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Comparative atomic force and scanning electron microscopy: An investigation of structural differentiation of hepatic stellate cells

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ABSTRACT

The molecular mechanism leading to the transdifferentiation of hepatic stellate cells (HSC) into myofibroblast-like cells following liver injury is not well understood. The state of cultured rat HSCs was determined using primarily fluorescence microscopy (UV), immunofluorescence (IF) (Glial fibrillary acidic protein (GFAP), Desmin, alpha-smooth muscle actin (alpha-SMA), F-actin) and immunocytochemistry (ICC) (GFAP, Desmin, alpha-SMA, Fibulin-2). Additionally, tapping-mode atomic force microscopy (TM-AFM) and field-emission scanning electron microscopy (FE-SEM) with low-resistivity indium-tin-oxide (ITO) thin-film were performed to observe the micro-morphological character of cells during HSC differentiation. Quiescent HSCs changed to the activated state were identified via UV, IF, and ICC observations. Normal rat HSCs (NHSCs) and thioacetamide-induced rat HSCs (THSCs) were demonstrated to be UV^-, $GFAP^+$, $Desmin^+$, alpha-SMA^+ and $Fibulin\text{-}2^-$. After F-actin staining, lamellipodia and filopodia were found in both NHSCs and THSCs, but membrane ruffles were only seen in THSCs. The micro-structures of lamellipodia and filopodia in both NHSCs and THSCs were confirmed using FE-SEM and TM-AFM with ITO; in contrast, the micro-projection was not found. Moreover, "aerial root" structures were observed for the first time in the filopodia of THSCs using TM-AFM. These results reveal that HSC transdifferentiation to a myofibroblastic-like cell (activated HSC) from thioacetamide-induced rat HSC induces extensive changes in the cytoskeleton.

1. Introduction

Hepatic stellate cells (HSCs), also known as Ito cells, fat-storing cells, or lipocytes, are non-parenchymal cells of mesenchymal origin. With little proliferative activity. HSCs are conventionally classified as perisinusoidal cells involved in the storage and metabolism of vitamin A (Blomhoff and Wake, 1991). HSCs are located in the Space of Disse around the sinusoids and surround the endothelial lining of liver sinusoidal capillaries (Geerts, 2001); they are believed to serve as pericytes for hepatic capillaries called sinusoids. HSCs comprise about 5–8% of total liver cells and 15–23% of non-parenchymal cells in the normal liver (Geerts, 2001) and are the key fibrogenic progenitor cell type (Gressner and Bachem, 1995). HSCs possess abundant elongate dendrite-like processes extending like a star's points from their cell bodies to encircle the sinusoids. Once liver damage (viral infection, aflatoxin B1 intoxication, chronic alcohol abuse or drug-induced injury) and inflammation occur, HSCs undergo a process of activation (the transdifferentiation of quiescent (retinoid-storing) cells into proliferative, extracellular matrix (ECM) protein-producing cells (Li et al., 1990; Rockey et al., 1992; Friedman, 1993; Geerts, 2001)), develop extensive cytoplasmic fibers, lose their perinuclear droplets, and increase in size as contractile myofibroblastic-like cells (Rockey and Friedman, 1992). In particular, myofibroblastic-like cells secrete high levels of the interstitial collagens I and III (Knittel et al., 1992) as well as several matrix metalloproteinases (MMPs) (Knittel et al., 1999b) and tissue inhibitors of matrix metalloproteinases (TIMPs) (Knittel et al., 1999b; Vyas et al., 1995), resulting in a dense and rigid network of matrix constituents which exerts physical stress on surrounding cells. The fibrogenic features of HSCs with induced ability to synthesize and deposit extracellular matrices (Friedman, 2003) represent a key cellular event in the genesis of liver cirrhosis.

Atomic force microscopy (AFM) has been highly valuable in the study of many biological samples, from single molecules to cells. Two basic applications of AFM include structural imaging and force–distance measurements with a sub-micrometer scale spatial

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resolution and 10 pN force resolution, respectively (Fang et al., 2000; Li and Logan, 2004). AFM and field-emission scanning electron microscopy (FE-SEM) provide a broad spectrum of phenotypic changes involving alterations in the extent and nature of disruption of cell–cell adhesion, directionality of motility, cell polarity and shape, and protrusion dynamics. The tomography images of ultra-structure, including microtubules, microfilaments and the other components of the cytoskeleton, are more frequently observed with FE-SEM than with AFM (de Souza et al., 2006; Jaber et al., 2007). However, AFM can provide not only instant high-resolution 3D imaging of cells, but can also examine the micro-mechanical properties of cells (A-Hassan et al., 1998; Braet et al., 1998; Lekka et al., 1999; Muller et al., 1997; Ohshiro et al., 2000; Radmacher, 1997; Ricci et al., 1997; Schneider et al., 1997a,b; Vinckier and Semenza, 1998). For example, the comprehensive surface structures of live endothelial cells (ECs), including rigid cytoskeletal elements along the flat filamentous membrane structures, have been investigated at the nano-scale by AFM (Kienberger et al., 2003; Pesen and Hoh, 2005). The topography of live ECs, including the induced shape/volume changes (Braet et al., 2001; Han et al., 2003; Oberleithner et al., 2003), elasticity, adhesion, surface structure and swarming behavior, has been extensively studied (Beckmann et al., 2006; Benoit et al., 2000; Costa et al., 2006; Liu et al., 2006; Pelling et al., 2006; Puech et al., 2006). Recently, AFM has been applied delicately to detect vacuoles through mechanical measurements in whole cells that might accelerate the investigation of fluid-filled organelles closer to physiological conditions (Riethmuller et al., 2007). The micro-morphological appearance of HSCs that undergo phenotypic changes are often characterized as "myofibroblastic activation" (Hautekeete and Geerts, 1997), however, these micro-morphological changes of HSCs have yet to be explored using AFM. The present work employed both TM-AFM and FE-SEM to characterize the micro-morphological changes during the transdifferentiation of HSCs.

2. Materials and methods

2.1. Preparation of cells

Isolation and culture of liver HSCs from male Sprague–Dawley rats (350–400 g, National Laboratory's Animal Center, Taipei, Taiwan) were performed as described previously (Knook et al., 1982). Rats were intraperitoneally anaesthetized with Urethane (0.3 g/100 kg BWt; Sigma, Louis, MO, USA). The abdominal cavity was opened, and portal and dorsal veins were exposed. The liver was first perfused (30 mL/min, MasterFlex 77240-10, Vernon Hills, IL, USA) through the portal vein in situ with 500 mL of HBSS (Hank's balanced salt solution without Ca^{2+} and Mg^{2+}). Once livers became pale-yellow, they were then re-perfused (30 mL/min, MasterFlex 77240-10) with 1 L of HBSS plus Ca^{2+} and Mg^{2+} containing 0.27 g/L protease E (Sigma cat #P5147) and 0.1 g/L collagenase (Sigma cat #C5138). At the end of the incubation period, the

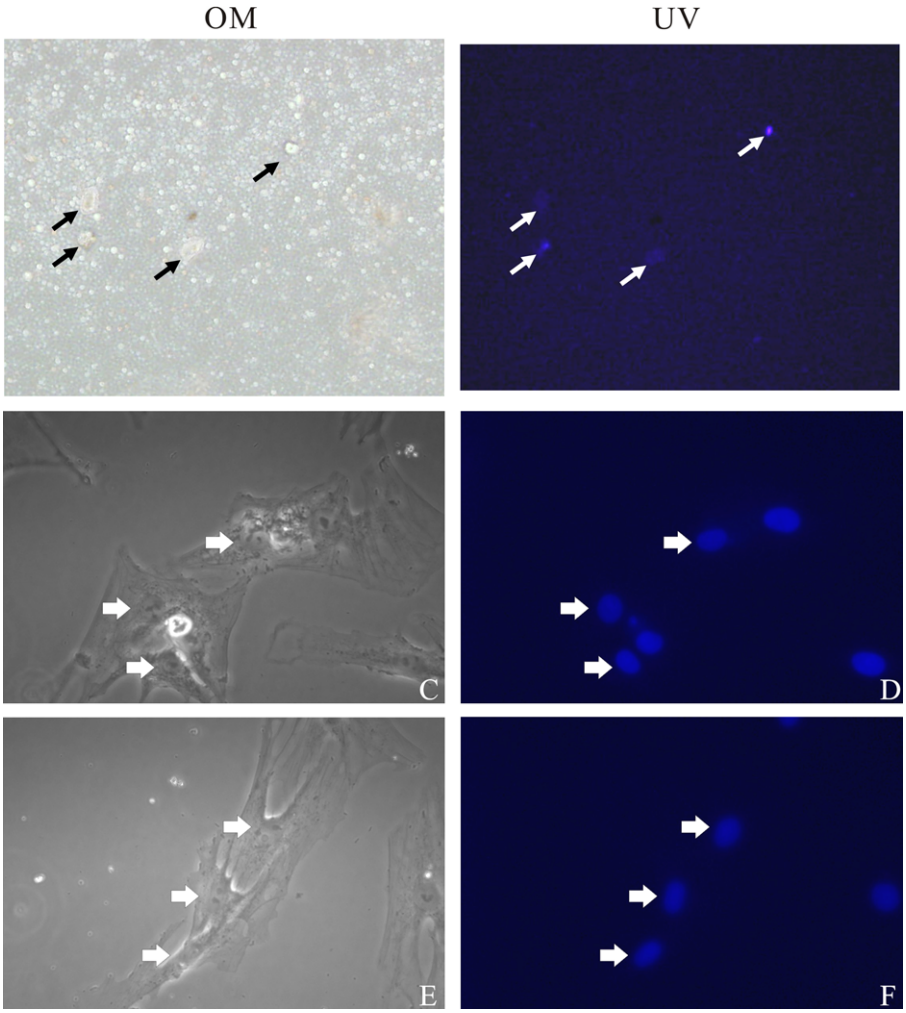

Fig. 1. The appearance of freshly-isolated HSCs using (A) optical microscopy (OM) and (B) ultraviolet (UV^+) irradiation to detect vitamin A. 100×. The nucleus of a HSC (C and E) stained with DAPI (D and F) and a HSC with one or more nucleoli (400×). White arrows indicate various structures in the different panels.