Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6298

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (2):** 1695-1699 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Paracrine Interactions of Hepatoma Cells with Endothelial Cell and Fibroblastic Cells

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Received: 1.03.2018 | Revised: 25.03.2018 | Accepted: 7.04.2018

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most lethal human cancers with a high mortality rate. Various factors can contribute to the genetic alteration of hepatic cells into hepatoma cells, thus leading to HCC. Besides the genetics of tumor cells, tumor microenvironment plays an important role in HCC development and progression. In the current study, we investigated the interactions between hepatic tumor cell lines with endothelial and fibroblastic cells. Results showed that paracrine factors such as platelet-derived growth factor (PDGF) from endothelial cells enhance the proliferation of hepatoma cells. Thus, targeting endothelial cells in HCC could represent a promising therapeutic strategy for HCC.

Key words: *Hepatocellular carcinoma (HCC), Endothelial cells, Tumor microenvironment, Paracrine factors, Platelet-derived growth factor*

INTRODUCTION

HCC is one of the most common and lethal cancers worldwide. It represents approximately 4% of all malignancies. Different etiological factors such as hepatitis B and C virus, alcohol and diabetes cause liver injury followed by inflammation, necrosis and hepatocyte proliferation, thus leading to HCC. The microenvironment is an important component for HCC initiation and development[1,2].

Microenvironment components are known to both inhibit and augment the activity of cells of tumor cells[3]. The stromal environment of HCC in liver consists out of several cell types, including endothelial cells (ECs) hepatic stellate cells (HSCs), macrophages and immune cells[1]. ECs and HSCs in liver have been reported to play active roles by interacting with the tumor cells. Both these cells are known to regulate tumor growth through contact dependent and independent mechanisms. These contacts promote tumor angiogenesis metastasis, stemness, and epithelial-to-mesenchymal transition[4-6]. A study of these interactions is crucial to design novel holistic therapies for the treatment of HCC. In the current study, we studied effect of endothelial cells and stellate cells on the growth of hepatoma cells by addressing in vitro studies.

Cite this article: Siddiqui, H., Rawal, P., Kaur, S., Paracrine Interactions of Hepatoma Cells with Endothelial Cell and Fibroblastic Cells, *Int. J. Pure App. Biosci.* **6(2):** 1695-1699 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6298

MATERIAL AND METHODS

Cell cultures: HepG2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) with 10% FBS (Hyclone) and 100µg/ml streptomycin and 100 IU/ml penicillin at 37℃ in humified atmosphere containing 5% CO_2. LX2 cells (Hepatic stellate cells) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) with 2% FBS (Hyclone) and 100µg/ml streptomycin and 100 IU/ml penicillin at 37℃ with 5% CO_2. HUVECs cells (Human umbilical venules endothelial cells) were grown in Endothelial medium (HiMedia Laboratories) with growth factors and 1% antibiotics on gelatin coated plates.

Coculture of cells: To study how endothelial cells and stellate cells modulate the tumorigenic behaviour of HePG2, we performed the assays in indirect co-cultures. About 1 million Huh7 cells were treated with conditioned media (CM) from HUVECs/LX2 cells. CMs were prepared after serum starvation of these cells for 24h and then collecting the supernatants after centrifugation to remove cell debris. After 24 and 48 hours of CM co-cultures, we counted the absolute number of HepG2 cells per well per field. HepG2 cells alone were used as controls.

MTT assays: The MTT Cell Proliferation Assay measures the cell proliferation rate. For the assay, we plated 100,000 HepG2 cells per well in a 96-well plate and incubated with media alone of CM from HUVECs or LX2 cells for 24 or 48h. Then, we removed the medium and washed cells with PBS. MTT was then added in medium to a final concentration of 0.5 mg/mL and incubated for 3 hours at 37°C, until intracellular purple formazan crystals were visible under microscope. Finally MTT was removed and solubilizing solution was added. Absorbance was taken at 570nm after incubation at room temperature or 37°C for 30 minutes to 2 hours, until cells had lysed and purple crystals had dissolved.

ELISA Assays: HUVECs, LX2 cells and hepatoma cells were cultured with serum-free medium for 24 hours. The supernatant was collected and ELISA for vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) was performed using ELISA kits (Thermofisher Scientific) as per manufacturer's protocol. The optical density values were measured at 450 nm wavelength in fluorescence microplate reader (Synergy /H1). Standard curve was plotted to calculate the exact concentrations of VEGF and PDGF in the culture supernatants.

Statistical Analysis: All quantitative data have been expressed as mean ± standard deviation. Student's unpaired t-test was used to analyze difference between two groups. Statistical significance between groups was accepted for $p<0.05$(*)

RESULTS AND DISCUSSION

Increased proliferation of HepG2 cells after co-cultures with HUVECs: We first cultured the hepatoma cells, HUVECs and LX2 cells individually (Fig 1A). There was no significant change in the morphology of the hepatoma cells after the co-cultures (Fig 1B). The number of hepatoma cells in HUVEC co-cultures was significantly increased after both 24h and 48h ($P< 0.05$ for both 24h and 48h, Fig 1C). MTT assays also validated our observations and there was an increased absorbance in HepG2 cells which were co-cultured with CM form HUVECs after 24h and 48h ($P< 0.05$ for both, Figure 2). Results suggest that there is a mutual crosstalk between tumor cells and their microenvironment, which is essential in for both normal tissue homeostasis and for tumor growth. HUVECs would be secreting some paracrine factors which would be enhancing the growth of HepG2 cells. Recent studies by Chiew et al, 2015 have shown in co-culture models that physical supports from HepG2 cells are indispensable for the differentiation and remodeling of endothelial cells (7). In our study, we report that endothelial cells also stimulate the growth of tumor cells in a paracrine manner even in the absence of a physical contact with the hepatoma cells. We however did not observe significant effect of CM from LX2 on the growth of hepatoma cells (Fig 1C and 2). Previous studies have reported that normal fibroblasts restrict the

growth and progression of cancer in both contact dependent and independent manner while cancer associated fibroblasts can promote tumor growth via paracrine interactions (8-10). We however did not observe any growth inhibition or stimulation of hepatoma cell growth in our study.

HUVECs secrete PDGF in culture supernatants: To study the factors secreted by HepG2 cells, HUVECs and LX2 cells, we performed ELISA assays. In comparison to HepG2-CM, there was enhanced production of PDGF from the HUVECs-CM ($P < 0.05$). VEGF was also enhanced in the HUVEC-CM but maximum increase was observed in the LX2-CM as compared to the HUVEC-CM ($P < 0.05$, Fig 3). This implies that PDGF secreted by endothelial cells has a growth promoting function under the in vitro conditions. PDGF is a cytokine that plays a crucial role in tissue regeneration, stimulating cell growth, cell motility, and morphogenesis (11, 12). VEGF is known to have both growth stimulating and inhibiting properties for hepatocytes (13-15). It is known to promote the tumor progression through angiogenesis. We did not observe a significant increase of hepatoma cell proliferation in the LX2 co-cultures. Also, there was no substantial secretion of VEGF in CM from HUVECs. This may be due to the fact that we measured the factors only in the basal conditions and did not give them any other activation stimuli. Thus our culture conditions did not simulate the tumor microenvironment in vivo which is far more complex. Also, besides VEGF and PDGF, there may be other paracrine factors that may be involved in the activation of hepatoma cells.

In summary, our study describes that the PDGF released by neighboring endothelial cells increases proliferation of tumor cells under in vitro conditions. It would be worthwhile to validate these findings under in vivo settings where the tumor microenvironment is much more complex, as macrophages, neutrophils and lymphocytes are also recruited to the tumor stroma.

Figure Legends

Figure 1A: Cultures of different types of cells. HUVECs: Human umbilical vein endothelial cells. LX2 or HSCs: Hepatic stellate cells. B. Co-cultures of HepG2 cells with conditioned media (CM) from HUVECs and LX2 cells. C. Bar diagram showing the number of cells in control (HepG2 cells cultured with media alone), HepG2 cells with CM from HUVECs and LX2 cells. Data is represented as mean $\pm$ SD $P < 0.05$ is significant.

Figure 2: Bar diagram showing MTT assay OD at 540nm in HepG2 cells alone (control), HepG2 cells with CM from HUVECs and HepG2 cells with CM from LX2 cells. Data is represented as mean $\pm$ SD. $P < 0.05$ is significant.

Figure 3: Bar diagram showing the levels of VEGF and PDGF secreted in culture supernatants from HepG2 cells, HUVECs and LX2 cells. Data is represented as mean $\pm$ SD. $P < 0.05$ is significant.

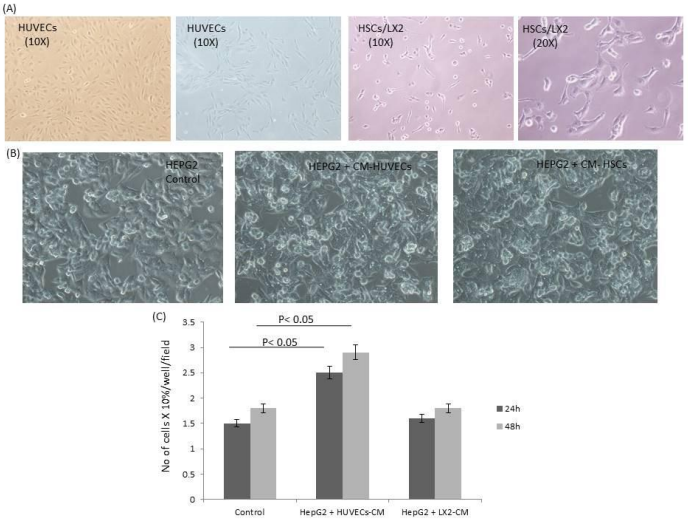

Fig. 1:

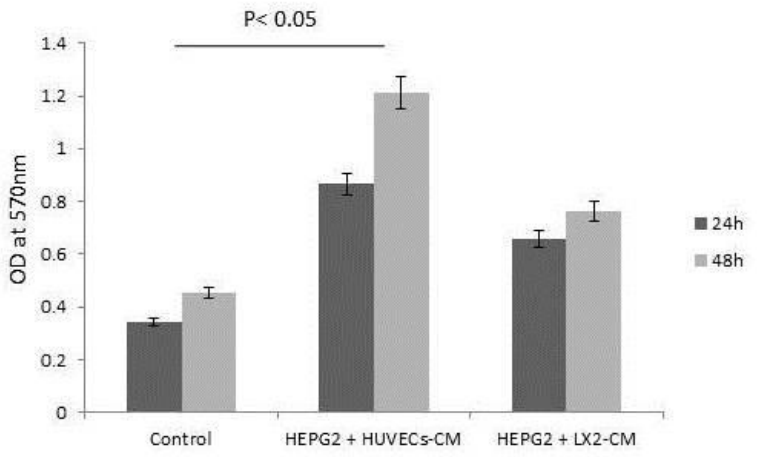

Fig. 2:

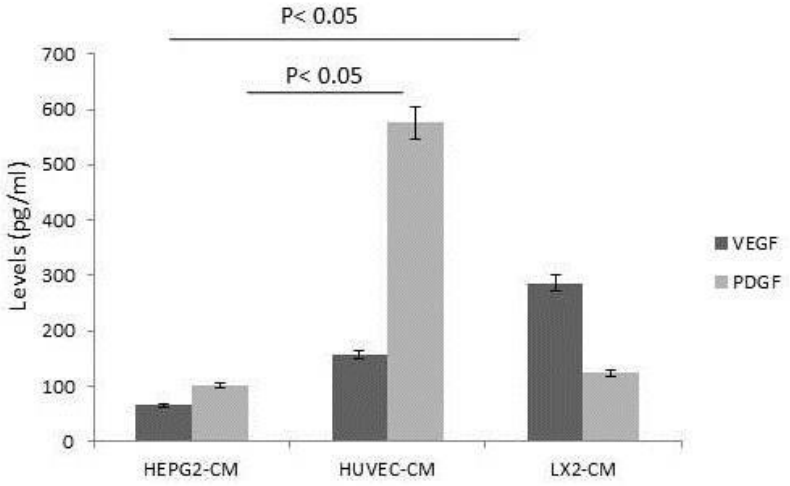

Fig. 3:

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