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Cytokeratin 18-mediated disorganization of intermediate filaments is induced by degradation of plectin in human liver cells

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ABSTRACT

Plectin is a cross-linking protein that organizes the cytoskeleton into a stable meshwork that helps maintain the uniform size and shape of cells. As cells of hepatocellular carcinoma are morphologically different from healthy human hepatocytes, we hypothesized that plectin deficiency and cytoskeletal disorganization underlies this pleomorphic transformation. To test this hypothesis we induced apoptosis as the most accessible pathway for creating plectin deficiency status *in vivo*. We analyzed expression levels and organization of plectin and other cytoskeletal elements, including intermediate filaments, microfilaments, and microtubules, after staurosporine-induced apoptosis in human Chang liver cells. The results revealed the expression of plectin and cytokeratin 18 were downregulated in hepatocellular carcinoma tissues *in vivo*. The expression of actin and tubulin, however, were not altered. *In vitro* analysis indicated that plectin and cytokeratin 18 were cleaved following staurosporine-treatment of human Chang liver cells. Time course experiments revealed that plectin was cleaved 2 h earlier than cytokeratin 18. The organization of plectin and cytokeratin 18 networks collapsed after staurosporine-treatment. Conclusively, degradation of plectin induced by staurosporine-treatment in liver cells resulted in cytoskeleton disruption and induced morphological changes in these cells by affecting the expression and organization of cytokeratin 18.

1. Introduction

The shape and structural integrity of human hepatocytes, like other animal cells, are maintained by the cytoskeleton. The cytoskeleton is composed of three organelles: intermediate filaments (IFs), microfilaments (MFs), and microtubules (MTs) [1]. IFs, with a diameter of 10 nm, are ropelike fibers consisting of members of a large heterogeneous family of proteins that includes cytokeratins (CKs) [2]. In human hepatocytes, the IF polymers are formed by a specific CK pair: CK8 (type II, molecular weight 52 kDa) and CK18 (type I, molecular weight 45 kDa) [3]. MFs are two-stranded helical polymers composed of actin with a diameter of 5–9 nm [4]. MTs are long, hollow cylinders composed of multiple isotypes of α- and β-tubulin with an outside diameter of 25 nm [5].

Proper cross-linking organization among cytoskeleton structures is critical for establishing the internal architecture and overall morphology of cells. Several cross-linking proteins that mediate the interaction between IFs and other cytoskeletal networks have been identified [2]. Among these, plectin is the most versatile. Plectin has binding sites for IF proteins, tubulin, and actin [6], and it is expressed in a variety of tissues and mammalian cell types [7,8]. At least one human disease has been attributed to altered plectin function; mutations in the plectin gene are the molecular basis of epidermolysis bullosa with muscular dystrophy [9]. The role of plectin deficiency in human cancer development, however, is largely unknown.

Abbreviations: MT, microtubule; MF, microfilament; IF, intermediate filament; CK, cytokeratin; IFAP, intermediate filament associated protein; HCC, hepatocellular carcinoma; HRP, horseradish peroxidase; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; siRNA, small interfering RNA; PBS, phosphate-buffered saline; FITC, fluorescein-conjugated isothiocyanate; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; STS, staurosporine; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Hepatoma cells are morphologically distinctive from healthy liver cells. Therefore, we speculated that plectin deficiency might affect critical cytoskeletal elements, resulting in cytoskeletal disorganization and transformation of human liver cells. In our previous *in vivo* study, modulation of CK18 in human hepatocellular carcinoma (HCC) was established [10], and recently we found that plectin protein levels were reduced in human HCC tissues [11]. Based on RNA interference (RNAi), plectin knockdown in Chang liver cells decreases CK18 expression and results in the CK18 shrinkage phenotype; furthermore, actin-rich stress fibers are increased but the organization of microtubule networks is unaltered [12]. Based on these data, we confirmed that plectin regulates cytoskeletal organization and the overall architecture of human liver cells.

In considering the causes of plectin deficiency, apoptosis might be the most accessible pathway for creating plectin deficiency in cells. Several studies have demonstrated the degradation of plectin during apoptosis. For example, plectin can be a major early substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis [13]. In staurosporine (STS)-treated apoptotic HaCaT keratinocytes, plectin, desmoplakin, and periplakin are cleaved [14]. Another study has shown that during apoptosis, CK8/18 is reorganized into granular structures that facilitate the rapid collapse of the cytoskeletal architecture [15], and it has been recently demonstrated that colchicine-induced apoptosis results in cytoskeleton alterations [16]. These studies have indicated that plectin and the cytoskeleton are involved in apoptosis.

We previously found that STS-induced apoptosis in human Chang liver cells results in plectin cleavage and CK18 modulation as well as an unstable IF organization [17]. In that study, however, it was not apparent whether STS-treatment resulted in cleavage of plectin and CK18 or if CK18 cleavage occurred downstream of plectin degradation following STS-induced apoptosis. To clarify, in this study we observed the time course of plectin and CK18 cleavage during STS-induced apoptosis from 30 min to 7 h. In addition, we aimed to identify the influence of plectin degradation by STS-treatment on the expression and organization of the primary cytoskeletal elements (IFs, MFs, and MTs) and the overall morphology of liver cells. Human Chang liver cells were used as the experimental *in vitro* model. We also determined the *in vivo* expression of plectin, CK18, actin, and tubulin in human HCC and liver tissues. We also evaluated the *in vitro* and *in vivo* data to determine whether the effects of STS-induced plectin degradation were comparable to the *in vivo* status of plectin downregulation in human HCC.

2. Materials and methods

2.1. Tissue samples and antibodies

Fresh surgically resected HCC tissues from four patients with liver cancer and one patient with liver trauma were studied. All HCC cases were histopathologically diagnosed as grade II. These samples were stored at −80 °C before protein extraction and Western blot analysis. In addition, paraffin blocks from 10 cases of human grade II HCC were also obtained. The following commercial primary and secondary antibodies were used for immunohistochemistry, immunoblotting, and immunofluorescent assays: anti-CK18, anti-actin, and anti-tubulin monoclonal antibodies were purchased from Zymed Laboratories (South San Francisco, CA, USA); anti-plectin monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-mouse and anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-mouse and anti-goat IgG was conjugated to biotin for immunohistochemistry, to horseradish peroxidase (HRP) for Western blot analysis, and to rhodamine or fluorescein isothiocyanate (FITC) for immunofluorescent staining.

2.2. Immunohistochemistry

The deparaffinized and rehydrated sections were treated with 3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. Nonspecific binding sites were blocked with bovine serum albumin for 10 min. The sections were incubated with monoclonal antibodies against plectin (1:50 dilution), CK18 (1:100 dilution), actin (1:200 dilution), and tubulin (1:100 dilution) for 1 h at room temperature. The biotinylated anti-mouse and anti-goat IgG were added (1:400 dilution, for 1 h at room temperature), and the final signal was expressed using the avidin–biotin peroxidase technique in the presence of hydrogen peroxide [18]. These sections were evaluated under a light microscope (BX51; Olympus, Tokyo, Japan).

2.3. Cell culture and apoptosis induction

Chang human non-tumor liver cells were obtained from The American Type Culture Collection (ATCC; Rockville, MD, USA) number CCL-13. The cells were cultured in Dulbecco's minimum essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and streptomycin, and 200 mM L-glutamine. The medium was replaced every 2 days. The cells were subcultured as follows: for the immunoblotting analysis, 3×10^5 cells were seeded into 6-well tissue plates and for the immunofluorescence assay, 3×10^4 cells were grown on coverslips in 24-well tissue plates. At approximately 80% confluence, the medium was exchanged with new test medium containing 1 μM STS in dimethyl sulfoxide (DMSO), and the cells were incubated for 0.5, 1, 2, 3, 4, 5, 6, and 7 h for induction of apoptosis. Medium without STS was used as the negative control.

2.4. Quantification of apoptotic cells

The STS-treated cells on coverslips were washed with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. After washing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min followed by three washes with cold PBS. The cells were then stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature. After staining, the coverslips were washed three times with cold PBS, mounted on slides, and observed under a fluorescence microscope (BX51; Olympus, Tokyo, Japan). The fragmental nuclei of apoptotic cells were calculated from a total of 200 cells. We repeated the same experiments at least three times for quantitative analysis.

2.5. Immunofluorescence

Prior to staining, cells were washed with ice-cold PBS and fixed for 20 min with 3.7% paraformaldehyde/PBS at room temperature. After fixation, cells were washed three times with PBS and permeabilized with 0.1% Triton X-100/PBS for 2 min at room temperature. Cells were washed three times in PBS and double-stained for 60 min with primary antibodies at room temperature and then washed with PBS again. Cells were then incubated with rhodamine-conjugated anti-mouse IgG and FITC-conjugated anti-goat IgG secondary bodies for 30 min at room temperature. Finally, the unbound antibodies were removed by washing twice for