Biomaterials 31 (2010) 1894–1903

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Hepatitis B virus induced coupling of deadhesion and migration of HepG2 cells on thermo-responsive polymer

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ARTICLE INFO

Article history:
Received 24 September 2009
Accepted 13 November 2009
Available online 27 November 2009

Keywords:
Deadhesion
Migration
Biophysics
HBV

ABSTRACT

The unique physical property of thermo-responsive polymer (TRP) has recently prompted its increasing applications in tissue engineering. On the other hand, TRP has not been exploited for potential applications in quantitative cell screening against external stimulations. In this study, TRP is applied as a model system for elucidating the effect of HBV replication on the biophysical responses of HepG2 cells transfected by wild type HBV genome. Moreover, mutant HBV genome is designed to assess the specific activity of the SH3-binding domain of HBx during HBV replication. The adhesion contact recession and geometry transformation of HepG2 cells transfected with empty vector (pcDNA3.1 cells), wild type HBV (wtHBV cells) and mutant HBV genome (mHBV cells) are probed during the thermal transformation across lower solution critical temperature of TRP. In comparison with pcDNA3.1 cells and mHBV cells, the initial rate of reduction in degree of deformation and average adhesion energy for wtHBV cells is significantly increased. Interestingly, migration speed and persistence time of cells are found to be correlated with the cell deadhesion kinetics. Immuno-fluorescence microscopy demonstrates that HBV replication reduces the actin concentration and focal adhesions at cell periphery during the initial 30 min cell deadhesion. The results strongly suggested that HBV infection triggers the dynamic responses of HepG2 cells through the cytoskeleton remodeling and subsequent mechanochemical transduction. Overall, it is shown that TRP provides a convenient platform for quantifying biological stimulations on adherent cells.

1. Introduction

Cell adhesion onto extracellular matrix (ECM) or biomaterial is best described by a two-step mechanism including passive adhesion and specific recognition between cell and opposing surfaces. On the other hand, cell deadhesion phenomena remains to be less explored compared to cell adhesion. Recently, poly(*N*-isopropylacrylamide) or PIPAAm has emerged as a promising thermo-responsive polymer (TRP) for regeneration medicine [1,2]. The acute transformation of the hydrophobicity of PIPAAm across its low critical solution temperature (LCST), i.e. the PIPAAm surface becomes more hydrophilic below LCST, enables the detachment of cell sheets without any enzymatic treatment [3,4]. Intuitively, cell deadhesion from PIPAAm surface below LCST is correlated to the cellular signaling cascades such as tyrosine phosphorylation and cytoskeleton reorganization [5,6]. Furthermore, traction and contractile forces originated from the cytoskeletal network likely drives the subsequent cell deadhesion and detachment. The cellular forces as mentioned above have been shown to detach ECM proteins such as collagen, laminin and fibronectin from tissue culture grade polystyrene (TCGP) dish [7]. Thus PIPAAm offers attractive properties for serving as a standard experimental system for elucidating the influence of pathophysiological stimulations on the behavior of anchorage-dependent cells.

Hepatitis B virus (HBV) infection affects more than 350 million people worldwide and often leads to fatal diseases such as liver cirrhosis and liver cancer [8]. It is well known that HBV infection disrupts the cell–cell adhesion [9,10] and down-regulates the expression of α1 and α5 subunits of integrin receptors [11]. Among the four proteins encoded by the HBV genome, HBx is a 154-unit amino acid with a molecular weight of 17.5 kDa expressed dominantly in cytoplasm [12]. It is known that HBx dictates the transcriptional activation and viral replication of HBV in the nucleus and cytoplasm of inflected cells, respectively [13,14]. Furthermore, HBx enhances the expression of two matrix metalloproteinases including MMP9 and MMP3 which indirectly moderates cell adhesion [15]. Recently, HBx has been suggested to

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doi:10.1016/j.biomaterials.2009.11.031

Table 1
Primer sequences of site-directed mutagenesis in proline-rich region of HBx.

B mut A sense (from 1443 bp to 1487 bp):
E S R G R P L P G P L G A L P
5' GAA TCC CGC GGA CGA GCC CTC CCG GGG GCG CTT GGG GCT CTA CCG 3'
A A
B mut A anti-sense:
5' CGG TAG AGC CCC AAG CGC CCC CGG GAG GGC TCG TCC GCG GGA TTC 3'
B mut B sense (from 1491 bp to 1532 bp):
A L P P A S P P I V P T D H
5' GCT CTA CCG CCC GCT TCT GCG GCT ATT GTA CCG ACC GAC CAC 3'
A A
B mut B anti-sense:
5' GTG GTC GGT CGG TAC AAT AGC CGC AGA AGC GGG CGG TAG AGC 3'

The two prolines (P) in translational amino acid sequences on *B mut A* sense and *B mut B* sense primer sequence were mutated into alanines (A).

play a role in cell apoptosis through the disruption of cytoskeleton [16]. Nevertheless, the intricate effect of HBV replication on the mechanochemical transduction of adherent cells remains largely unknown. For instance, it has been shown that the cell adhesion kinetics on ECM protein coated surface is affected by HBV replication [17,18].

In this study, the PIPAAm system was applied to probe the effect of HBV replication on the biophysical responses of HepG2 cells. First, the whole HBV genome was introduced into the HepG2 cells for simulating HBV infection. Then confocal reflection interference contrast microscopy (C-RICM) is applied to probe the adhesion contact and cell geometry of various types of HepG2 cells during the thermally triggered process induced by the proven PIPAAm system. Conventional RICM based on epi-fluorescence microscope collects lights from different focal planes reflected from the specimen, while C-RICM only collects light from a fixed focal plane at the cell–substrate interface. In effect, C-RICM achieves a more controlled and limited depth of focus. Therefore C-RICM enhances the overall accuracy in the measurement of the adhesion contact area and deformation profile compared to conventional RICM [19,20]. Laminin is an ECM protein which regulates cell adhesion, migration, proliferation, differentiation and apoptosis through interaction with cell surface receptor such as integrins and syndecans [21–23]. Laminin was chosen as model ECM protein herein because it is found in the basal lamina underlying hepatocyte. Moreover, cell migration and cytoskeleton organization under HBV replication is probed with time-lapse florescence microscopy and epi-fluorescence microscopy, respectively.

2. Materials and methods

2.1. Preparation of PIPAAm-grafted surfaces

Glass coverslips were cleaned with Piranha solution (30% v/v H_2O_2 and 70% v/v H_2SO_4) for 1 h, rinsed thoroughly with doubly distilled water (resistance: 18 MΩ, Sartorius, Germany) and then dried in vacuum oven at 22 °C. The preparation of PIPAAm-grafted surfaces using the atom transfer radical polymerizations (ATRP) reaction have been previously depicted in detail [24]. In brief, glass coverslips were first immersed in 30 ml of chloroform (Sigma–Aldrich Inc., USA) followed by the addition of 0.5 ml of triethylamine (Sigma–Aldrich Inc., USA) and 2 ml of 4-(chloromethyl)-phenyltrichlorosilane (Alfa Aesar Pte. Ltd., Singapore). After 24 h of reaction, the coverslips were thoroughly rinsed with acetone and dipped in acetone for another 30 min in order to remove the non-reacted silane. After being dried in air, the silanized coverslips were dipped into 15 ml of *N*-isopropylacrylamide (NIPAAm) (Sigma–Aldrich Inc., USA) solution with a concentration of 0.23 g/ml, followed by 20 mg of CuCl and 4 mg of $CuCl_2$ (Sigma–Aldrich Inc., USA). The solution mixture was degassed with argon gas for 20 min. Finally, 5 μl of 1,1,4,7,10,10-hexamethyl-triethylenetetramino (Sigma–Aldrich Inc., USA) was added to the mixture and the reactor was sealed with ParaFilm for the interfacial polymerization of NIPAAm. To stop the polymerization, the glass substrates were taken out from the solution mixture and washed consecutively with abundant amount of DMSO (Sigma–Aldrich Inc., USA) and double distilled water (Sartorius, Germany), and then dried in vacuum oven. The root-mean-square roughness of PIPAAm-grafted surface at 22 °C and 37 °C is 1.7 ± 1.8 nm and 1.1 ± 0.8 nm, respectively [25]. The water contact angle of PIPAAm-grafted surface is 61 ± 3.1° and 79 ± 3.6° at 22 °C and 37 °C, respectively, as reported elsewhere [24,25].

2.2. Preparation of laminin-coated PIPAAm surface

400 μl of 0.1 mg ml^{-1} laminin solution, which was diluted from the original laminin solution (Sigma–Aldrich Inc., USA, 1.0 mg ml^{-1} in water) was directly loaded onto the PIPAAm surface and incubated at 4 °C for 24 h. The surface was then thoroughly washed with deionized water to remove the excess laminin. After being sterilized with 70% ethanol, the surface was stored in 4 °C refrigerator before further experiments.

2.3. Preparation of mutant HBV genome

The design of DNA primers for mutation analysis of the genotype B SH3 binding domain from prolines to alanines within HBV genome is shown in Table 1. This set of four primers which was used to generate HBx mutated in the proline-rich region within full length HBV genome is cloned in pcDNA3.1+ vector with QuikChange® XL Site-Directed Mutagenesis Kit (QIAGEN, USA) (Table 1). "B mutant A" means the double mutated region A (P39 and P42), while "B mutant B" represents the double mutated region A (P43 and P46) of HBV genome genotype B. After the two-step mutation, the mutant plasmids, wild type plasmids and empty vector were amplified by transformation into *Escherichia coli* and then extracted using Plasmid Midi Kits (QIAGEN, USA) for cell transfection. The success of mutation generation was confirmed by DNA sequencing.

2.4. Cell culture and transfection

Three cell types including: 1. HepG2 cells transfected with empty pcDNA3.1 vector (pcDNA3.1 cells); 2. HepG2 cells transfected with a replicative wild type HBV genome cloned in pcDNA3.1 (wtHBV cells); 3. HepG2 cells transfected with a replicative mutant HBV genome cloned in pcDNA3.1 (mHBV cells) have been employed for our current investigation. The replicative HBV genome was constructed by cloning a linear form of viral genome into mammalian expression vector pcDNA3.1. Replicative genome consisting of ~1.1 × HBV genome in length with terminal redundancy was constructed to reflect the *in vivo* environment, whereby the pregenome RNA is the template for HBV replication [8].

HepG2 cells (ATCC, USA) were maintained in Gibco Dulbecco's minimal essential medium (MEM) (Invitrogen Inc., USA), complemented with 10% fetal bovine serum (Invitrogen Inc., USA) and 1% anti-mycotic (Invitrogen Inc., USA) under 37 °C and 5% CO_2. To maintain their viability, the cells were passaged by trypsinization with 2×Trypsin-EDTA/PBS at 37 °C and pH 7.2 (Invitrogen Inc., USA) every 3–4 days.

Transfection of HepG2 cells was carried out with Effectene transfection reagent (QIAGEN, USA) according to manufacturer's instructions. In brief, 6×10^5 cells were seeded and cultured on a 60 mm polystyrene dish (Nunc Inc., USA)

Table 2
Primer sequences specific for *HBcAg*, *HBsAg* and *β-actin* gene in RT-PCR.

HBcAg forward: 5′-ATCTCCTAGACACCGCCTCA-3′
HBcAg anti-sense: 5′-TTCCAAATTATTACCCACCC-3′
HBsAg sense: 5′-TCACCATATTCTTGGGAACAA-3′
HBsAg anti-sense: 5′-GTTTTGTTAGGGTTTAAATG-3′
β-actin sense: 5′-CTTAGTTGCGTTACACCCTTTC-3′
β-actin anti-sense: 5′-ACCTTCACCGTTCCAGTTTT-3′