on collagen-coated substrate. Considering that Dox is one kind of chemotherapy drugs, and Lat-A & Cyto-D at 5 μ M or 10 μ M concentration disrupt microfilament organization [Spector et al., 1989 and Peterson et al., 1999], the two concentrations for the three drugs are selected in this study. Fig. 6.2 shows the phase contrast images of HepG2 cell at 2 and 120 minutes after the addition of Dox at 5 or 10 μ M into HepG2 cells adhering on collagen-coated substrate. After two minutes of 5 μ M Dox incubation, there is no significant change of morphology of HepG2 cells in comparison with that of untreated cells (Fig. 6.1). After 120 minutes of Dox addition into HepG2 cells, the size of lamellipodium at the cell periphery is significantly reduced. At the same time, cells become round in shape with smooth contour. At higher concentration of Dox (10 μ M), the size of lamellipodium is rapidly reduced towards 2 minutes of Dox treatment. Two hours of 10 μ M Dox treatment induce significant round-up of the cells. It is evident that Dox restrains HepG2 cell-spreading compared with normal HepG2 seeding on collagen substrate without addition of Dox. The results demonstrate that Dox addition of HepG2 induces reorganization of actin filament dependent on concentration of doxorubicin, which leads to lamellipodium change. Therefore significant changes in morphology of the HepG2 cells and in proteins distribution occur after adding Dox at 5 and 10 μ M. The impairment observed in the actin filaments after adding Dox in Fig.6.16 is likely to cause HepG2 cell dysfunction, which affects tension development and favors cell shrinkage [Chen and Chien, 1999]. In addition, Dox is known to be a chemotherapy drug that causes the damage of DNA [Yan et al., 2004], while it affects actin polymerization that is directly associated with cell spread and morphology [Colombo et al., 1984]. It is possible that cell-spreading is

correlated with the redistribution of the response of cytoskeletal components against the addition of Dox [Molnari et al., 1990].

Fig. 6.2. The phase contrast images of HepG2 cell on collagen substrate at 2 and 120 min after adding Dox of 5 and 10 μ M.

The properties of drug likely lead to different cellular responses and change cellular morphology. Fig. 6.3 shows the phase contrast images of HepG2 cells on collagen-coated substrate after the addition of Cyto-D (5 and 10 μ M) at 2 and 180 minutes of incubation. At both concentrations of Cyto-D, HepG2 cells form large numbers of small cystic vacuoles at the periphery of the cell membrane. At lower concentration of Cyto-D, there is no significant change of cell morphology from 2 to 180 minutes of drug incubation. On the other hand, prolonged exposure of HepG2 to higher concentration of Cyto-D (10 μ M) leads to change in morphology including cell

round-up and development of large cystic vacuoles. The result is consistent with that of normal rat fibroblast and entamoeba invaders exposed to Cyto-D [Brett and Godman 1986; Makioka et al., 2004]. It is likely that the formation of cystic vacuoles is related to the chemical structure specific for Cyto-D, which has the central perisohydroindole core rings [Makioka et al., 2004]. Also, the phenomenon is associated with the mechanism of Cyto-D interaction with actin and the concentration of Cyto-D. Since Cyto-D binds to the barbed end of the actin filament with high affinity and inhibits both association and dissociation of monomers at this end [Flanagan and Lin 1980]. Cyto-D at higher concentration binds two actin monomers and promotes the hydrolysis of the bound ATP, generating low affinity ADP-actin and a consequent important rise in the critical concentration which suppresses cell spreading or extension [Goddette and Frieden 1986]. On the other hand, the formation of structure is possible due to flow of fluid within cell after HepG2 cell treatment of Cyto-D [Cuvingham 1995]. Fig. 6.4 shows the phase contrast images of HepG2 cells on collagen-coated substrate at different times after the addition of Lat-A (5 and 10 μM). At lower concentration (5 μM) of Lat-A, small cystic vacuoles rapidly form at the periphery of the cell after 2 minutes. Generally, the cell edge retracts during the three hours of Lat-A incubation. Interestingly, there is certain degree of heterogeneity among the cell population as shown by the progressive spreading of some cells against incubation time on the surface (indicated by arrows). The result implies that lower concentration of Lat-A does not inhibit actin polymerization of certain cells. Moreover, the heterogeneity as mentioned above is not observed at a higher Lat-A concentration. At higher Lat-A concentration (10 μM), the most significant change in cell morphology is detected after two minutes of incubation as shown by the formation of numerous budding membrane structures all over the cell membrane. At later stage of Lat-A incubation, the number of the budding

structures is significantly reduced. The results demonstrate that the influence of Lat-A on cell spreading on collagen depends on the concentration of drug. It is because Lat-A adding into HepG2 cell not only sequesters actin monomer but also inhibits nucleotide exchange and actin polymerization [Giganti and Friederich 2003]. Furthermore, Lat-A might modulate the activity of monomer-binding proteins such as thymosin β 4 and profilin that are frequently associated with increased cell motility [Yarmola et al., 2000; Clark et al., 2000]. In addition, the budding structure represents ballooning out of the plasma membrane, as shown in Fig. 6.4. It is possible that the fluid-driven expansion of the cell membrane is sufficiently rapid to initially outpace the local rate of actin polymerization [Cunningham, 1995]. The results for all three disruptive or suppressive reagents as mentioned above strongly indicate that Dox, Cyto-D and Lat-A impair integrin-collagen interaction and lead to the retraction of originally spread cells.

Fig. 6.3. The phase contrast images of HepG2 cells on collagen substrate at 2 and 180 min after adding Cyto-D of 5 and 10 μ M.

Fig. 6.4. The phase contrast images of HepG2 cells on collagen substrate at 2 and 180 min after adding Lat-A 5 and 10 μM . The arrowed cells still spread on collagen after adding Lat-A of 5 μM .

Confocal-reflection interference microscopy (C-RICM) has been proved as an effective tool for probing the contact mechanism of model biomaterial on non-deformation substrate and for elucidating the effect of subtle molecular interactions on the bio-adhesion energy [Chan and Wan 2002]. The application of C-RICM has been extended to investigate the adhesion dynamics of cells on ligand-coated substrate *in situ* during the initial stage of cell seeding [Yin et al., 2003]. In this study, it is hypothesized that Dox, Cyto-D and Lat-A would influence the cell adhesion dynamics on ECM via

the disruption or suppressive of actin microfilaments and subsequent signal transduction and interaction of receptor and ligand. The adhesion contact dynamics of HepG2 cells seeding on collagen-coated surfaces after drug incubation is explored by using novel technique of C-RICM. Fig. 6.5 shows a series of C-RICM images for a typical HepG2 cell on collagen substrate after the addition Dox at concentration of 5 μM (A) and 10 μM (B). From the C-RICM images, strong adhesion contact between the cell membrane and collages substrate appears as dark region due to the destructive interference of the two reflected laser beams [Fang et al., 2002]. Before 5 μM Dox addition, the adhesion contact area of the cell is 246.60 μm^2 . Immediately following 2 minutes of Dox incubation, the adhesion contact area is reduced by 15 %. From 2 to 120 minutes after Dox incubation, the adhesion contact area of the cell is reduced to 197.29 μm^2 . The % change of cell adhesion area is increased to 48% from 20% against the increase of Dox concentration. Fig. 6.6 shows a series of C-RICM images for a typical HepG2 cell on collagen substrate after the addition Cyto-D at concentration of 5 μM (A) and 10 μM (B). From 0 to 180 minutes after addition of 10 μM Cyto-D, the adhesion contact area of HepG2 cell is reduced to 70 μm^2 from 210 μm^2 . The percentage of cell adhesion area is 67% apparently higher than 40% that is the percentage of cell adhesion area of HepG2 cell induced by 5 μM of Cyto-D incubation from 0 to 180 minutes. Fig. 6.7 shows a series of C-RICM images for a typical HepG2 cell on collagen substrate after the addition Lat-A at concentration of 5 μM (A) and 10 μM (B) at different time. The percentage change of cell adhesion contact area is consistent about 10%, regardless of concentration of Cyto-D at 5 or 10 μM adding HepG2 cells from 0 to 2 minutes. Furthermore, addition of 10 μM of Cyto-D into HepG2 cells lead to the reduction of adhesion contact area from 456.73 μm^2 to 229.37 μm^2 from 0 to 180 minutes. It is evident that the reduction of contact area is higher than

which contact area of HepG2 cell from 451.49 μm^2 to 336.32 μm^2 is caused by 5 μM Cyto-D incubation during the time. The results indicate that Dox, Cyto-D, and Lat-A diminish adhesion contact area of HepG2 cell on collagen substrate over time. Moreover, the three types of drugs at high concentration further enhance the ability of decrease of adhesion contact area.

Fig. 6.5. A series of C-RICM images of a typical HepG2 cell during initial seeding on collagen after adding Dox 5 μM (A) and 10 μM (B).

Fig. 6.6. A series of C-RICM images of a typical HepG2 cell during initial seeding on collagen after adding Cyto-D 5 μ M (A) and 10 μ M (B).

Fig. 6.7. A series of C-RF images of a typical HepG2 cell during initial seeding on collagen substrate after adding Lat-A 5 μ M (A) and 10 μ M (B).

Fig. 6.8. The average percentage change of 2-dimensional spreading area for a population of HepG2 cells after addition of (5 μ M and 10 μ M) Dox, Lat-A and Cyto-D from 0-2 hours (for Dox, Student's *t*-test: $p<0.0057$) and 0-3 hours (for Lat-A and Cyto-D, Student's *t*-test: $p<0.0036$ and 0.01, respectively).

Cell deformation reflects biomechanical transformation of cell through the receptor-ligand interaction, cytoskeleton reorganization, signal transduction, etc. In this study, the percentage change of two-dimensional spreading area can be obtained after cell spreading area is measured by using an image analysis software under addition of Dox, Cyto-D and Lat-A. Fig. 6.8 shows the average percentage change in the 2-dimensional spreading area for a population of HepG2 cells addition of (5 μ M and 10 μ M) Dox, Lat-A and Cyto-D from 0-2 hours (for Dox) and 0-3 hours (for Lat-A and Cyto-D). The average percentage change of 2-D spread area for HepG2 cells after addition of 5 μ M and 10 μ M of Dox is -20.9% and -23.92%, respectively, after 2h of cell seeding (Student's *t*-test: $p<0.0057$). The average percentage change of 2-D spread area for HepG2 cells after addition of 5 μ M and 10 μ M of Lat-A is -10.5% and -

11.63%, respectively, after 3h of cell seeding (Student's *t*-test: $p < 0.0036$). The average percentage change of 2-D spread area for HepG2 cells after addition of 5 μM and 10 μM of Cyto-D is -15.4% and -18.36%, respectively, after 3h of cell seeding (Student's *t*-test: $p < 0.01$). The results evidently display the reduction of cell spreading on collagen substrate after addition of the three-drugs. In comparison with addition of low concentration (5 μM) of the three-drugs, we can see the reductions of adhesion contact area and percentage change of 2-D spread area are more apparently enhanced by the higher concentration (10 μM) of the three-drugs.

Fig. 6.9. The average cell deformation of a population HepG2 cells against time after adding Dox.

Based on the two dimensional spread area and adhesion contact area from phase contrast microscopy and C-RICM, the degree of cell deformation (a/R) of HepG2 cells towards the addition of Dox, Cyto-D and Lat-A can be determined from our unique

biophysical approach. Fig. 6.9 shows averaged a/R against time after Dox addition for a population of HepG2 cells adhering on collagen-coated substrate. During the initial ten minutes of Dox incubation, the averaged a/R of HepG2 cells after addition of 5 μM of Dox decreases more rapidly than that of Dox 10 μM . Nevertheless, average cell deformation is equal at 60 minutes after addition of 5 and 10 μM of Dox, which is 0.67. From 60 minutes onward, averaged a/R at higher Dox concentration (10 μM) is lower than that at lower Dox concentration (5 μM). After two hours of Dox incubation, a/R reaches 0.64 and 0.62 at 5 μM and 10 μM , respectively. Fig. 6.10 shows averaged a/R against time after Cyto-D addition for a population of HepG2 cells adhering on collagen coated substrate. In contrast to the result of Dox incubation, the value of a/R for HepG2 cells at Cyto-D concentration of 10 μM is lower than that of CytoD concentration of 5 μM during the initial ten minutes of Cyto-D incubation. The value of a/R is 0.70 and 0.67 for HepG2 cell after addition 5 and 10 μM Cyto-D, respectively. At 180 minutes after addition of Cyto-D, average a/R is reduced to 0.51 from 0.58 against increment of Cyto-D concentration. Apparently, percentage change in the reduction of a/R over the 3 hours of Cyto-D incubation is from 23% to 32% at 5 μM and 10 μM Cyto-D.

Fig. 6.11 shows the average a/R of a population HepG2 cells seeding on collagen substrate against time after addition of Lat-A at concentrations of 5 and 10 μM . The overall trend demonstrates that the average a/R at Lat-A concentration of 10 μM is larger than that of 5 μM . It is obvious that the temporal behavior of a/R for HepG2 cells caused Lat-A addition significantly differs from that induced by Dox and Cyto D. First of all, the initial rate of reduction of a/R for HepG2 induced by Lat-A of 5 and 10 μM is -0.013 min^{-1} and 0.003 min^{-1} lower than 0.030 min^{-1} and 0.011 min^{-1} & 0.010 min^{-1} and 0.025 min^{-1} of initial rate of reduction of a/R for HepG2 cells induced by

Dox and Cyto-D of 5 and 10 μM , respectively. Secondly, the overall reduction of a/R against the entire period of Lat-A of 5 and 10 μM incubation is 5.5% and 6.8%, which is clearly lower than that under Cyto-D of 5 and 10 μM incubation. The lower extent of a/R reduction under Lat-A perturbation observed herein orchestrated the phenomena that some HepG2 cells have strong resistance against Lat-A in cell edge recession under phase contrast microscopy. Simultaneously, paired Student's t -test is used to compare the two sets of data of the three drugs. For Dox, Student's t -test: $p < 0.006$, 0.026, 0.039, and 0.009 at 20, 60, 90, and 120min, respectively. For Cyto-D and Lat-A, Student's t -test: $p < 0.001$, 0.003, 0.002 and 0.005 & $p < 0.012$, 0.032, 0.016, and 0.04 at 20, 80, 140, and 180min, respectively. It is obvious that the statistical significance is displayed in the comparison between HepG2 cells after adding these drugs at 5 and 10 μM by applying Student's t -test. In short, the results indicate complex interplay between drug property, drug concentration and biomechanical responses.

Fig. 6.10. The average cell deformation of a population HepG2 cells against time after adding Cyto-D.

Fig. 6.11. The average cell deformation of a population HepG2 cells against time after adding Lat-A.

Contact mechanics model can be used to calculate cell adhesion energy through a/R and elastic modulus [Wan and Liu, 2001]. It is useful to access the effect of ligand-receptor recognition on the adhesion energy of HepG2 cells seeding on collagen-coated substrate after drug incubation. Fig. 6.12 shows averaged adhesion energy against time after Dox addition for a population of HepG2 cells adhering on collagen-coated substrate. Addition of 5 μM Dox into firmly adherent HepG2 cells immediately results in the reduction of adhesion energy and the reduction rate of adhesion energy is faster than the addition of 10 μM Dox during initial 10 minutes. However, 60 minutes after addition of Dox, the adhesion energy induced by 10 μM of Dox is lower than that by 5 μM of Dox. During the entire period of Dox incubation, the adhesion energy is reduced by 73.6% and 77.9% at 5 μM and 10 μM of Dox after 2 hours (Student's t -test:

$p < 0.007$), respectively. Fig. 6.13 shows averaged adhesion energy against time after Cyto-D addition for a population of HepG2 cells adhering on collagen-coated substrate. The reduction of adhesion energy caused by addition of 10 μM Cyto-D is always higher than by addition of 5 μM Cyto-D over time. During the entire period of Cyto-D incubation, the adhesion energy is reduced by 80% and 90% at 5 μM and 10 μM of Cyto-D after 3 hours (Student's t -test: $p < 0.002$), respectively. Fig. 6.14 shows averaged adhesion energy against time after Lat-A addition for a population of HepG2 cells adhering on collagen-coated substrate. In comparison with the results of Dox or Cyto-D addition, big fluctuation in adhesion energy emerges during the entire period of Lat-A incubation. The general trend of adhesion energy is that the reduction of adhesion energy against time induced by Lat-A of 5 μM is smaller than by Lat-A of 10 μM . During the entire period of Lat-A incubation, the adhesion energy is reduced by 23% and 29% at 5 μM and 10 μM of Lat-A after 3 hours (Student's t -test: $p < 0.040$), respectively. The results general indicate that the overall extent in the reduction of averaged adhesion energy is increased against the increase of drug concentration. The fluctuation of adhesion energy of HepG2 cells after adding Lat-A emerges is likely attributed to the resistance of HepG2 cells towards Lat-A compared to that towards Dox and Cyto-D. The results are supported by reduction of traction force and stiffness against exposure to these drugs [Wakatsuki et al., 2000]. Also, change in adhesion energy shows the same concentration dependency as shown in cell deformation. Furthermore, it is important to correlate the adhesion contact dynamics and adhesion energy with the cytoskeleton organization for HepG2 cells. The immunostaining images in Fig. 6.16 clearly demonstrate the effect of Dox, Cyto-D and Lat-A on actin filaments, which restrain cell spreading, decrease adhesion contact area, as well as attenuate adhesion strength of HepG2 cell on collagen-coated surface.

Fig. 6.12. The average adhesion Energy of a population HepG2 cells against time after adding Dox.

Fig. 6.13. The average adhesion Energy of a population HepG2 cells against time after adding Cyto-D.

Fig. 6.14. The average adhesion Energy of a population HepG2 cells against time after adding Lat-A.

Actin filaments are one of the major network structures of the cytoskeleton in HepG2 cells and are involved in cell location, cell morphology change, membrane ruffling, and formation of lamellipodia [Abercrombie 1980]. In order to investigate the change in the organization of actin filaments after the exposure of HepG2 cell to Dox, Cyto-D and Lat-A, actin filaments are labeled with FITC-phalloidin. It is important to access the structure of actin network for firmly adhered HepG2 cells before any drug perturbation. Fig. 6.15 shows typical confocal fluorescence images of normal HepG2 cell after two hours of seeding on collagen-coated surface. The result indicates high concentration of actin filaments under the plasma membrane after HepG2 cells seeding on collagen-coated substrate for 120 minutes. The bundles and networks of actin

filament distribute under cell membrane and between cell membrane and nucleus, respectively. The pattern is caused by actin polymerization and interaction of actin filaments and other proteins associated with the membrane, as well as proteins in signaling pathways. Also, the result supports that cytoskeleton reorganization triggered by integrin-collagen recognition coincides with the adhesion contact evolution during HepG2 cell seeding on the substrate.

Fig. 6.15. Image of the structure of actin filaments of HepG2 cells seeding on coverslip coated with collagen substrate at 120min.

After adding Dox or Cyto-D or Lat-A of 5 μ M into firmly adherent HepG2 cells, the confocal fluorescence images of actin network of HepG2 cell seeding on collagen at 5 and 150 minutes are shown in Fig. 6.16. After adding Dox into HepG2 cells for 5 minutes, the bundles of actin filaments localize at the periphery of cell and lies between

membrane and nucleus. Following 150 minutes of Dox incubation, the bundles of actin filaments apparently disappear but filamentous actin network associated with plasma membrane emerges. At the same time, the image reveals that there is a high concentration of actin filaments in the center of nucleus labeled by FITC-phalloidin. The result is consistent with that nucleolar staining masked in brightly labeled K-562 cells contains substantial amount of F-actin [Grzanka et al., 2004]. The observation suggests that actin could be involved in chromatin reorganization after adding Dox into HepG2 cells. Also, Dox does not damage the structure of actin network of HepG2 but makes it less condense. It is believed that the time for Dox acting on HepG2 cell is too short only 150 minutes or concentration of Dox is relatively low. After 5 minutes of HepG2 cells exposure to Lat-A, bundles of actin filaments form and distribute under cytoplasm membrane. Following 150 minutes of Lat-A incubation, less dense peripheral actin bundles with interruption and intracellular punctate actin filaments emerges. The observation verifies that Lat-A has the ability of depolymerizing actin filaments in consequent to sequestration of monomeric actin, resembling the result that Lat-A employed in studies of fibroblast NIL8 cells [Spector et al., 1989]. However, whether Lat-A could affect intracellular actin filaments is unclear. Addition of Cyto-D of 5 μ M into HepG2 cells promotes formation of bundles of actin filaments under cytoplasm membrane at 5 minutes of incubation. Simultaneously, Cyto-D disrupts actin networks in cytoplasm causing the appearances of clumps of actin filaments. The available data show that Cyto-D has an actin-filament-severing activity, which has been proved by Hartwig and Stossel [Hartwig and Stossel 1979]. Our experimental result is in agreement with the result that Cyto-D disrupts actin bundles of MDCK cell and F-actin filaments of HT-29 cell, which are replaced with large focal aggregates of F-actin within the cytoplasm [Stevenson and Begg 1994; Colombo et al., 1990]. Interestingly,

actin filaments reorganize to a small ring-like structure at 150 minutes after adding Cyto-D. From the immunostaining images, we can distinctly see Dox does not damage actin filaments in contrast to Lat-A and Cyto-D. It is because that the addition of these drugs not only disrupts the structure of actin filament but also reduces peripheral membrane proteins, many of which are actin-binding protein, such as α -catenin, β -catenin, α -actinin and vinculin [Yamada et al., 2004]. Therefore, the drugs adding to HepG2 cell disrupt or suppress actin filament structure resulting in decreased cell size, cell adhesion contact, cell adhesion energy, and cell deformation.

Fig. 6.16. The images of the structure of actin filaments of HepG2 cells on collagen after addition of Dox, Lat-A and Cyto-D of 5 μ M.

6.3 Conclusion

In summary, we explore the influence of Dox, Cyto-D and Lat-A on cell adhesion dynamics and contact mechanics of HepG2 cells on collagen substrate by employing Confocal reflection interference contrast microscopy in conjunction with phase contrast microscope, fluorescence microscope, and immunostaining technique. In this study, the kinetics of cell detachment and actin dynamics of live HepG2 cells following the addition of the three drugs is probed with confocal reflectance interference contrast microscopy (C-RICM) and fluorescence confocal microscopy. First, it is shown that the reduction in two-dimensional spread area of HepG2 cells is 10.5 %, 15.4 % and 21.9 % under the influence of 5 μ M of Lat-A, Cyto-D and Dox, respectively. Secondly, C-RICM demonstrates that the recession of adhesion contact against time of cell seeding upon the addition of the three drugs. Thirdly, the initial cell detachment rate and extent of change in degree of cell deformation (a/R) are dependent on both the drug types and concentration. Lastly, oscillation-like responses of a/R and adhesion energy is uniquely found in Lat-A induced cell detachment. Overall, our biophysical approaches have been proven as a highly quantitative platform for screening drug interactions with targeted cells.

Chapter 7: Conclusions and Future Work

7.1 Conclusions

In this work, biophysical experiments for elucidating the effect of GFP-actin transfection, HBV infection, and cytoskeleton disruptive drugs on adhesion dynamics of HepG2 cells on model ECMs have been performed. My thesis aims to understand the intricate interplay between receptor-ligand interaction, cytoskeleton alteration, HBV infection, drug interaction, adhesion dynamics and biomechanical responses of cells. Indeed, our unique biophysical approach demonstrated the more complex binding between collagen/fibronectin and the integrin receptor influences cytoskeleton organization, signal transduction cascades, gene expression, etc, resulting in changes in cellular morphology, cell deformation and spreading, as well as cell adhesion dynamics. From these experiments with confocal reflection interference contrast microscopy (C-RICM) in conjunction with GFP-protein expression, immunostaining, and other molecular biology techniques, we make the conclusions as shown below:

1) Understanding the biophysical responses of cell towards a change of cytoskeleton property is important for mechanochemical transduction. The initial rate of increase in the degree of cell deformation (a/R) and adhesion energy of intact HepG2 cell seeded on fibronectin- or collagen-coated coverglass are higher than that of transfected HepG2 cells. Moreover, GFP-actin expression increases and maintains the average adhesion energy of the cells on collagen- and fibronectin-coated glass, respectively, at steady state compared with that of the normal cells. In addition, GFP-actin fusion proteins are successfully integrated into actin filaments. Therefore, actin filaments are available for real time observation in the cytoplasmic cortex. More

importantly, this study provides a benchmark in the application of GFP-actin expression for studying the cell-biomaterials interaction *in vitro*. The exact biophysical responses of GFP-actin expressing cells towards receptor mediated adhesion on engineered biomaterials can be used for engineering cytoskeleton structures and biological functions in engineered tissue equivalents.

2) HBV infection affects the reorganization dynamics of actin microfilament and leads to the alteration of cell morphology. HBV infection adversely affects cell spreading and adhesion processes of normal HepG2 cells on collagen and fibronectin coated substrates. Our result further shows that an important adaptor protein in the cytoskeleton network, vinexin, directly interacts with HBX protein induced by HBV infection. In addition, the increase of adhesion energy of HBV-infected cells is smaller than that of normal HepG2 cells. Also, during early stage of cell seeding, only exogenous vinculin is detected in cells infected by HBV while vinculin-associated focal complexes are found. In addition, the increase of adhesion energy of HBV-infected cells is smaller than that of normal HepG2 cells. Finally, the kinetics of adhesion contact evolution is transformed from intermediate reaction-limited rate on the stick cell surfaces of normal HepG2 cells to other reaction regimes upon HBV infection or/and GFP-actin expression. Overall, highly intricate interplay between HBV infection, cytoskeleton dynamics, adhesion contact dynamics, cell spreading, GFP-actin expression is demonstrated in HepG2 cell adhesion.

3) In the investigation on the influence of cytoskeleton disruptive drugs on cell adhesion recession and contact mechanics of HepG2 cells on collagen coated substrate, our results indicate that change in degree of cell deformation and adhesion energy is dependent on the dosage of drugs. Cell morphology is rapidly transformed after

treatment of HepG2 cells with Lat-A, CytoD and Dox. These drugs stimulate the depolymerization kinetics of actin monomer and result in the reorganization and redistribution of actin microfilament network. Moreover, confocal fluorescence microscopy shows that Dox does not disrupt but compress the structure of actin filaments in contrast to Cyto-D and Lat-A. More importantly, C-RICM in conjunction with other techniques is proven as an effective platform of screening the effect to drugs on cells/tissues.

7.2 Contributions

a. One of my main contributions in my thesis is to extend our unique biophysical techniques for probing the mechanochemical transduction of live cells under different stimulus.

b. During the course of my thesis, I have developed the confocal-reflection interference contrast microscopy (C-RICM) for real-time imaging of live cell on model substrate. I demonstrated the use of C-RICM to probe the adhesion contact kinetics of seeded cells. At the same time, we make use of the contact mechanics model of adherent cell (set by NTU) to quantitatively analyze our C-RICM data.

c. In the first part of my investigation, I successfully integrated C-RICM and confocal fluorescence microscopy to probe the adhesion kinetics, cell spreading and cytoskeleton organization simultaneously. Along the way, I have developed HepG2 cell line with GFP-actin transfection which enables cells to express GFP-actin for fluorescence imaging. I discovered that GFP-actin expression modulates the adhesion contact kinetics of HepG2 cells, e.g., delay of adhesion contact evolution. Most importantly, my study provides a benchmark in the application of GFP-actin expression

for studying the cell-biomaterials interaction in real time. For example, the biophysical responses of GFP-actin expressing cells towards receptor mediated adhesion on engineered biomaterials must be used with cautions for deducing the actual properties of non-transfected or intact cells.

d. In addition, I provided biophysical/molecular evidences that HBV infection influences the kinetics of HepG2 cell adhesion (e.g., adhesion energy and degree of cell deformation) to model ECM. At the same time, HBV replication modulates initial formation of focal adhesion complex as well as cytoskeleton reorganization. Our new molecular data further showed that the modulation of adhesion kinetics is correlated with the binding between HBx protein, a HBV protein and vinexin, a cytoskeleton protein. Therefore new insight into the biophysical cascades of HBV infection has been obtained in my study.

e. Lastly, I have demonstrated that our biophysical approaches are a highly quantitative experimental approach for screening drug interactions with targeted cells. Specifically, we exploit the influence of Dox, Cyto-D and Lat-A on initial adhesion mechanism of HepG2 cells on collagen-coated substrate by our unique biophysical approaches. In this study, the kinetics of adhesion contact recession and actin dynamics of live HepG2 cells following the addition of the three drugs are determined. It is shown that the biophysical parameters during cytoskeleton disruption are dependent on drug type, concentration and actin organization.

7.3 Future work

Based on biophysical studies as mentioned above, the future work should focus and cover the following areas:

a. Research on the adhesion dynamics and cytoskeleton alteration of cell on biomaterials by using C-RICM.

Apply various polymers to construct scaffold for cell adhesion, C-RICM can be used to investigate the mechanochemical transduction and adhesion dynamics of cells in biomaterial surfaces for designing new tissue engineering devices.

b. Probing the protein expression level of cells against the change of cell-biomaterial interactions by applying two-dimensional gel electrophoresis and RT-PCR.

Overall, new insights into the intricate interplay between cell biophysics, biomaterial design and biological functions of cells will be developed for tissue engineering.

c. Investigate outside-in or inside-out signal transduction during cell adhesion on ECM model or biomaterials by using Weston blotting method.

During cell adhesion, outside-in or inside-out signal transduction plays very important role in cellular processes. FAK and Src will be explored in adhering cells by using Western blotting method.

d. Explore integrin dynamics and their role of cell during adhesion on ECM model or biomaterials

Cyan fluorescent protein-fused and yellow fluorescent protein-fused α and β cytoplasmic domains of integrin receptor will be produced by gene transfection techniques. The cytoplasmic conformational changes in the integrin in cell can be investigated by fluorescent resonance energy transfer (FRET).

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological macromolecules.

New information on the integrin dynamics can be obtained, such as at resting state (cell just seeding on dish), during ligand binding state, and under external force stimuli.

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