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Original Research

Trafficking mechanism of bone marrow-derived mesenchymal stem cells toward hepatocellular carcinoma HepG2 cells by modulating Endoglin, CXCR4 and TGF-β

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Abstract: Mesenchymal stem cells (MSCs) display differential migration ability toward different tumor-released factors. Migration of MSCs is highly important in induction of proliferation and invasiveness of hepatocellular carcinoma (HepG2) cells. In this study, the role of CXCR4/CXCL12 axis and TGF-βR signaling were evaluated in the migration of MSCs toward HepG2 cells. The MSCs were incubated with SDF-1α (CXCL12), antagonists of CXCR4, TGF-βR, and co-receptor of TGF-β, (endoglin) for 48h. Then, the migration of these cells toward HepG2 cells was analyzed using in vitro migration assay. SDF-1α at a concentration of 100nM MSCs revealed the highest migration rate toward the conditioned medium (1.62 fold compared to the migration of un-treated MSCs; $p<0.05$). Applying combination of the antagonists against CXCR4, TGF- βR, and co-receptor of TGF-β decreased the migration rate significantly (4.51 fold; $p<001$). Western blot analysis confirmed that RhoA activity is a core modulator in migration pathway. This study demonstrated that CXCR4 and TGF-βR signaling are important for migration of MSCs toward HepG2 cells. Identifying the key mediators in the migration of MSCs toward hepatocellular carcinoma cells and then development of the therapeutic inhibitors against these factors can be considered as an essential strategy in suppression of tumor progression and metastasis.

Key words: Mesenchymal stem cells, Migration, SDF-1α, TGF-β, CD105, HCC.

Introduction

Mesenchymal stem cells (MSCs), as a lineage of adult stem cells, have been known to reside in various tissues including bone marrow, adipose tissue, umbilical cord blood, and placenta (1-4). Inherent capacity of MSCs for self-renewing and differentiation is the ability makes them attractive candidates for cell therapy and regenerative medicine (5). Besides, the migratory capacity of MSCs toward tumor-derived factors has been shown for different tumor cells (6, 7). There are two possibilities in migration of MSCs toward tumor microenvironment. First, recruitment of MSCs into tumor area induces tumor cells proliferation and aggressiveness. It has been shown that conditioned medium from MSCs induces proliferation of HepG2, Hela, and MCF-7 cells (8, 9). MSCs also accelerate hepatocellular carcinoma (HCC) metastasis and invasiveness both in vivo and in vitro via induction of epithelial-mesenchymal transition (EMT) (10). Then, blockade of MSCs migration toward tumor microenvironment can be considered as a valuable therapeutic strategy to limit cancer progression. The migratory capacity of MSCs can compromise these cells as carriers for the delivery of biological anti-tumor agents into tumor sites (11). For this purpose, modified MSCs were applied to express anti-tumor factors against key modulators of tumor progression. MSCs transfected with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for activating external apoptotic pathway, herpes simplex tyrosine kinase (HSV-tk) for targeted effect of ganciclovir, interleukin-12 (IL-12) for enhanced cell mediated immunity against tumor, and sodium iodide symporter (NIS) for iodine radionuclide accumulation in tumor area have been used on rodent hepatocellular carcinoma models (12-15). Stromal derived factor-1α (SDF-1α) or CXCL12 is a well-known chemokine and highly important for homing of MSCs especially in bone marrow. MSCs express both SDF-1α and its receptor, CXCR4 suggesting an autocrine regulation of activity (16, 17). Transforming growth factor-β (TGF-β) which is produced by alternatively activated macrophages and some tumors, can induce tumor angiogenesis and attenuate immunity against tumor (18). TGF-β has been verified to mediate the trafficking of bone marrow-derived MSCs toward glioma cancer stem cells. Moreover, the relative role of endoglin (CD105) as one of TGF-βR co-receptors in the migration of MSCs toward glioma stem cells has been identified(19). Whereas hepatocellular carcinoma cells and HepG2 cells were known to secrete TGF-β. MSCs express TGF-βR, which is important as a key participant in the migration of MSCs toward HCC (20-22). Despite, various studies suggest tumor-derived factors as mediators for recruitment of MSCs toward tumors microenvironments; the distinct mechanism by which MSCs migrate toward HCC is unclear (23). This study addressed the role of CXCR4/CXCL12 and TGF-βR/CD105 signaling as key potent axis in the migration of MSCs toward HepG2 cells.

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Materials and Methods

Materials

Human bone marrow-derived MSCs and HepG2 cells were purchased from Pasteur Institute Cell bank (Tehran, Iran). CXCR4 inhibitor (AMD3100), TGF-βR inhibitor (GW788388), CD105 inhibitor (SB431542) and ECM gel coated insert were obtained from (Sigma-Aldrich St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) Medium, penicillin–streptomycin and fetal bovine serum (FBS) were gained from Invitrogen (Auckland, New Zealand). Cell culture inserts were provided from (SPL life sciences, Korea). Rabbit polyclonal anti p-RhoA (Ser 188) was obtained from Santa Cruz Biotechnology (USA).

Cell culture

The human hepatocellular carcinoma (HepG2) cells were provided from Pasteur Institute cell bank (Tehran, Iran). Cells were cultured in DMEM medium containing 10 % fetal bovine serum, 100 U/ml penicillin and 100 lg/ml streptomycin at 37_ C in a humidified 5 % CO2 atmosphere. Two million cells were seeded in a 25 cm^2 flask with growth medium for at least 48hr. Starvation medium (DMEM containing 0.1% BSA, ≥96% fatty acid free) was added for the last 48 hr before the migration experiment.

Conditioned media collection

HepG2 cells were cultured in 10 cm dishes. After reaching 90% confluence, the media was removed and the dishes were washed by phosphate buffered saline (PBS). Ten ml of DMEM containing 1% bovine serum albumin (BSA; fatty acid free) was added and after 24 hours of incubation, the media was removed. The cells were washed with PBS and then 10 ml of DMEM containing 1% BSA was added. After 24 h incubation, the conditioned media was collected followed by a cell counting for each dish. Collected conditioned media was centrifuged in 1,500 rpm to remove any debris and filtered using 0.22µm filter (JET-Biofil, Korea) and stored at -20˚C(24).

Transwell migration assay

The collected HepG2 conditioned media or starvation media (DMEM containing 0.1% BSA) were placed in the lower chamber of 6.5 mm cell culture inserts (SPL life sciences, Korea). ECM gel coated insert (SIGMA, St. Louis, MO) was used at a final concentration of 0.7 mg/ml in DMEM. MSCs (2×105) were plated in upper chamber with 200 µl of starvation medium or increasing concentrations of SDF-1α (SIGMA, St. Louis, MO) up to 100nM. After 48h of incubation, the inserts were picked up and the ECM gel was scraped gently using a cotton swab and the inserts were washed with PBS and exposed to paraformaldehyde (3.7% v/v) for 5 min followed by a 30 min exposure to crystal violet (0.05% g/v in distilled water and 0.45µm filtered). The inserts were washed using PBS twice and the migration was evaluated by counting the number of migrated cells per 10 high power fields and calculating the average (x 400) by Cell Counter software (Borland Software Corporation, Scotts Valley, CA, USA). Each experimental group was repeated three times.

Real-time quantitative PCR (RT-qPCR)

The total RNA was extracted from cultured MSCs using Trizol reagent according to the manufacturer's protocol. The amount of RNA was measured by optical density (A260/A280 ratio) with Nano Drop 1000 Spectrophotometer (Wilmington,DE,USA). Amplification of each cDNA was performed for the 25 cycles that permitted detection of basal mRNA levels in the linear range of each mRNA. Real time PCR amplification was carried out for 35 cycles using the following protocol: 95˚C for 1 min, 94˚C for 15 s, 52.5˚C for 20 s, 72˚Cfor 20s and 72˚Cfor 5 min. specific primers for RhoA forward primer (5'- CCATCATCCTGGTTGGGAAT-3') and reversed primer (5'- CCATGTACCCAAAAGCGC -3': were recycled for PCR. The PCR products were exploited for electrophoresis on agarose gel and optimized with internal control GAPDH.

Western blot analysis

To detect phosphorylated RhoA levels, cells at a density of $5x10^5$ were incubated for 24h. Then, they treated with the same conditions mentioned in the RT-qPCR. After that, cells lyzed at 4°C in a buffer containing 50mM Tris, 20mM NaCl , 200 µl Np40 in a final concentration of 20ml (pH=8). Fifteen µl of protease inhibitor cocktail (7x) was mixed with 750 µl of lysis buffer and then (1X) lysis buffer was added to each flask. Cells were detached by scrapper, and then placed on rotator for 25min dissected by centrifugation for 20 min. The supernatant were collected, and the protein concentrations were determined using BCA protein assay kit (pierce, Rockford, IL). Equal quantities of protein (35 µg per sample) were disconnected by electrophoresis in 12.5% SDS–PAGE and relocated to nitrocellulose membrane. After blocking with 10% skimmed milk for 1h, proteins, incubated with rabbit phosphate polyclonal antibodies of p-RhoA and β-actin at 4 °C overnight. The membranes further incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Finally, immunoreactive protein bands were developed with the ECL system. Normalization of western blot was ensured by β-actin as a loading control. Western blot quantification was performed using Image J software 1.48.

Statistical analysis

Data were exhibited as mean ± standard deviation of three independent experiments. A one way analysis of variance (ANOVA) followed by tukey's multiple comparison test were performed to determine the significance of differences between control and treatment groups, $p< 0.05$ was considered as statistically significant.

Results

SDF-1α enhanced the migration BM-MSCs to HepG2 cells

The migration of un-treated BM-MSCs toward HepG2 CM (positive control) was significantly higher than that toward starvation medium (negative control; $p<0.001$). Treatment of BM-MSCs with SDF-1α increased the migration rate toward HepG2 CM in a dose dependent manner. SDF-1α (100 nM) caused the highest

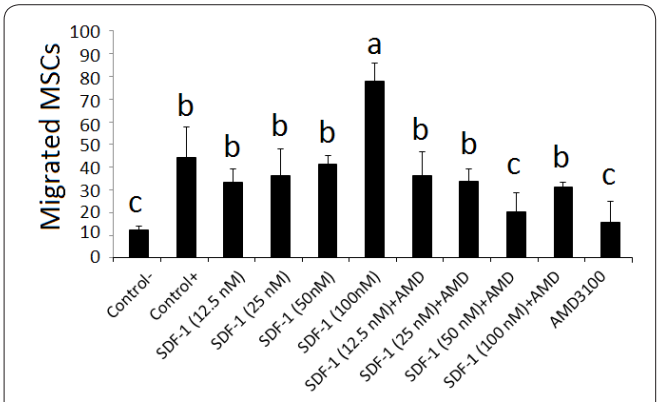

Figure 1. Effect of interactions on SDF-1α/CXCR4 axis on the migration of BM-MSCs toward HepG2 cells. Negative control: migration of un-treated MSCs toward starvation medium; positive control: migration of un-treated MSCs toward conditioned medium of HepG2 cells; AMD or AMD3100: CXCR4 inhibitor.

migration rate toward conditioned medium (Figure 1).

Endoglin, CXCR4 and TGF-β inhibitors involved in trafficking BM-MSCs to HepG2 cells

AMD3100 as a CXCR4 inhibitor, decreased the recruitment of MSCs toward conditioned medium to compared the negative controls ($p<0.05$). The administration of TGF-βR antagonist, GW788388, and CD105 antagonist, SB431542, attenuated the movement of MSCs toward the conditioned medium of HepG2 cells. The combination of the three inhibitors (AMD3100+GW788388+SB431542) caused a marked decrease in the migration rate of MSCs toward HepG2 cells ($p<0.001$). However, there was no significant difference in migratory effect of these cells with negative control group (Figure1, 2). In addition changes in quantification of MSCs investigated under light microscopy. Exposure of cells to 100 nM SDF-1α for 24 hours resulted in increased the number of cells and noticeable effect on the cellular granularity in the populations of MSCs (Figure3.e). When MSCs were treated with combination of the three inhibitors, we observed significantly decreased in number cells (Figure3.f). However, the number of migration MSCs toward HepG2 cells was significantly higher than that toward to starvation medium (Figure3.c, d).

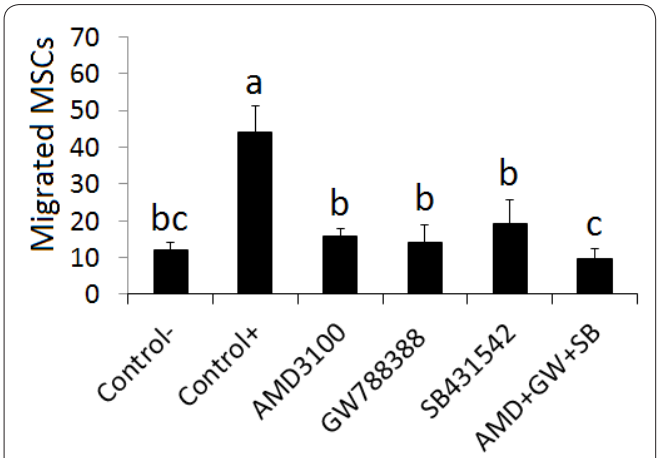

Figure 2. Effect of interactions on CXCR4, TGF-βR, and CD105 on the migration of BM-MSCs toward HepG2 cells. Negative control: migration of un-treated MSCs toward starvation medium; positive control: migration of un-treated MSCs toward conditioned medium of HepG2 cells; AMD or AMD3100: CXCR4 inhibitor; GW or GW788388: TGF-βR inhibitor; SB or SB431542: endoglin inhibitor.

The effects of inhibitors on the mRNA levels of RhoA

Our results from real time RT-PCR showed that applying neither SDF-1α nor inhibitors caused significant effect on RhoA mRNA expression. However, there was a trended decrease in the expression levels of RhoA gene when MSCs treated with GW788388 and triplex mixture of inhibitors ($P\geq0.05$) (Figure 4a).

SDF-1α provoked p-RhoA levels in western blot analysis

Western blot study demonstrated that SDF-1α at 100nM markedly increased the p-RhoA level (Figure 4b). In addition, AMD3100, SB431542 and GW788388

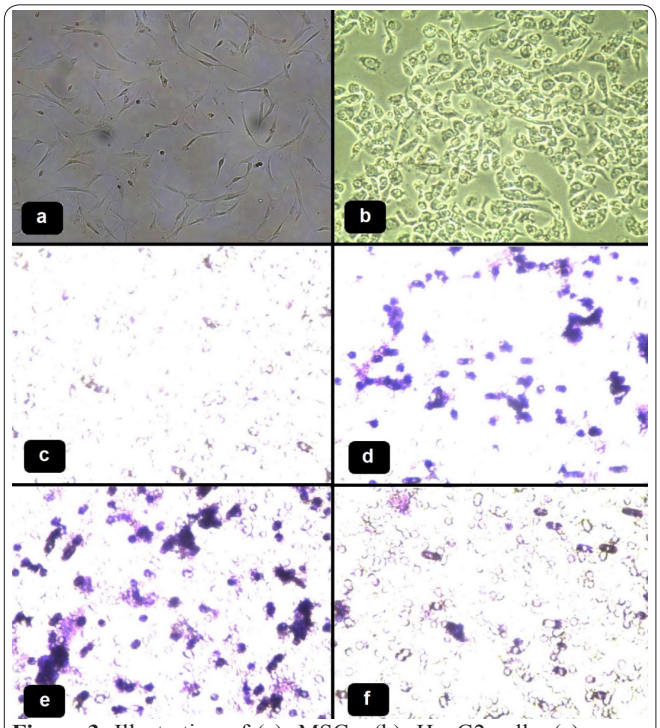

Figure 3. Illustration of (a): MSCs; (b): HepG2 cells; (c): negative control or migration of un-treated MSCs toward starvation medium; (d): positive control or migration of un-treated MSCs toward HepG2 CM; (e): migration of MSCs treated with SDF-1α at 100nM toward HepG2 CM; (f): migration of MSCs treated with the combination of AMD3100, GW788388 and SB431542 toward HepG2 CM. (c-f, crystal violet stained; x400).

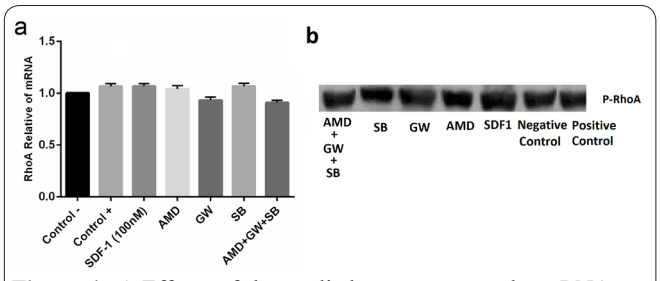

Figure 4. a) Effects of the applied traetments on the mRNA expression levels of RhoA. Neither SDF-1 nor the inhibitors caused signifcant change in relative expression of RhoA when GW788388 and the mixture of the inhibitors caused a trended decrease in the expression of RhoA. **b)** Western blot analysis of RhoA activity `Negative control: MSCs cultured in starvation medium; positive control: MSCs incubated with the conditioned medium of HepG2 cells; AMD or AMD3100: CXCR4 inhibitor; GW or GW788388: TGF-βR inhibitor; SB or SB431542: endoglin inhibitor.

did not cause significant change compared to control groups. Furthermore, we observed a significant decrease in p-RhoA level when treated with the mentioned inhibitors simultaneously ($p<0.05$).

Discussion

MSCs have been emerged as a novel and promising target population for therapy of various degenerative diseases, immune disorders and cancer progression protocols due to their immunosuppressive, pro-angiogenic and metastatic properties. In response to tumor stimuli, MSCs migrate to cancer tissue sites to participate in development and metastatic progressions**.** Although MSCs have been investigated in pre-clinical studies for immunomodulation therapy after liver transplantation (25), signal pathway of the potential effect of MSCs on HCC progression in tumor microenvironment is still need to be understood. Recent studies revealed that migration of MSCs toward HepG2 cells was mediated by SDF-1α/CXCR4 and TGF-β/TGF-βR axes. The chemokine SDF-1α, is one of the most important factors for MSCs homing as it has been known as the key mediator of MSCs migration in wound healing (26). In tissue repair, genetically modified MSCs with overexpression of CXCR4 as a SDF-1α receptor, have been shown to migrate to injury sites more efficient than the normal MSCs (27). In addition, several in vitro and in vivo studies showed that TGF-β is the key modulator in the recruitment of BM-MSCs toward glioma cancer stem cells (28). The cellular migration involves numerous intracellular reactions which finally eventuate in some rearrangements of cytoskeleton proteins and actin polymerization in response to the (29). TGF-β and CXCR4 activate Rho A, a member of small GTPase molecules leading to active downstream molecule ROCK-1, one of the best known Rho associated kinase are recognizing pathways exerting changes in cytoskeleton and actomyosin assembly. On the basis of present study, the proposed signaling for trafficking of MSCs toward HepG2 cells is schematically illustrated in figure 5. Rho A is activated in downstream of G-protein coupled receptors (GPCR) and through non-SMAD signaling of TGF-βR. The mitogen-activated protein kinase-1 pathway which can be triggered in response to GPCR or TGF-βR activation, causes cytoskeleton rearrangement but probably this pathway is not as important as the RhoA pathway (30, 31). Although it has been revealed that MSCs may support cancer invasiveness and stimulate cancer metastasis, further study on the detailed role of MSCs in tumor progression and its mechanisms is still required to explore in in vitro and animal models. In this study we investigated the role of TGFß, CXCR4 and endoglin in migration BM-MSCs to HepG2 cells and revealed that blocking cross-talk between MSCs and tumor cells have beneficial effects on clinical prognosis. It has been shown that zoledronic acid decreases breast cancer metastasis by inhibiting cancer cells and MSCs interaction (32). To investigate the differentiation potential of BM-MSCs, we applied inhibitors of TGFß, CXCR4 and endoglin for determination of mechanism of action in migration to cancer cells. Although the increase in the migration rate of the cells when they were incubated with SDF-1α (up to 50 nM) was not statistically significant, SDF-1α (100 nM) caused the highest migration rate toward conditioned medium (Figure 1). Co-treatment SDF-1α and AMD3100 led to no significant differences in comparison to AMD3100 administration solely, except for the concentration of 50 nM SDF-1α which diminished the migration level markedly. SB431542as a specific inhibitor of endoglin, decreased the migration level milder than GW788388 as TGF-βR inhibitor or AMD3100 as CXCR4 chemokine receptor inhibitor. Figure 3 represents the number of the migrated MSCs in different conditional treatment and the stained inserts for striking groups. Cristal violet staining MSCs demonstrated the minimum number cells in treatment with combination of AMD3100, GW788388 and SB431542 (Figure3.f). Previous studies already showed that applying AMD3100, GW788388 and SB431542 down-regulates RhoA, ROCK-1 and MLC signaling pathways (33, 34). However, we investigated RhoA gene expression as a core modulator of this pathway. The applied antagonists did not significantly decrease the mRNA expression levels of RhoA which suggests that post translational modification mechanisms such as phosphorylation may be involved in the activation of RhoA. To better understand the molecular mechanism responsible for the RhoA activation, our western blot analysis confirmed that TGF-βR and CXCR4 signaling pathways culminated in RhoA phosphorylation. Our finding demonstrated that combination of these factors are necessary to arrest migration and require to more understand the molecular mechanism involve between MSCs and tumor progression. In Conclusion, it is expectable that inhibiting MSCs' migration toward HCC by blocking CXCR4, TGF-βR, and CD105 serve as efficient adjuvants along with other pharmaceutical therapeutics in order to alleviate the negative effect of MSCs on HCC condition. Our results suggest that identifying the patients with high SDF-1α and TGF-β expression and then stable inhibition of these key factors can be applied as effective adjuvants to improve the efficacy of chemotherapeutic agents in cancer patients.

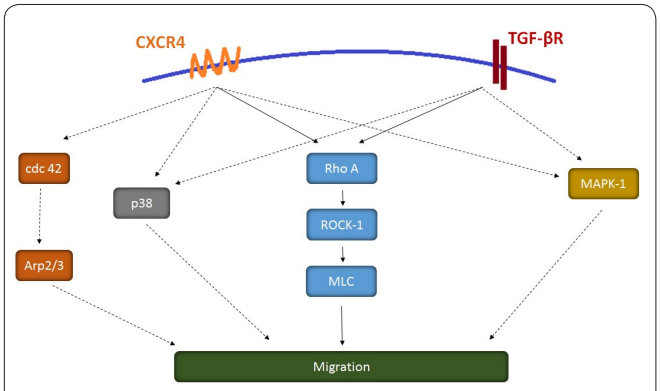

Figure 5. Schematic illustration of signaling pathways involving in the recruitment of MSCs toward HCC HepG2 cells**.** The pathway involving the RhoA GTPase and ROCK-1 is of high importance and MAPK-1, p38, and Arp2/3 pathways may have relative roles in the migration of MSCs toward HCC HepG2 cells.

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