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***Basic Study***

**Effect of adipose derived mesenchymal stem cells on hepatocellular carcinoma: *In vitro* inhibition of carcinogenesis**

Serhal R *et al*. ADMSCs inhibits hepatocarcinogenesis

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**Abstract**

***AIM***

To investigate the effect of adipose derived mesenchymal stem cells (ADMSCs) and their conditioned media (CM) on hepatocellular carcinoma (HCC) cells tumorigenesis.

***METHODS***

The proliferation rate of HCC cancer cells, HepG2 and PLC-PRF-5 were measured using the trypan blue exclusion method and confirmed by the cell counting kit 8 (CCK-8). The apoptosis level was detected by flow cytometry using annexin V-FITC kit. Protein and mRNA expressions were quantified by means of ELISA and real time PCR respectively. Migration and invasion rates were performed by transwell migration and invasion assay. Wound healing test was assessed to endorse the data obtained with the transwell migration kit.

***RESULTS***

Our data demonstrated that when co-culturing HCC cell lines with ADMSCs or treating them with ADMSC CM the proliferation rate of cells was significantly inhibited with an increase in the apoptosis level of the two cancerous cell lines. The decrease in the proliferation rate was further accompanied with an upregulation of the mRNA level of P53 and Retinoblastoma and a downregulation of the mRNA levels of c-Myc and hTERT. More notably, ADMSCs and their CM suppressed the expression of the two important markers of carcinogenicity in HCC, alpha-fetoprotein (AFP) and Des-gamma-carboxyprothrombin (DCP). In addition, the migration and invasion levels of HepG2 and PLC-PRF-5 cells were significantly diminished possibly through increased expression of the tissue inhibitor metalloproteinases TIMP-1, TIMP-2 and TIMP-3.

***CONCLUSION***

These findings shed a new light on a protective and therapeutic role for ADMSCs and their CM in controlling the invasiveness and carcinogenesis of HCC.

**Key words:** Hepatocellular carcinoma; Adipose derived mesenchymal stem cells; Adipose derived mesenchymal stem cells conditioned media; Proliferation; Apoptosis; Migration; Invasion

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**Core tip:** In this study, we reported the *in vitro* effect of adipose derived mesenchymal stem cells (ADMSCs) on liver cell lines, HepG2 and PLC-PRF-5. It is the first study to demonstrate that ADMSCs and their respective conditioned media inhibited the two markers of cancer alpha-fetoprotein and Des-gamma-carboxy-prothrombin and decreased the invasiveness of the cancer cells by increasing the mRNA expression of tissue inhibitor metalloproteinases, TIMP-1, TIMP-2 and TIMP-3. In addition, ADMSCs significantly reduced the proliferation rate, the invasiveness and the migration of the cancerous cells while inducing their apoptosis.

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**INTRODUCTION**

Hepatocellular carcinoma (HCC) is the most common primary hepatic cancer that accounts for approximately 70%-80% of all primary liver cancers[1]. It is now considered as the second cause of cancer related mortality worldwide[2]. HCC development results from an imbalance between excessive cell growth and apoptosis, which is mainly regulated by P53, a tumor suppressor gene. The alterations in the expression or activation of this gene have been extensively reported in HCC and were related to hepatocarcinogenesis[3,4].

The detection of HCC at an early stage is crucial but difficult due to the presence of inflammation and liver damage. Several markers, such as Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP) (AFP-L3), Des-gamma-carboxy-prothrombin (DCP), Dickkopf-1, Midkine and microRNA are recommended as biochemical indicators in the diagnosis of primary liver cancer at different phases[5]. AFP however is used for monitoring liver cancer recurrence after treatment[6]. Late stages of HCC, more specifically HCC metastasis, is associated with upregulation of matrix metalloproteinases (MMP)[7,8], as these proteins are implicated in matrix degradation thus allowing malignant growth and cancer cell invasion(14, 15).

The treatment of HCC entails liver transplantation and/or other palliative modalities such as liver resection, local ablation, transarterial chemoembolisation (TACE), and systemic cytotoxic chemotherapy. Those treatments are limited by their toxicity towards normal tissues, by multifocal development and tumor[9](8). Hence, the development of new targeted therapies is needed to prevent HCC in a cirrhotic liver, or to restrain metastasis and abolish cancer invasiveness in this killer disease.

Recent accomplishments in stem cell (SC) research provide a new prospective in cell based therapy and tissue regeneration. Indeed, the interaction between mesenchymal SCs (MSCs) and cancer has been extensively studied. MSCs are adults, multipotent, non-hematopoietic cells having an auto renewal capacity and a multilineage potential. MSCs can be isolated from different sources such as bone marrow[10], umbilical cord[11], peripheral blood[12], placenta[13], and adipose tissue[14]. Adipose tissue remains the most abundant source. SCs are called intrinsic drug store, not only because of their differentiation capacity but because of their paracrine and trophic effects. Indeed, the exact role(s) that MSCs play in tumors modulation remains controversial. It was reported that MSCs promote cancer via immune suppression[15,16], the promotion of vasculature or angiogenesis[16,17], the stimulation of epithelial-mesenchymal transition[18], and their contribution of tumor microenvironment[19,20]. The use of bone marrow derived MSCs in a model of Kaposi sarcoma has been shown to exert anti-tumorigenic and pro-apoptotic effects via the suppression of Akt activity upon direct cell-cell contact[21]. In addition, it has been demonstrated that co-culturing of glioma cancer cells with cord blood MSCs induced apoptosis of the cancer cells[22]. Emerging evidence has established that MSCs may serve as vehicles to deliver therapeutic agents, such as cytokines, apoptosis inducers and prodrugs, and that they can be genetically engineered to produce antitumor molecules such as interferon β (INF β) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL)[23]. However, the antitumor properties of MSCs and their secretions are not yet clear. The role of MSC on HCC remains too controversial, and few reports study the effects of adipose derived MSCs (ADMSCs) on HCC.

The present work aims to investigate the effect of human ADMSCs and their conditioned medium on the carcinogenesis of HCC cell lines, through the modulation of proliferation, apoptosis, tumor markers expression, migration and invasion.

**MATERIALS AND METHODS**

***Cell lines and culture conditions***

The human HCC cell lines (HepG2/C3A/HB-8065, PLC-PRF-5/CRL-8024) were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States) in 2015. All cells were cultured in cancer cell media (CCM) as suggested by ATCC at 37 ℃ in low glucose DMEM media (1g/L glucose) (Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (Sigma Aldrich, Germany) in a humidified atmosphere containing 5% CO2 at passages 1 to 5.

***MSC isolation, primary culture***

Adipose tissues were obtained from healthy donors undergoing an elective liposuction procedure from abdominal, hip or thigh regions after consent in the department of plastic surgery, Hotel Dieu De France hospital, Beirut, Lebanon. Briefly, lipoaspirates were washed extensively with a saline solution then digested with type I collagenase solution (Sigma Aldrich, Germany) for 1-2 h at 37 ℃. After centrifugation, cell pellet was washed and filtered to remove debris. The synovial vascular fraction obtained was plated onto tissue culture flasks in DMEM nutrient mix F12 (Sigma Aldrich, Germany) containing 10% FBS (Sigma Aldrich, Germany) and 1% PS-amphotericin (PSA) then incubated at 37 ℃ and 5% CO2. After 48 h, non-adherent cells were removed; a fresh DMEM was added and replaced every 2-3 d. The isolated ADMSCs at passage 1 were used for ulterior experiences.

***Morphological observation***

ADMSCs and HCC cell lines morphology before and after coculture was observed under inverted microscope. Photos are taken at a magnification of 100 ×.

***Characterization of MSCs***

**Morphology and immunophenotyping:** ADMSCs at passage 1 have a fibroblast like morphology and were characterized by immunophenotyping using flow cytometry analysis. The following PE conjugated mAbs were used: anti CD73, anti CD29, anti CD44, anti CD45, anti CD31, anti CD106, anti CD34, anti CD90, and anti CD105 (BD, Biosciences, San Jose, CA, United States). Appropriate isotype controls were used at the same concentrations as the test antibody to determine non- specific staining.

**Multilineage differentiation of ADMSCs:** ADMSCs showed a differentiation capacity to become adipocytes, osteocytes, and chondrocytes. Adipogenic differenciation, osteogenic differentiation and chondrogenic differentiation was performed in the lab similarly as per our previous publication[24].

***Coculture of ADMSCs with HCC cell lines***

To determine the effect of ADMSCs on HCC cell lines, the HepG2 and PLC-PRF-5 were cultured directly in 6 well plates with ADMSCs and indirectly in inverted transwell cell culture insert for six-well plates (1-μm pore poly (ethylene terephthalate) (Corning, United States) at 1:1 and 2:1 ratio; in case of indirect coculture, ADMSCs were seeded in the apical compartment while the cancer cells in the basal compartment. In the direct co-cultured the number of cells seeded are mentioned in each specific experiment. In each and all conditions, cells were grown in DMEM F12 supplemented with 10% FBS in humidified atmosphere at 37 ℃ and 5% CO2. After 48 h, the media were removed and replaced with fresh DMEM F12. Finally, the supernatant is collected after 48 h and stored at -80 ℃ for ulterior ELISA analysis.

***Preparation of ADMSC*** ***conditioned media***

ADMSCs were grown in 75 cm2 flasks (Sarstedt, United States) with 10% FBS DMEM F12 (Sigma Aldrich, Germany) at 37 with serum free media. After 24 h, the conditioned media (CM) was collected, centrifuged, filtered and conserved at -80 ℃ until used.

***Treatment of cancer cells with ADMSC CM***

In all experiments where we used CM, cancer cell lines, HepG2 and PLC-PRF-5 cells were seeded in six well plates in CCM as described earlier [low glucose DMEM media (1g/L glucose) supplemented with 10% FBS and 1% PS]. After adherence, the supernatants of HepG2 and PLC-PRF-5 were removed and replaced with prepared ADMSC CM at different dilutions (1:1, 1:2, 1:4, 1:5, 1:10, 1:25, 1:50, 1:100, 1:200, 1:400) for 48 h. All dilutions were significant in respect to cancer markers and morphology. After 1:25 dilution, no differences in results were observed (data not shown). Thus, in all experiments ADMSC CM were diluted at (1:1, 1:5 and 1:25).

***Cell count assay***

HCC cells were harvested and counted with the trypan blue exclusion method using a hemocytometer.

***Proliferation test***

The effect of ADMSCs and ADMSCs CM on the proliferation of HCC cells was evaluated using a cell-counting kit 8 (CCK-8) (Sigma Aldrich, Germany) according to the manufacturer recommendations. The tetrazolium salt or WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium, monosodium salt] is cleaved into formazan by succinate-tetrazolium reductase, an enzyme that exists only in the mitochondrial respiratory chain and is active only in viable cells. The formazan production is proportional to the number of living cells in the culture. Briefly, HCC cells were cocultured with ADMSCs directly in 6 well plates, indirectly in 6 well plates 24-mm transwells (Corning, United States) or treated with ADMSC CM for 48 h. Then, the WST-8 was added into the wells after removal of transwells in case of indirect coculture. Finally, the absorbance was measured in triplicates at the absorbance 450 nm of reduced WST-8.

***Flow cytometry analysis of apoptosis***

To study the effect of ADMSCs on inducing apoptosis of HCC cells, an annexin V-FITC kit (MiltenyiBiotec, Germany) was used according to the manufacturer's instructions. Briefly, HCC cells were cocultured indirectly with ADMSCs in 6 well plates 24-mm transwells (Corning, United States) or were treated with ADMSC CM for 48h. Next, the transwell was removed and 106 of freshly obtained HCC cells were washed and resuspended in binding buffer. The cells were stained with annexin V-FITC, incubated in the dark for 15 min then binding buffer and propidium iodide solution were added. For each sample, 106 cells were analyzed by flow cytometry using MACSQuant analyzer device. Apoptosis was analyzed using the computer program MACSQuant software, and the percentage of apoptosis was determined and plotted.

***AFP AND DCP ELISA***

To test the level of AFP and DCP in cells supernatants, quantitative enzyme linked immunosorbent assay kit (HUMAN, Germany and CUSABIO, United States, respectively) were performed as per manual instructions. The optical density is determined using an ELISA plate reader at 450 nm.

***Wound healing assay***

For monolayer wound healing assay, ADMSCs (negative control), HCC cells (positive control), and HCC cells which are directly cocultured with ADMSCs or treated with ADMSC CM and all are seeded and cultured until > 90% confluence in 10% FBS DMEM F12 in 6 well plates (Corning, United States). By scratching the cells with a 20 μL pipette plastic tip, we stimulated three wounds per well. After washing the wells gently with PBS, the migrated cells into the wounded areas were monitored and photographed at 0 h, 6 h, 12 h, 24 h and 48 h. The distance of cell migration was measured using image J 1.48v software (Wayne Rasband, National Institutes of Health, United States) by comparing the images from time 0 to the last time point. The relative migration distance of cells is measured by the distance of cell migration/the distance measured at 0 h.

***Transwell migration assay***

The assay of HepG2/C3A and PLC/PRF/5 cell migration was performed using a Boyden chamber in a 24-well plate designed by CellBiolabs (United States) according to the manufacturer's recommendations. Briefly, for each condition, 106 cells were suspended in 1 ml of serum free DMEM. Then, 3 × 106 cells were added in the upper chamber of each well. The same medium supplemented with 10% serum was added to the lower chamber of each well as a chemo-attractant solution. After 24 h, the cells that migrated to the lower chamber of the wells were stained using a crystal violet cell staining solution. The stain is instantly dissolved once the kit extraction solution is added. The solution was then transferred to a 96-well microtiter plate, and the absorbance was measured at 560 nm using a plate reader.

***Transwell invasion assay***

The invasion ability of the HepG2/C3A and PLC/PRF/5 cells was assayed using a Boyden chamber in a 24-well plate designed by CellBiolabs (United States). According to the manufacturer's recommendations, all cells were incubated in serum free DMEM overnight. For each condition, 106 cells were suspended in 1 ml serum free DMEM. Then, 3 × 106 cells were added in the upper chamber of each well. The same medium supplemented with 10% serum was added to the lower chamber of each well as a chemo-attractant solution. After 48 h, the cells that invaded the bottom of the membrane were stained using a crystal violet cell staining solution. An extraction solution was then added, and the mixture was transferred to a 96-well microtiter plate. Finally, the absorbance was measured at 560 nm using a plate reader.

***RNA extraction and real time PCR***

Total RNA from cell cultures was extracted using QIAamp RNA extraction kit (Qiagen, Valencia, United States). cDNA was generated from 500 ng of total RNA with iScript Reverse Transcription Kit (Bio-Rad laboratories, CA, United States). Quantification of gene expression was conducted using iQ SYBR Green Supermix (Bio-Rad laboratories, CA, United States). The reverse transcription (RT) product was used to measure the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a positive housekeeping gene, AFP, TIMP-1, TIMP-2, TIMP-3, P53, RB, hTERT and c-Myc using real time PCR (qPCR) technique and specific primer sequences (Table 1). The reaction conditions were as follow: pre-denaturation at 95 ℃ for 3 min; 40 cycles of denaturation at 94 ℃ for 20 s, annealing and acquiring at 60 ℃ for 60 s. The threshold cycle (Ct) value for triplicate reactions was averaged, and the relative genomic expression was calculated by 2-∆∆Ct value [∆Ct= Ct (gene) - Ct (GAPDH)][25]. Melting curves were performed to ensure only a single product was amplified.

***Statistical analysis***

For immunophenotyping experiments, the values are presented as the mean ± standard error mean of the mean (SEM). For other experiments, data were expressed as mean ± standard deviation (SD). The differences between the groups were analyzed by student *t*-test using Graphpad prism online software and *P* < 0.05 was considered significant.

**RESULTS**

***Characterization and differentiation of ADMSCs***

The isolated ADMSCs at passage 1 showed a fibroblastic-like morphology (Figure 1A). Flow cytometry analysis showed a high expression of the positive markers CD73 (82.02% ± 4.84%), CD29 (90.28% ± 2.24%), CD44 (88.3% ± 1.78%), CD90 (93.19% ± 1.65%), CD105 (59.63% ± 8.13%), and a subtotal lack of expression of the endothelial and hematopoietic markers CD34 (2.17% ± 0.34%), CD31 (2.27% ± 0.43%), CD45 (2.18% ± 0.31%), and CD106 (2.31% ± 0.11%) (Figure 1B). Furthermore, our cells were capable to differentiate into adipocytes, chondrocytes and osteocytes (Figure 1C).

***ADMSCs inhibits the proliferation of HCC cell lines***

Unregulated cell proliferation is a fundamental abnormality in cancer development. Previous studies were controversial and demonstrated that MSCs either suppress or induce cell growth[26,27]. Here we aim to determine the effect of our ADMSCs and their secreted soluble factors on the proliferation and growth of cancer cells. Therefore, we cultured HCC cell lines, with different conditions: HepG2 and PLC-PRF-5 alone (control) or with ADMSCs directly or indirectly (at a ratio HCC: ADMSCs of 1:1 or 2:1) or treated them with different dilutions of ADMSC CM for 48h. Morphological observation revealed a considerable inhibition in the cell numbers of the two types of cancer cells, in the presence of ADMSCs, either in direct or indirect cocultures. This inhibition was less remarkable when cancer cells were treated only with ADMSC CM (Supplementary Figure 1). Using cell count assay and WST-8 proliferation test, our results showed that ADMSCs in indirect coculture reduced the number of HCC cells (Figure 2A) and inhibited their proliferation (Figure 2B) as compared to control cells with *P* < 0.001.

In direct coculture, we couldn't discriminate between the proliferation of HCC cells and ADMSCs (data not shown), knowing that the microscopic observation shows an enormous inhibition of cancerous cell number (Supplementary Figure 1). Similarly, the ADMSC CM, undiluted or diluted 5 × or 25 ×, significantly reduced the number of HepG2 and PLC-PRF-5 cells (Figure 2A), and inhibited the proliferation of HepG2 cells (Figure 2B) with *P* ≤ 0.001, while only the undiluted ADSMC CM was capable of reducing the proliferation of PLC-PRF-5 cell (Figure 2B) with *P* = 0.001.

***Effect of ADMSCs on of HepG2 and PLC-PRF-5 cell apoptosis***

Resistance to cell death or apoptosis is a crucial process in malignant cells. It has been shown that BMSCs (bone marrow derived MSCs) induce apoptosis and cell cycle arrest in G0/G1 phase[27]. In order to elucidate the mechanism of growth suppression by ADMSCs, HepG2 and PLC-PRF-5 cells were cultured alone (control), indirectly co-cultured with ADMSCs or treated with ADMSC CM. Apoptosis was assessed by flow cytometry after removal of ADMSCs in the case of coculture. Our results showed that ADMSCs significantly increased the apoptotic rate of HepG2 (21.54% ± 4.1% *vs* control = 1.94% ± 0.3%, *P* < 0.05) and of PLC-PRF-5 (2.91% ± 0.2% *vs* control = 0.5% ± 0.1%, *P* < 0.001). As shown in Figure 2C-E, the apoptosis of HepG2 and PLC-PRF-5 was also significantly increased when treated with undiluted ADMSC CM, as compared to control cells (11% ± 0.5% *vs* control, *P* < 0.01 and 3.8% ± 0.15% *vs* control, *P* < 0.01, respectively).

***Effect of ADMSCs on the expression of AFP and DCP***

To monitor the malignant status of HCC cell lines, the levels of biochemical markers AFP and DCP, were measured in the supernatant of ADMSCs (negative control), HCCs cells (positive control), and HCCs cells co-cultured directly with ADMSCs or treated with ADSMCs CM. The ADMSCs in the negative control did not express AFP neither DCP (data not shown). As illustrated in Figure 3, we found that the AFP protein level is dramatically declined upon co-culturing HepG2 and PLC-PRF-5 cells with ADMSC as compared to control cells (*P* < 0.001). Similar results were obtained when HepG2 cells and PLC-PRF-5 cells were treated with the different concentrations of ADMSC CM (*P* < 0.001 and *P* < 0.05, respectively, Figure 3A).

Next, we verified if the AFP was also repressed at the mRNA level. Our data show that ADMSCs and their CM reduce significantly the mRNA AFP expression in HepG2 and PLC-PRF-5 cells (*P* ≤ 0.001 and *P* < 0.05 respectively, Figure 3B).

In addition to AFP, we also assessed the level of DCP in HCC cells alone (control) co-cultured with ADMSCs or treated with ADMSC CM. We observed that ADMSCs significantly decreased the levels of DCP in HepG2 and PL-PRF-5 cells (*P* < 0.001). ADMSC CM, undiluted or diluted 5 times, significantly reduced the DCP secretion by HepG2 cells *(P* < 0.001). On the other hand, only the undiluted ADMSC CM decreased significantly the level of DCP in PLC-PRF-5 cells only at a dilution 1:1 (*P* < 0.001) (Figure 3C).

***ADMSCs and ADMSCs CM reduce migration and invasion of HCC cell lines***

Cell migration and invasion are important processes in tumor development and metastasis. Bone marrow derived MSCs are previously found to promote microvascular HCC[28]. Conversely, they are showed to inhibit tumor invasion[26]. Thus, we tested whether ADMSCs could alter the migration of liver cancer cells using the wound healing assay. The migratory rate of wounded cells was measured at different times (0 h, 6 h, 12 h and 24 h). As shown in Figure 4A and B, the migration rate of HepG2 and PLC-PRF-5 cells was inhibited dramatically when co-cultured directly with ADMSCs for 24 h as compared to when cultured alone (control) (*P* < 0.001). As well, the ADMSCs CM, undiluted or diluted 5 times significantly reduced the migration rate of HepG2 (*P* < 0.001). However, the ADMSCs CM had no effect on the migratory rate of PLC-PRF-5 cells (Figure 4A-C).

The effect of ADMSCs on cell migration was confirmed using the transwell migration technique. As shown in Figure 4D, the ADMSCs and their CM decreased significantly the migration rate of HepG2 cells (*P* < 0.01). ADMSCs inhibited also the migration rate of PLC-PRF-5 (*P* < 0.001, Figure 4D).

Using the transwell invasion assay, our data show that the invasive capacity of HepG2 cells was significantly reduced when co-cultured with ADMSCs at 1:1 and 1:2 ratios and ADMSC CM, as compared to control cells (*P* < 0.05). Conversely, ADMSCs had no significant effect on the invasion capacity of PLC-PRF-5 cells (Figure 4E).

***Tissue inhibitor metalloproteinases are overexpressed by HCC cell lines***

Migration and invasion are initially regulated by the dysregulated expression of MMPs and tissue inhibitor metalloproteinases (TIMPs). To examine if TIMPs contribute to the decrease in the migration and invasion capacity of HepG2 and PLC-PRF-5 cells upon their culture with ADMSCs or ADMSC CM, we studied the expression of TIMP-1, -2 and -3 by real time PCR. We observed that ADMSCs and their cultured media increased significantly the mRNA level of TIMP-1, -2 and -3 in HepG2 (Figure 5A) and of only TIMP-1 and -3 in PLC-PRF-5 cells (*P* < 0.05, Figure 5).

***Expression of P53, RB, c-Myc and hTERT***

Excessive proliferation and resistance to cell death are regulated by deactivation of tumor suppressor genes and activation of oncogenes. P53 and RB are two tumor suppressor genes implicated in the regulation of apoptosis and cell cycle[29,30]. C-Myc, a proto-oncogene and growth regulator, is overexpressed in HCC[31]. Human telomerase reverse transcriptase (hTERT), the catalytic unit of telomerase, is highly expressed in HCC[32]. In order to assess the influence of ADMSCs and their CM on tumor suppressors genes and growth regulators, HepG2 and PLC-PRF-5 cells were cultured alone (control), cocultured with ADMSCs or treated with ADMSC CM and the mRNA levels of their P53, RB, c-Myc and hTERT were measured using RT-PCR. In HepG2 cell lines, we found that ADMSCs and their CM significantly induced the expression of RB, and P53 genes while decreasing significantly mRNA expression of hTERT (*P* < 0.05). The mRNA expression of c-Myc remained unchanged (Figure 6A). In PLC-PRF-5 cells, however, ADMSCs and their CM significantly upregulated the mRNA levels of c-Myc and RB while reducing the mRNA expression of hTERT (*P* < 0.05), and unchanging the mRNA level of P53 unchanged as compared to control cells (Figure 6B).

**DISCUSSION**

HCC is a malignant condition with higher incidence and no effective treatment[33]. Adipose derived SCs have been proven therapeutic efficacy in many types of diseases[34–37]. Their CM is shown to inhibit proliferation and increase apoptosis in HCC[38]. Adipose derived SCs secrete anti-inflammatory cytokines and growth factors and have immunomodulatory effects however many controversies have been noted concerning their role in cancer. Therefore, the effect of ADMSCs and their CM on cancer is not clear. In our study, we have investigated the role of ADMSCs and their CM in HCC through two cell lines HepG2 and PLC-PRF-5. ADMSCs were capable of inhibiting cell proliferation, hindering the diagnostic cancer markers AFP and DCP and promoting apoptosis. In addition, ADMSCs were able to decrease migration and invasion of cancerous cells through the increase in TIMPs. Thus, we suggest that ADMSCs might offer an alternative cell-based therapy for patients enduring from HCC.

There are a lot of findings concerning the effect of MSCs on cancer cells, sometimes contradictory within the same type of cancer or between varying types of cancer[39]. ADMSCs can be recruited by prostate cancer cells and stimulate tumor growth through increasing tumor vascularity[40]. It was reported that the interaction of MSC with tumor cells contribute to gastric carcinoma[41] in addition the co-culture or co-injection of MSCs with osteosarcoma cells enhance tumor growth in mice and promote proliferation of osteocarcinoma. Furthermore, ADMSCs support breast tumor growth and progression[42] but can inhibit proliferation of pancreatic cancer cells *in vitro* and *in vivo*[40]. MSC can also inhibit tumor growth in Kaposi’s sarcoma[43], colon cancer[44,45], hepatoma[46,47], prostate[48,49], pancreatic[50,51] , lung cancer[47] and other tumor models[52]. Similar controversies were reported concerning the effect of the SCs secretome. For example bone marrow-MSC CM has either anti-tumor effects on non-small lung cancer cells[53] or stimulatory effects on myeloma cells[54]. Whereas adipose tissue SC CM (ADMSC CM) had no effect on human glioblastoma cancer SCs subpopulations[55]. In addition it has been demonstrated that human umbilical cord embryonic SC CM (hUCESC–CM) has an anti-tumor effects on proliferation, apoptosis and tumor-cell invasiveness[56]. These findings confirm that in certain type of cancer MSC could enhance tumor growth and in some others it can inhibit the invasiveness and metastasis[26,57,58]. This might be explained by the complexity of MSC source, the malignant cell type being involved, and the interaction between the MSCs and the tumor cells. The number and the microenvironment of MSCs might also influence the growth or the inhibition of the tumor cells. A recent review by Hill *et al*[59] focused mainly on the key mechanisms in which MSCs are differentiated into tumor associated MSCs (TA-MSCs) and cancer associated fibroblasts (CAFs) promoting pro-metastatic and growth states when in contact with tumor microenvironment. Despite the described pro-metastatic role of MSCs when in contact with a tumor microenvironment, many other studies reported that when LEAD MSCs are in contact with cancer cells, it might reduce tumorigenecity[60,61]. The paracrine effects of SCs, their trophic effects when in contact with a stimulatory environment might provide them the potential to be anti-tumorigenic. We think that the type of cancer might dictate the secretions profile of SCs. Therefore, the microenvironment, cell to cell interaction, and origin of MSCs contribute and direct MSCs to be tumorigenic or anti-tumorigenic.

No studies involve the direct effect of ADMSCs on the proliferation and apoptosis of HCC. In our study, ADMSCs caused the inhibition of proliferation and cell death of the cancerous cells when co cultured with HepG2 and PLC-PRF-5. Zhao W *et al*[38], reported that the ADMSCs CM, inhibited the proliferation and increased apoptosis of HCC cell lines. In our data, the inhibition was more significant and was approximately abolished with ADMSCs more than the CM. We think that this is due to a mechanism underlying cell to cell contact, suggesting that the interactions of receptors ligands on ADMSCs and cancer cells contribute to the inhibition of proliferation and thus trigger cell death. This mechanism of cell-cell interaction should be further investigated. In addition, the upregulation of the tumor suppressor gene P53 and RB and down regulation of c-Myc and hTERT might also contribute to the overall scene of tumor suppression. However, tumor suppression is not confined only to inhibition of proliferation by cell to cell interaction and the CM of SCs, it is more complicated and cancer liver cell killers are ready for invasiveness and metastasis. Metalloproteinases play major roles in the progression and metastasis of numerous cancers including HCC[62]. This could suggest that inhibiting metalloproteinases might be one of the possible ways to decrease invasiveness and metastasis of HCC.

We, solely, report in this present study the increase in the secretion of the tissue inhibitor metalloproteinases TIMP-1, -2, and -3, by ADMSCs or ADMSC CM which might be responsible in part to the decrease in migration and invasion of HCC cells. The inhibition of invasion is also explained by the inhibition of the secretion of TGF-β (data not shown) which is well known to be associated with the decrease of metastasis and invasiveness[63] by a feedback mechanisms. We aim in future studies to demonstrate the exact role of these factors especially TIMPs on migration and invasion of cancer cells using specific siRNA.

The increase of secretion of TIMPs in the presence of ADMSCs or ADMSC CM might be a part of the strategic fight that SCs exert to restrain tumor invasion. Other important steps in ADMSCs strategic fight is the stimulated decrease in AFP and DCP. AFP and DCP have been used as serum markers in patients with HCC and as detectors of tumor progression and malignant proliferation respectively. When ADMSCs were co-cultured with HCC cell lines both AFP and DCP were significantly decreased which might be an indication of SCs attempt to work on the proliferation and tumor progression. This is the first report to demonstrate a decrease in DCP in HCC cell lines. Our *in vitro* results will be confirmed subsequently in an *in vivo* study. The primary results of the pilot study prove the effect of ADMSCs on tumor growth and AFP level (data not shown).

To conclude our study, report novel molecules contributing to the effect of adipose derived SCs on HCC. The increase in TIMPs and in apoptosis, the inhibition in proliferation and invasiveness, the decrease in AFP and DCP are all processes and coordination attempt from SCs as a strategic management, utilizing all resources, here the microenvironment and cell to cell contact to accomplish their mission of inhibiting tumor cell proliferation, progression and invasiveness.

**ARTICLE HIGHLIGHTS**

***Research background***

Hepatocellular carcinoma (HCC) is a malignant condition with higher incidence and no effective treatment. Mesenchymal stem cells (MSCs) secrete cytokines and growth factors known to have paracrine, trophic and immunomodulatory effects. Due to their paracrine and differentiation potential, adipose derived SCs have been proven therapeutic efficacy in many types of diseases. Their conditioned media (CM) is shown to inhibit proliferation and increase apoptosis in HCC. However, many controversies have been noted concerning their role in cancer.

***Research motivation***

Many studies showed the effect of SCs or their CM on cancer, some reports showed suppressing and inhibiting effect on tumor growth. Other reports showed enhancing tumor growth and proliferation of cancerous cells. No known study has reported the effect of adipose derived MSCs (ADMSCs) on the proliferation and apoptosis of HCC. Thus, our aim was to investigate the therapeutic effects of Adipose derived SCs and their CM on HCC. In Specific the ADMSC effect on cancer cell markers, on the proliferation and metastatic potential of cancerous cells, and their effect on modulating cancer cell death.

By discovering that adipose derived SCs and their CM have the ability to modulate cancer markers, the proliferation and metastasis of liver cancer cells, we might open a new path for research on the mechanism of action by which MSCs can affect cancer. If the results were to increase and stimulate cancer cells, then further investigations need to be pursued on two levels: (1) to study the behavior of SCs along with the factors contributing to the stimulatory effects; and (2) try to inhibit the pathways leading to this progressive effect. In another hand, if the ADMSC were to inhibit cancer and induce apoptosis then ADMSC could be a potential therapy for this deadly disease, when today no cure is yet has been discovered. To achieve this goal in vivo animal models and clinical studies needs to be pursued.

***Research objectives***

In our study our main objective was to investigate the role of ADMSCs and their CM in HCC using two cell lines HepG2 and PLC-PRF-5. In specific, we wanted to study the effect of ADMSCs on alpha-fetoprotein (AFP) and Des-gamma-carboxyprothrombin (DCP), the capacity of ADMSC to modulate metastasis or proliferation of the above cancerous cell lines, in particular we studied TIMPs, P53, and RB. ADMSCs were capable of inhibiting cell proliferation, hindering the diagnostic cancer markers AFP and DCP and promoting apoptosis. In addition, ADMSCs were able to decrease the migration and invasion of the cancerous cells through the increase in TIMPs. Realizing these objectives, had shed light on a novel apoptotic and abolishing effect of MSCs on cancer. This will direct us, and other researchers to further investigate the effect on other cell markers playing role in cancer and the mechanisms by which the ADMSCs exert their anticancerous effects.

***Research methods***

HCC cell lines purchased from ATCC were cultured in low glucose DMEM media. Adipose derived MSCs isolated from lipoaspirates were cultured in DMEM nutrient mix F12. The isolation method of MSCs was modified and improved to obtain a high yield of living ADMSCs using a minimal quantity of fat and collagenases. Isolated ADMSCs were characterized to demonstrate their viability and capacity of multilineage differentiation.

The coculture conditions and treatment with ADMSC CM were extensively studied in order to determine the number of cells that should be used in all experiments. After co-culturing HCC with ADMSCs or stimulating it with ADMSC CM, the protein and mRNA levels of HCC cancer markers (AFP and DCP) were detected using ELISA kits and real time PCR respectively.

In addition, the proliferation level and apoptosis rate of HCC cells were measured using WST-8 proliferation test and annexin V-FITC kit respectively. Along with these tests, the mRNA levels of P53, RB, hTERT and c-Myc genes involved in the regulation of proliferation and apoptosis, were quantified using real time PCR.

Furthermore, using the wound healing assay and the migration and invasion test, we studied the effect of ADMSCs and their CM on the metastasis of HCC cell lines. In parallel, the mRNA level of tissue inhibitor metalloproteinases (TIMPs), was measured using real time PCR. TIMPs have been reported to play a major role in in inhibiting metastasis.

In all assays, the experiments were repeated at least three times in order to obtain statistically significant results.

***Research results***

In our study, ADMSCs inhibited cancer cell proliferation and increased cancer cell death when co cultured with HepG2 and PLC-PRF-5. This effect was more significant in case of direct co-culture probably due to cell-cell interactions. The upregulation of the tumor suppressor gene P53 and RB and down regulation of c-Myc and hTERT might be the factors responsible of the mentioned findings. The mechanisms of these results should be further investigated.

We, solely, reported the increase in the secretion of tissue inhibitor metalloproteinases TIMP-1, -2, and -3 which might be accountable in part by the decrease in migration and invasion of HCC cells. Future studies should be performed to confirm this relation, in addition further investigations are needed to study the involvement of the metalloproteinases (MMP-2 and MMP-9) in the inhibition of metastasis. We also found that ADMSCs and ADMSCs CM decreased the migration and invasion of HCC cell lines.

A decrease in AFP and DCP levels were found after coculturing HCC with ADMSCs or stimulating HCC with ADMSC CM. This might be an indication of SCs attempt to obliterate proliferation and tumor progression. These findings will be confirmed and used subsequently in an in vivo animal study.

***Research conclusions***

This Study reported many novelties in the effects of ADMSC on HCC. This is the first report to demonstrate a decrease in DCP in HCC cell lines. No other study investigated the direct effect of ADMSC on the proliferation and apoptosis of HCC. We reported novel molecules contributing to the effect of adipose derived SCs on HCC in particular, TIMPs. We reported that ADMSC when co cultured with HepG2 and PLC-PRF-5 has an anticancerous effect. This is explained by the inhibition of proliferation and cell death of the cancerous cells. We also showed for the first time the effect of direct cell to cell interactions which is a new mechanism by which ADMSC might be abolishing completely the proliferation of tumor cells. The indirect contact of ADMSC with HCC cell lines inhibited the proliferation, metastasis and increased the apoptosis to a lesser extent than the direct coculture suggesting that the paracrine effects of ADMSC contribute to the antitumor effects.

These findings confirm that in certain type of cancer MSC could enhance tumor growth and in some others, it can inhibit the invasiveness and metastasis. This might be explained by the complexity of MSC source, the malignant cell type being involved, and the interaction between MSCs and tumor cells. The number and the microenvironment of MSCs might also influence the growth or the inhibition of tumor cells.

***Research perspectives***

Our findings will lead to many perspectives investigations: (1) to investigate the mechanism of cell to cell contact by which ADMSCs inhibit cancer cells (autophagy); (2) to investigate what are the factors exerting the inhibitory effects. In particular, the effect of STC-1 and DKK-1 molecules known to have a role in the suppression of genes involved in proliferation, migration and invasion and in the overexpression of apoptotic genes. These results should be demonstrated by the WST-8 proliferation assay, apoptosis annexin /PI assay, and migration and invasion tests.

*In vivo* study will be pursued to confirm our results mainly the effect of ADMSC and their conditioned medium on tumor growth, apoptosis and metastasis, as well as the paracrine effects of ADMSC.

In order to understand and quantify the changes in hepatic cancer cell morphology when in direct contact with ADMSC, a study will be conducted in collaboration with the Department of Physics at the American University of Beirut. This will also help us determine the mechanism by which ADMSCs induce HepG2 cell death.

In summary, ADMSCs are cells with complex mechanisms having the capacity to interact with adjacent cells exerting trophic and paracrine effects thus altering the microenvironment. Their role in each disease must be vigorously studied in order to elucidate their therapeutic effects. In this study, we determined the inhibitory effects of ADMSC on cancer cell markers and on the key factors known to play a major role in inflammation, invasion and metastasis of cancer.

We think our study shed a new light on the role of ADMSC on HCC.

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**Figure 1 Characterization of adipose derived mesenchymal stem cells.** A: The microscopic observation (magnification 100 ×) showed that the isolated adipose derived mesenchymal stem cells (ADMSCs) have a fibroblast like morphology. B: Immunophenotyping of ADMSC using flow cytometry analysis. The flow cytometry histograms of the ADMSCs at passage 1 for a representative donor are displayed. The percentage of positively stained cells is indicated in the middle right section of each histogram. The green line indicates the positively stained cells; whereas the purple line indicates the isotype-matched monoclonal antibody control. Histograms showed that ADMSCs were positive for (B-a) CD73, (B-b) CD44, (B-c) CD90, (B-d) CD105, (B-e) CD29, and were negative for (B-f) CD45, (B-g) CD31, (B-h) CD106, (B-i) CD34. C: Differentiation capacity of ADMSCs incubated for 3 wk in adipogenic, chondrogenic and osteogenic medium. (C-a) Representative images of adipogenic differentiation marked by the formation of intracellular lipid droplets colored using Oil Red O compared to control. (C-b) Solid chondrogenic micromass colored by Alcian blue indicating the presence of glycosaminoglycans. (C-c) Representative images of osteogenic differentiation confirmed by the presence of calcium deposit colored by Alizarin Red. All immunophenotyping and differenciation experiments were repeated four times.

**Figure 2 Effect of adipose derived mesenchymal stem cells and their conditioned media on the proliferation and apoptosis of hepatocellular carcinoma cells.** Hepatocellular carcinoma (HCC) cells (2 × 105) were seeded in 6 well coculture plates in the presence or absence of adipose derived mesenchymal stem cells (ADMSCs) and ADMSC conditioned media (CM), undiluted or diluted 5 times and 25 times for 48 h. The proliferation of HCC cells HepG2 and PLC-PRF-5 were measured by (A) cell count assay and (B) WST-8 proliferation test. The apoptosis of HepG2 (C) and PLC-PRF (D) cells co-cultured as above, was measured by flow cytometry using Annexin V/PI test kit. C and D: Two representative experiments of apoptosis in HepG2 and PLC-PRF cells, respectively. E: The average rate of apoptosis in HepG2 and PLC-PRF-5 induced by ADMSCs, ADMSC CM, and data here is representative of three independent experiments, each repeated in triplicates. All data are represented as mean ± SD (a*P* < 0.05, b*P* < 0.01, c*P* < 0.001). CTR: Control; ADMSC: Adipose derived mesenchymal stem cell; CM: Conditioned media.

**Figure 3 Expression of hepatocellular markers in hepatocellular carcinoma cells.** Hepatocellular carcinoma (HCC) cells (2 × 105) were seeded in 6 well coculture plates in the presence or absence of adipose derived mesenchymal stem cells (ADMSCs) and ADMSC conditioned media (CM), undiluted or diluted 5 times and 25 times for 48 h. The protein levels of alpha-fetoprotein (AFP) (A) and Des-gamma-carboxyprothrombin (DCP) (C) was measured in the cell supernatant by ELISA. B: The mRNA of AFP was assessed by quantitative PCR. Results were displayed as percentage of controls. Data are represented as mean ± SD of five independent experiments, each repeated in triplicates (a*P* < 0.05, b*P* < 0.01, c*P* < 0.001). CTR: Control; ADMSC: Adipose derived mesenchymal stem cell; CM: Conditioned media.

**Figure 4 Effect of adipose derived mesenchymal stem cells and their conditioned media on migration and invasion of hepatocellular carcinoma cells.** Hepatocellular carcinoma (HCC) cells (2 × 105) were seeded in 6 well co-culture plates in the direct presence or absence of adipose derived mesenchymal stem cells (ADMSCs) (at ADSMCs: HCC ratio of 1:1 or 1:2) or ADMSC conditioned media (CM), undiluted or diluted 5 or 25 times. The migration level of (A) HepG2 and (B) PLC-PRF-5 cells was assessed by wound healing assay. The migration rate at 24 h is represented in (C). D: A transwell migration assay was performed to confirm the results of wound healing assay. E: The invasiveness of HCC cells was measured by transwell invasion assay. In the transwell migration and invasion assay, 3 × 105 HCC cells alone, co-cultured with ADMSCs, or treated with ADMSC CM were seeded into the apical chamber of transwell plates and allowed to migrate or invade through the uncoated polycarbonate membrane or collagen coated polycarbonate membrane respectively (8 μm pore size) to the lower chamber for 24 and 48 h respectively. Then the migratory or invasive cells were stained by crystal violet cell stain solution and extracted using an extraction solution provided in the kit. The level of migration and invasion was measured using a plate reader at the absorbance of 560 nm. Values shown are representative of five independent experiments each repeated in triplicates. Data are represented as mean ± SD of five independent experiments, each repeated in triplicates (a*P* < 0.05, b*P* < 0.01, c*P* < 0.001). CTR: Control; ADMSC: Adipose derived mesenchymal stem cell; CM: Conditioned media.

**Figure 5 Effect of adipose derived mesenchymal stem cells and adipose derived mesenchymal stem cells conditioned media on mRNA levels of tissue inhibitor metalloproteinases in hepatocellular carcinoma cells.** Hepatocellular carcinoma (HCC) cells (2 × 105) were seeded in 6 well co-culture plates in the presence or absence of adipose derived mesenchymal stem cells (ADMSCs) or of undiluted ADMSC conditioned media (CM). The mRNA level of TIMP-1, TIMP-2, and TIMP-3 in HepG2 (A) and PLC-PRF-5 (B) cells after the removal of ADMSCs in the case of coculture was measured by quantitative-PCR. Data are represented as mean ± SD of five independent experiments, each repeated in triplicates (a*P* < 0.05, b*P* < 0.01, c*P* < 0.001).ADMSC: Adipose derived mesenchymal stem cell; CM: Conditioned media.

**Figure 6 Expression of tumor suppressor genes and oncogenes in hepatocellular carcinoma cells**. Hepatocellular carcinoma (HCC) cells (2 × 105) were seeded in 6 well co-culture plates in the presence or absence of adipose derived mesenchymal stem cells (ADMSCs) and undiluted ADMSC conditioned media (CM) for 48 h. The mRNA expression of tumor suppressor genes P53/RB, oncogene c-Myc and the enzymatic component of telomerase hTERT were assessed in (A) HepG2 and (B) PLC-PRF-5 cells after removal of ADMSCs in the case of co-culture by RT-PCR. Data are represented as mean ± SD of five independent experiments, each repeated in triplicates (a*P* < 0.05, b*P* < 0.01, c*P* < 0.001). ADMSC: Adipose derived mesenchymal stem cell; CM: Conditioned media.

**Table 1 List of primers sequences for real time PCR**

|  |  |
| --- | --- |
| **Primer** | **Sequence** |
| GAPDH F | 5'- GCACCACCAACTGCTTAGCA -3' |
| GAPDH R | 5'- CTTCCACGATACCAAAGTTGTCAT -3' |
| AFP F | 5'- CAGCCACTTGTTGCCAACTC -3' |
| AFP R | 5'- GGCCAACACCAGGGTTTACT -3' |
| TIMP-1 F | 5'- GACCAAGATGTATAAAGGGTTCCAA -3' |
| TIMP-1 R | 5'- GAAGTATCCGCAGACACTCTCCAT -3' |
| TIMP-2 F | 5'- AGGCGTTTTGCAATGCAGAT -3' |
| TIMP-2 R | 5'- TCCAGAGTCCACTTCCTTCTCACT -3' |
| TIMP-3 F | 5'- CAGGACGCCTTCTGCAACTC -3' |
| TIMP-3 R | 5'- AGCTTCTTCCCCACCACCTT -3' |
| P53 F | 5'- CAAGCAATGGATGATTTGATGCT -3' |
| P53 R | 5'- TGGGTCTTCAGTGAACCATTGT -3' |
| RB F | 5'- GCAAATTGGAAAGGACATGTGA -3' |
| RB R | 5'- GAAACTTTTAGCACCAATGCAGAA -3' |
| C-Myc F | 5'- CACCACCAGCAGCGACTCT -3' |
| C-Myc R | 5'- TTCCACAGAAACAACATCGATTTC -3' |
| hTERT F | 5'- GACGTAGTCCATGTTCACAATCG -3' |
| hTERT R | 5'- CGTCCAGACTCCGCTTCATC -3' |