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Role of Albumin in Growth Inhibition in Hepatocellular Carcinoma

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Keywords
Albumin · Hepatocellular carcinoma · Growth inhibition · Prognosis

Abstract
Objective: Levels of serum albumin have recently emerged, together with C-reactive protein, as an important prognostic indicator for hepatocellular carcinoma (HCC). It has recently been reported that larger HCCs are associated with lower albumin levels. However, the albumin-mediated growth decrease has yet to be determined. ***Methods:*** We examined a large HCC cohort and then by direct exposure of HCC cells in vitro, the relationship of albumin levels to HCC growth. ***Results:*** We found that patients with lower albumin levels had significantly larger maximum tumor diameters, more portal vein thrombosis, more tumor multifocality, higher α-fetoprotein levels, and a lower survival than patients with higher albumin levels. Direct addition of exogenous albumin at physiological concentrations resulted in decreased growth in several HCC cell lines in vitro. We found a decrease in MAP kinase levels and in levels of Cdk2 and Cdk4, cyclinE, as well as in α-fetoprotein. ***Conclusion:*** These results indicate that in addition to its role as a monitor of systemic inflammation, albumin may have a direct role in HCC growth inhibition, either through modulation of α-fetoprotein or through its actions on growth-controlling kinases.

Introduction

Prognosis in patients with hepatocellular carcinoma (HCC) has been thought to be mainly influenced by both tumor factors such as size, number of tumors, portal vein thrombosis (PVT), and α-fetoprotein (AFP) as well as liver or microenvironmental factors such as bilirubin, and ascites [1, 2]. More recently, systemic inflammation indices have been found to be independent prognosticators for several tumor types [3–5], including HCC [6–10]. A widely used index has been the Glasgow Score [6–10] that consists of 2 factors, namely serum albumin and C-reactive protein levels. It has recently been reported that larger HCCs are associated with lower albumin levels [11, 12]. This could result from poor liver function from liver disease, liver destruction by growing HCC, or by systemic inflammation. In the current paper, we examined the

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Table 1. Comparisons between HCC patients with serum albumin dichotomized into <3.5 or ≥3.5 (g/dL), in the total cohort (**a**), and in patients with total bilirubin <2.0 mg/dL (**b**)

Parameter	Albumin <3.5 (45.7%)	Albumin ≥3.5 (54.3%)	*p**
a Total cohort			
Platelet count, $\times 10^9$/L	150.14±101.07	163.65±79.59	<0.0001
Hb, g/dL	11.73±2.33	13.61±1.84	<0.0001
GGTP, IU/mL	91.36±104.25	72.48±121.33	<0.0001
Bilirubin, mg/dL	2.37±3.57	1.14±1.61	<0.0001
PT	11.99±2.34	10.79±1.07	<0.0001
Albumin, g/dL	2.85±0.45	3.95±0.34	<0.0001
AST, IU/L	131.79±197.77	73.82±91.56	<0.0001
AFP, ng/dL	41,494.85±572,434.60	12,515.15±70,304.81	<0.0001
MTD, cm	6.11±4.39	4.90±3.80	<0.0001
PVT, n (%)	603 (31.92)	349 (15.51)	<0.001**
Nodules >3, *n* (%)	751 (39.76)	488 (21.69)	<0.001**
b Bilirubin <2.0 mg/dL			
Bilirubin, mg/dL	1.07±0.42	0.91±0.35	<0.0001
AFP, ng/dL	19,581.64±106,407.60	9,518.64±60839.03	<0.0001
MTD, cm	6.08±4.44	5.02±3.88	<0.0001
PVT, n (%)	311 (24.13)	288 (13.68)	<0.001**
Nodules >3, *n* (%)	423 (32.82)	427 (20.28)	<0.001**

Values are expressed as means ± standard deviation, unless otherwise indicated. HCC, hepatocellular carcinoma; Hb, hemoglobin; PT, prothrombin time; MTD, maximum tumor diameter; PVT, portal vein thrombosis; AFP, α-fetoprotein; GGTP, gamma-glutamyl transpeptidase; AST, aspartate aminotransaminase. * Wilcoxon rank-sum (Mann-Whitney) test. ** χ^2 test.

possible relationship between albumin and HCC growth and report that albumin levels in vivo correlated inversely with HCC growth and in vitro can antagonize HCC cell growth.

Materials and Methods

Patients

We retrospectively analyzed a prospectively collected 756 HCC patient database of prospectively-accrued patients as previously reported [13] who had full baseline tumor parameter data, including CT scan information on HCC size, number of tumor nodules, and presence of PVT and plasma AFP levels; complete blood counts; routine blood liver function tests (total bilirubin, GGTP, and albumin); demographics, and survival information. Database management conformed to legislation on privacy, and approval for this retrospective study on de-identified HCC patients was obtained by the institutional review board. This work was approved by the Institutional Review Board of the University of Pittsburgh for the retrospective analysis of deceased and de-identified patients with HCC and was in compliance with the Helsinki Declaration.

Statistical Analyses of Clinical Data

Mean and standard deviation for continuous variables, and relative frequency for categorical variables, were used as indices of centrality and dispersion of the distribution. The χ^2 and z test for proportions for categorical variables, the Kruskal-Wallis rank test, and Wilcoxon rank-sum (Mann-Whitney) test for continuous variables were used to test associations. Patient survival between categories was estimated with the Kaplan-Meier method, and comparison of survival was made with the Breslow (generalized Wilcoxon) test. The Breslow test was used, as opposed to the log-rank test, due to the large proportion of patients who died early. To evaluate the correlation between some parameters, we used the Spearman-rank correlation.

When testing the null hypothesis of no association, the probability level of α error, two tailed, was 0.05. All statistical computations were made using STATA 10.0 Statistical Software (Stata Statistical Software: release 10; StataCorp. 2007, College Station, TX, USA).

Cell Culture

Human HCC cell lines HuH-7, PLC/PRF/5, and Mahlavu were cultured in DMEM, and SNU-449 was cultured in RPMI-1640, as described previously [14]. For the conditions of albumin with different concentrations, bovine serum albumin (Amresco, 0332) was dissolved in medium and added to cells after filtration.

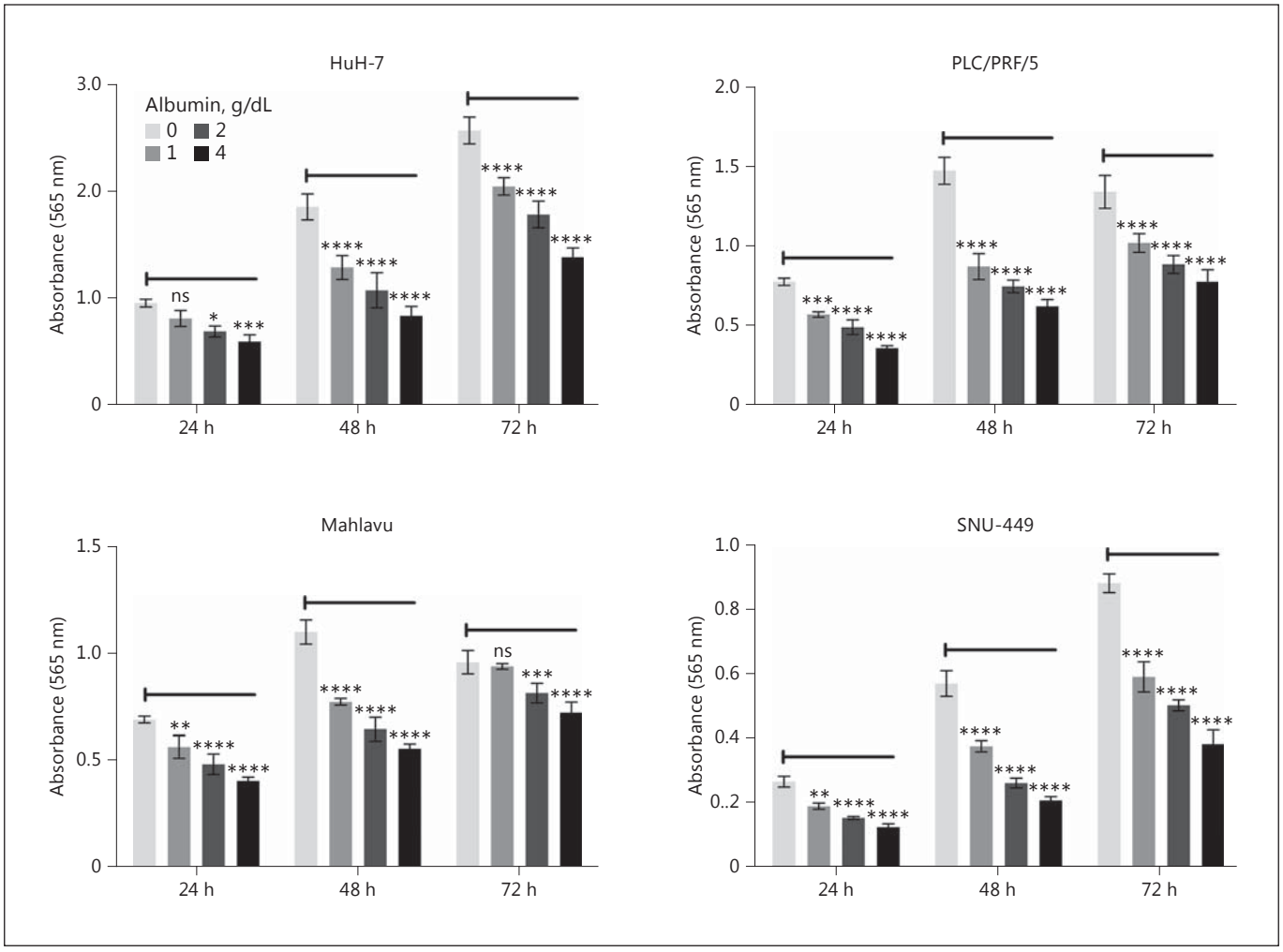

Fig. 1. Albumin-mediated HCC cell growth inhibition. HuH-7, PLC/PRF/5, Mahlavu, and SNU-449 HCC cell lines treated with albumin in different concentrations (1, 2, and 4 g/dL). After 24, 48, and 72 h of incubation, MTT assay was carried out, and the absorbance levels of each condition were detected ($n = 4$). * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$, **** $p < 0.0001$. Error bars indicate standard deviation. ns, not significant.

Cell Viability Assay

Cells were grown in 96-well plates (2×10^3 per well for HuH-7 and PLC/PRF/5, 1×10^3 per well for SNU-449 and Mahlavu) with the indicated concentrations of albumin, and after 24, 48, or 72 h, cell viability was assessed using MTT reagent (M5655; Sigma-Aldrich) according to the manufacturer's instructions.

Western Blotting

Total cell lysates were prepared from HCC cell lines with RIPA buffer. Protein concentrations of samples were determined by the bicinchoninic acid assay using the manufacturer's instructions (Pierce, IL, USA). Cell lysates were boiled with Laemmli buffer and loaded onto SDS-PAGE. Resolved proteins were transferred onto PVDF membranes for fluorescence applications (Immobilon-FL, IPFL00010). The membrane was blotted with primary antibodies against phospho-p44/42-MAPK (Erk1/2) (Thr202/Tyr204) (197G2), (cs-4377); ERK1 (C-16), (sc-93); Cdk2 (D-12), (sc-6248); Cdk4 (H-22), (sc-601); cyclinE, (BD Pharmingen 14591C); phospho-SAPK/JNK (Thr183/Tyr185) (G9), (cs-9255); JNK2 (56G8), (cs-9258); caspase-3 (8G10), (cs-9665); PARP, (cs-9542); LC3A/B (D3U4C), (cs-12741); phospho-Akt (Ser473) (D9E), (cs-4060); Akt, (cs-9272); AFP/AF01 (kindly provided by Prof. Bellet). Infrared fluorescence IRDye secondary antibodies were used (LI-COR) and detection was performed by infrared imaging system (Odyssey CLx). Equal loading and transfer were confirmed by probing for calnexin or beta-actin. Densitometric analysis of the bands was done using ImageJ software.

Statistical Analysis of in vitro Data

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., CA, USA). Statistical methods included analysis of variance (ANOVA). Differences between groups were considered at $p < 0.05$, ** $p < 0.01$, $p < 0.001$, and $p < 0.0001$.

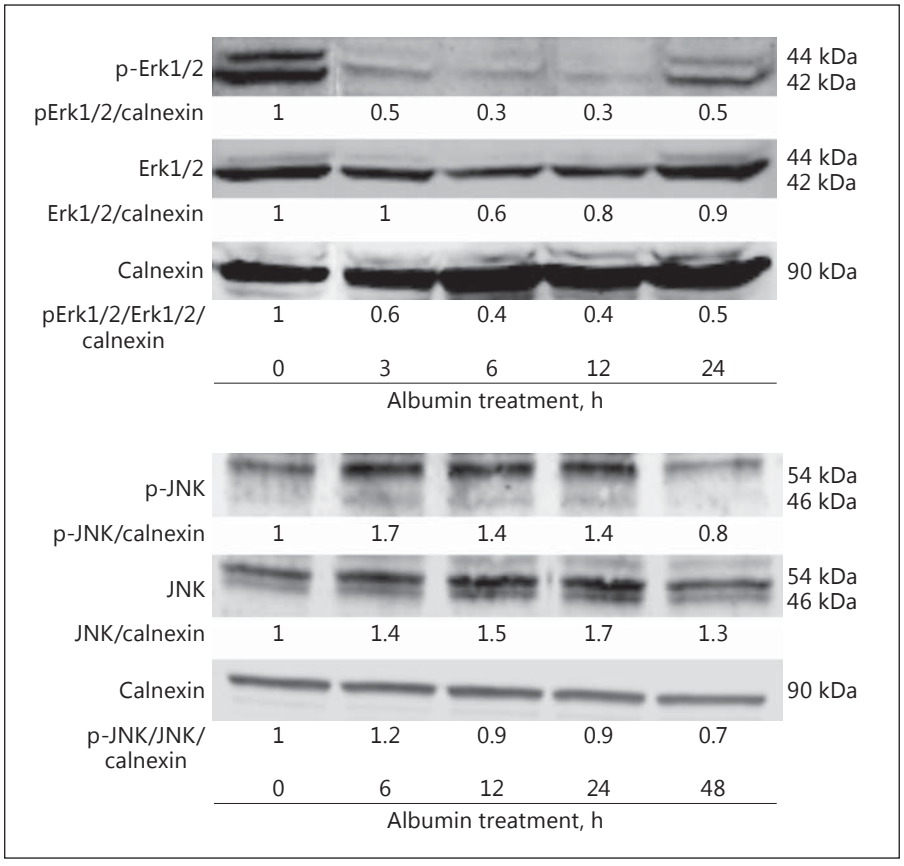

Fig. 2. HuH-7 cells were treated with 4 g/dL albumin for 3, 6, 12, 24 and 48 h. Expressions of phosphorylated and total Erk 1/2 and JNK were examined by western blot. Calnexin was used as a loading control. Densitometric analysis of each band was done by ImageJ software. The values obtained were divided by the values of the loading control. Zero condition was accepted as "1", and the others were rated upon it. Activation phosphorylation of the protein was calculated as the ratio of phosphorylated protein/total protein/loading control.

Results

Clinical Evaluation of Normal and Low Albumin Levels

Liver and tumor characteristics (maximum tumor diameter [MTD], number of tumor nodules, PVT, and blood AFP levels) were examined in the total cohort, after dichotomizing according to serum albumin levels (3.5 g/100 mL being the lower limit of normal). We found that patients with low albumin levels (n = 345 or 45.7%) had worse liver function (higher bilirubin levels) and significantly more aggressive tumors (higher MTD, PVT, number of tumors, and blood AFP levels) than patients with normal albumin (n = 411 or 54.3%) (Table 1a). Since albumin is a liver function test, we were concerned that our finding might reflect only poor liver function. The analysis was repeated only in patients who had normal bilirubin levels (Table 1b), and we found similar results to the total cohort. Furthermore, to evaluate the correlation between liver parameters and MTD, we used the Spearman-rank correlation and found that whereas albumin levels protected against tumor aggressiveness (negative correlation), there was only a positive correlation for aspartate aminotransferase or bilirubin levels, which did not protect (data not shown). Thus, the various liver parameters had unrelated associations with tumor size.

Albumin and HCC Cell Growth

To directly examine whether albumin had any relationship to HCC cell growth, HCC cell lines were cultured in different albumin concentrations in the culture medium, and cell growth was measured at specified times. We found that HCC cell growth was inhibited by higher albumin concentrations in the culture medium in each cell type (Fig. 1).

Possible Mechanisms for Albumin Growth Actions

In order to examine possible mechanisms for the effects of albumin in HCC cell line growth, we initially examined by western blot, lysates from cells treated with 4 g/dL albumin, and probed for several MAP kinases (Fig. 2). We found that addition of albumin to the cell cultures was associated with a decrease in phospho-ERK levels, as well as levels of phospho-JNK. We then exam-

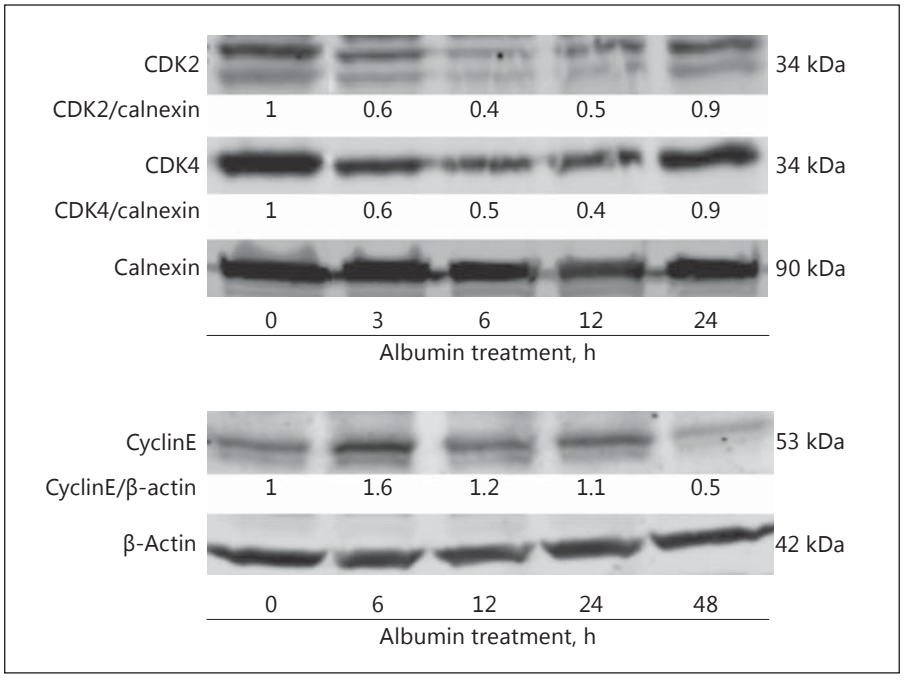

Fig. 3. HuH-7 cells were treated with 4 g/dL albumin for 3, 6, 12, 24 and 48 h. Expressions of Cdk2, Cdk4, and cyclinE were examined by western blot. Calnexin and β-actin were used as loading controls. Densitometric analysis of each band was done by ImageJ software. The values obtained were divided by the values of the loading control. Zero condition was accepted as "1", and the others were rated upon it.

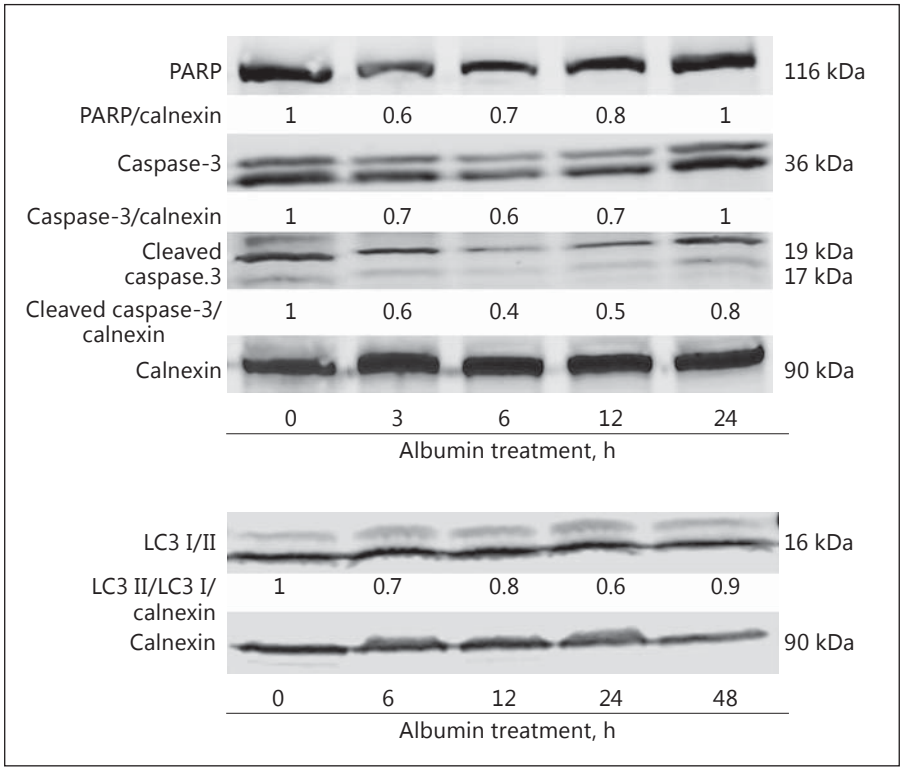

Fig. 4. HuH-7 cells were treated with 4 g/dL albumin for 3, 6, 12, 24 and 48 h. Expressions of PARP, caspase-3, and cleaved caspase-3 were examined by western blot. Calnexin was used as a loading control. Densitometric analysis of each band was done by ImageJ software. The values obtained were divided by the values of the loading control. Zero condition was accepted as "1", and the others were rated upon it.

ined the levels of cycline-dependent kinases (CDKs) and cyclinE, and found a decrease in levels of Cdk2 and Cdk4, as well as of cyclinE (Fig. 3), in association with the presence of albumin in the culture medium. To further examine the possible mechanisms for the decreased growth seen in Figure 1 after albumin addition to the cell cultures, we searched for evidence of apoptosis or autophagy (Fig. 4). However, we found only minor changes in the

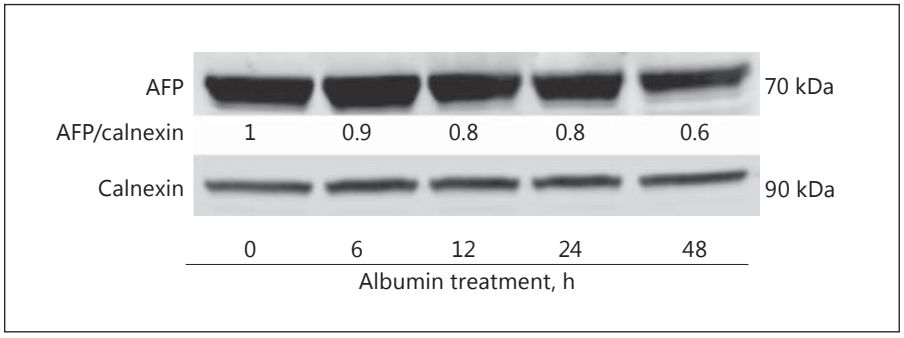

Fig. 5. HuH-7 cells were treated with 4 g/dL albumin for 6, 12, 24 and 48 h. Expression of AFP was examined by western blot. Calnexin was used as a loading control. Densitometric analysis of each band was done by ImageJ software. The values obtained were divided by the values of the loading control. Zero condition was accepted as "1", and the others were rated upon it.

levels of caspase-3 or cleaved caspase-3 and some decrease in PARP levels. There was only minor decrease in levels of the autophagy markers LC3 I/II. An inverse relationship has been described during hepatic development, between levels of mature albumin and fetal albumin or AFP. We therefore examined whether addition of albumin to our cultures might influence AFP levels (Fig. 5). We found that AFP levels were decreased at the higher, growth-inhibitory concentrations of albumin, suggesting one possible mechanism for growth inhibition by albumin.

Discussion

Albumin is one of the two components (together with C-reactive protein) of the Glasgow inflammation index for cancer, an important independent prognosticator for many cancers, including HCC. It has previously been reported to have direct growth inhibitory properties in vitro for breast cancer [15] and HCC [16], and there is also suggestive clinical evidence for a suppressive effect on HCC in patients [17]. Several studies have shown a decrease in albumin levels in human HCC cells compared to the non-HCC liver [18–20]. In the normal embryonic liver, as AFP levels decline and the albumin levels increase [21, 22]. However, the evidence from analbuminemic rats [20] is that the postnatal decline in AFP levels occurs despite persistently low albumin levels. Despite this genetically interesting rat cell line, there may be several mechanisms for the postnatal decline in AFP levels. In addition, albumin and AFP may or may not be linked in human HCC, as there is evidence that they are expressed in different cells [23]. The mechanisms for the cell growth-inhibiting actions of albumin are not fully elucidated, although some cell cycle-associated proteins have been found to be altered by its effects [16], as we confirm here. Albumin has been found to be decreased in both HCCs and peritumoral tissues, and the proliferation of HCC cells has been shown to be inhibited by overexpression of albumin [18]. A decrease in HCC albumin levels has also been the subject of other reports [20]. However, the reciprocal relationship between albumin and AFP has been reported by some [19] but not by others [24], although a relationship between AFP and tumor size has been reported [24]. Interestingly, albumin nanoparticles are being increasingly studied as carriers for cancer therapeutic agents [25–27].

In the experiments reported here, we show that in a large clinical cohort, lower albumin levels were associated with larger and more aggressive tumor parameters and higher AFP levels (Table 1). We then showed that addition of albumin to the culture medium of several HCC cell lines caused a decrease in cell growth, as well as a decrease in AFP protein (Fig. 1, 5). We found associated MAPK signaling pathway protein levels to be also decreased. However, the decrease in cell growth was not associated with significant induction of apoptosis or autophagy. This represents another example of HCC biology in relation to microenvironmental liver factors [28]. Whether the albumin-mediated growth decrease is a result of an inhibitory action on AFP or a direct effect on the cell proliferation machinery/signaling has yet to be determined. If these results are validated in another study, consideration might be given to albumin infusions as therapy for patients with hypoalbuminemia and HCC, as are already used for treating intractable ascites.

Disclosure Statement

The authors declare that there are no conflicts of interest to disclose.

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