肝星形细胞分泌的趋化因子CXCL1在肝癌转归中的作用

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摘要 该文探讨了人肝星形细胞(hepatic stellate cells, HSC)对肝癌细胞(HepG2、SMMC-7721)的迁移、侵袭能力和上皮–间质转化(epithelial-msenchymal transition, EMT)的影响及其机制。采用条件培养法培养肝癌细胞, 利用细胞划痕和Transwell实验分析肝星形细胞对肝癌细胞的迁移和侵袭作用。Western blot分析肝星形细胞自身及其分泌到培养液中趋化因子CXCL1[chemokine (C-X-C motif) ligand 1]和肝癌细胞的CXCL1受体2——CXCR2(CXCL1 receptor 2)的表达量, 以及条件培养下肝癌细胞中p-PI3K、p-AKT、p-GSK-3β和Snail的表达变化。激光共聚焦显微术和Western blot检测肝癌细胞上皮标志物E-cadherin、间质标志物N-cadherin和Vimentin的表达变化。结果显示, 在HSC中大量表达并分泌趋化因子CXCL1, 而肝癌细胞HepG2、SMMC-7721中高表达CXCR2。肝癌细胞通过条件培养后, 细胞形态改变, 细胞黏附下降, 细胞迁移和侵袭能力增强, 上皮标志物E-cadherin蛋白表达下调、间质标志物N-cadherin蛋白和Vimentin蛋白表达上调, PI3K/AKT信号通路中的关键成员PI3K和AKT磷酸化水平上调, p-GSK-3β和转录因子Snail表达上调。在肝癌细胞条件培养下加入CXCR2受体的特异性抑制剂(SB265610)后, 肝癌细胞上皮标志物E-cadherin蛋白表达上调、间质标志物N-cadherin蛋白和Vimentin蛋白表达下调, p-PI3K、p-AKT、p-GSK-3β和Snail的表达下调。以上结果说明, 肝星形细胞可能通过CXCL1/CXCR2轴活化PI3K/AKT信号通路并最终促进肝癌细胞上皮–间质转化。

关键词 肝癌; 上皮–间质转化; 肝星形细胞; 生长调节性癌基因-α; CXCL1受体2; 信号通路

The Effect of Chemotactic Factor CXCL1 Secreted by Hepatic Stellate Cells on Hepatic Carcinoma Outcomes

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Abstract This research investigated the influence of human hepatic stellate cells (HSC) on the migration, invasion and EMT of hepatocellular carcinoma (HCC) cells and underlying mechanism. HCC cells (HepG2, SMMC-7721) were co-cultured with conditional medium of HSC or HSC themselves. Cell migration and invasion were detected by methods of cell wound healing and Transwell chamber assays. Product of CXCL1 in HSC and

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HSC-conditional medium (HSC-CM), the expression of CXCR2 (CXCL1 receptor 2) in HCC cells, and p-PI3K, p-AKT, p-GSK-3β and Snail in conditional cultivated HCC cells were detected by Western blot. The changes of epithelial markers, E-cadherin, mesenchymal markers, N-cadherin and Vimentin in HCC cells were detected by laser scanning confocal microscopy (LSCM) and Western blot. The results showed that HSC produced much chemokines CXCL1, while HCC cells (HepG2, SMMC-7721) highly expressed CXCL1 receptor CXCR2. Moreover, their morphology changed, adhesion ability decreased, migration and invasion ability enhanced, the expression of epithelial marker E-cadherin was downregulated and mesenchymal markers N-cadherin and Vimentin were up-regulated in conditional cultivated HCC cells. Furthermore, the phosphorylation levels of the important members of PI3K/AKT signal pathway, PI3K and AKT were up-regulated, as well as levels of p-GSK-3β and transcription factor Snail under conditional cultivation. In contrast, the expression of epithelial marker E-cadherin was up-regulated, but mesenchymal markers N-cadherin and Vimentin were down-regulated, and intracellular p-PI3K, p-AKT, p-GSK-3β and Snail were down-regulated, when the conditional cultivated HCC cells were treated by CXCR2 inhibitor (SB265610). Our results suggested that hepatic stellate cells promoted epithelial-mesenchymal transition of HCC cells through activating PI3K/AKT signaling pathway by CXCL1/CXCR2 axis.

Keywords hepatocellular carcinoma; epithelial-mesenchymal transition; hepatic stellate cells; growth-related oncogene-alpha; CXCL1 receptor 2 (CXCR2); signaling pathway

肝癌转移和复发严重影响术后生存率, 是临床常见并急需解决的难题。肝星形细胞位于肝窦周Disse腔隙内, 约占肝脏所有非实质细胞的13%[1]。在正常的肝组织中, 肝星形细胞处于静息状态。肝脏一旦受到炎症、化学、病理等因素刺激后, 肝星形细胞被激活。活化的肝星形细胞作用于肝癌细胞, 促进肝癌细胞生长、迁移和侵袭[2-5]。它与肝癌细胞进行双向调控: 一方面, 活化的肝星形细胞诱导肝癌发生及其转移侵袭; 另一方面, 肝癌细胞又作用于肝星形细胞使其进一步活化, 进而加剧肝癌恶化[6]。从HSC收集条件培养基(HSC-conditional medium, HSC-CM)培养单层肝癌细胞, 可显著诱导肝癌细胞增殖和迁移; 在共培养体系中, HSC促进肝癌生长和侵袭并减少中央坏死的程度[4]。此外, 研究还证明, 癌内肝星形细胞与肝癌细胞共培养后, 使肝癌细胞活力和生存力、迁移和侵袭能力增加并伴随获得上皮–间质转化(epithelial-mesenchymal transition, EMT)的表型[7]。

趋化因子是诱发EMT的关键分子, 癌细胞发生EMT, 是其获得迁移浸润能力的关键步骤[8-9]。趋化因子CXCL1[chemokine (C-X-C motif) ligand 1]又称为黑色素瘤生长刺激因子(melanoma growth stimulating activity, MGSA)或生长调节性癌基因α(growth-related oncogene α, GROα), 是一类与细胞相关的ELR$^+$(吸引中性粒细胞及诱导血管新生)CXC族趋化因子, 与CXC趋化因子受体2(CXCR2)的结合力最强。研究表明, 趋化因子CXCL1与PI3K/AKT信号通路关系密切, CXCL1可结合并激活G蛋白偶联受体CXCR2, 活化PI3K信号通路, 通过酪氨酸激酶磷酸化激活AKT和下游蛋白GSK-3β/Snail, 最终导致上皮细胞间质化[10-13]。此外, 很多肝脏疾病均可诱导肝细胞和肝星形细胞高度表达CXCL1, 最终导致肿瘤发生、发展、侵袭、转移以及不良预后[14]。本研究主要是通过肝星形细胞体外条件培养肝癌细胞来研究趋化因子CXCL1活化PI3K/AKT信号通路并诱导肝癌细胞上皮–间质转化。

1 材料与方法

1.1 细胞株及培养所需试剂抗体

人肝癌细胞系HepG2、SMMC-7721购于中国科学院上海生命科学研究院细胞库。肝星形细胞LX2购于香港大学临床实验中心。肝癌细胞培养用DMEM(dulbecco's modified eagle's medium)培养基(HyClone公司)加10%胎牛血清(FBS, Gibco公司)。肝星形细胞用RPMI-1640(Gibco公司)加10% FBS, 均按1:3比例传代培养至细胞长满。二甲基亚砜(DMSO)购自Sigma公司。聚偏二氟乙烯膜(PVDF膜)、Transwell小室购自Millipore公司。兔抗人E-cadherin抗体、N-cadherin抗体、Vimentin抗体、GROα抗体、CXCR2抗体、PI3K(Phospho-Y467/Y199)抗体、

AKT(Phospho-S473)抗体、GSK-3β(phospho-S9)抗体及SNAI1(A242)抗体均系多克隆抗体, 且均购自Bioworld公司(1:500稀释)。鼠抗人GAPDH抗体系单克隆抗体购自Bioworld公司(1:10 000稀释)。CXCR2受体抑制剂SB265610购自Sigma公司(5 mg, 使用浓度为1 μmol/L)。Martrigel胶购自BD公司。

1.2 肝星形细胞条件培养基的收集和细胞间接共培养

肝星形细胞条件培养基(HSC-CM)的收集是将HSC铺在Corning培养皿中并置于37 ℃、5% CO_2孵箱内进行常规培养, 待细胞长至80%密度后, 弃旧培养液, 加无血清的RPMI-1640, 培养48 h后, 1 000 r/min离心5 min, 收集上清, 0.22 μm过滤, 并冷冻在–20 ℃(一星期内使用)。细胞共培养采用Corning公司的六孔板及配套0.4 μm孔径细胞培养小室。培养基是无血清的RPMI-1640。上层接种HSC, 密度为5×10^4/mL, 下层分别接种HepG2和SMMC-7721细胞, 密度均为2.5×10^5/mL。HSC细胞分别与HepG2、SMMC-7721细胞共培养72 h, 共培养后的肝癌细胞用于迁移、侵袭实验和Western blot分析。

1.3 肝癌细胞的迁移实验

细胞的迁移实验将设三个组, 分别是常规组、条件培养基组和共培养组, 将已长到80%的各组细胞消化计数, 均匀的铺到24孔板(1×10^5/孔), 每组设3个复孔。次日, 待细胞长满后, 用消毒过的直尺垂直于24孔板的竖线, 用20 μL的加样枪头紧贴直尺轻轻划一痕。用PBS洗2次, 除去划痕上的细胞, 加新鲜不含血清培养基常规培养。显微镜下在0, 24, 48, 72 h各拍照一次, 观察细胞的迁移情况。

1.4 肝癌细胞的侵袭实验

细胞的侵袭实验分组同迁移实验, 小室上层Matrigel胶: DMEM培养基以1:6比例(最佳比例)冰上稀释, 100 μL/孔, 铺成单层, 37 ℃温箱40 min。将条件分组培养好的细胞消化制备细胞悬液, 密度为5×10^5/mL。取细胞悬液每孔400 μL加入Transwell小室孔径8 μm, 24孔板中。下室加600 μL含10% FBS的培养基, 放入孵箱培养24~28 h后, 用棉签在小室内层轻轻擦试胶, 用甲醇固定10 min, 苏木精染核8~10 min, 伊红染胞质3~5 min, 最后用70%乙醇洗涤2次。在20倍显微镜下拍照, 每个小室至少拍3~5个视野, 计数穿越的细胞个数, 取均数±标准差作统计分析。

1.5 激光共聚焦免疫荧光实验

激光共聚焦扫描显微镜专用的玻底皿用多聚赖氨酸包被24 h, 将常规和HSC-CM培养后的肝癌细胞计数, 每个皿传1×10^5细胞, 用DMEM加含10% FBS培养。2~3 d后, 用4%多聚甲醛进行室温固定20 min。预冷的PBST洗3次, 5 min/次。3% BSA室温封闭1 h, 对非特异性位点进行了封锁。预冷的PBST洗3次, 5 min/次, 加入一抗(用BSA分别按不同比例进行稀释), 4 ℃孵育过夜。次日用PBS洗涤3次后, 用带有荧光基团的二抗温育2 h, 用DAPI(4,6-二脒基-2-苯基吲哚, Sigma公司)染色细胞核15 min。预冷的PBS洗2次, 5 min/次; 加入500 μL PBS, 4 ℃避光保存。激光共聚焦扫描显微镜进行观察并保存、分析。

1.6 Western blot分析

采用Centricon离心超滤管(Millipore公司)对肝星形细胞条件培养基进行浓缩。另外, 细胞总蛋白的提取是将细胞裂解缓冲液和蛋白酶抑制剂混合(Beyotime公司)后裂解细胞10 min。用BCA浓度测量法(Beyotime公司)对提取的细胞总蛋白样品进行蛋白定量。SDS-PAGE凝胶电泳后转至PVDF膜, 5%脱脂奶粉室温摇床封闭1 h, 加一抗稀释液4 ℃孵育过夜。洗膜后加入二抗稀释液室温孵育2 h, 膜上均匀加入ECL发光液, 放入曝光仪内进行检测, 膜显影后获取图像。用Quantity One凝胶图象处理系统分析目的条带, 标记并获取目的条带的灰度值。本实验中蛋白质标准化的内参使用GAPDH, 目的蛋白的灰度值/内参蛋白的灰度值表示蛋白的相对表达量。

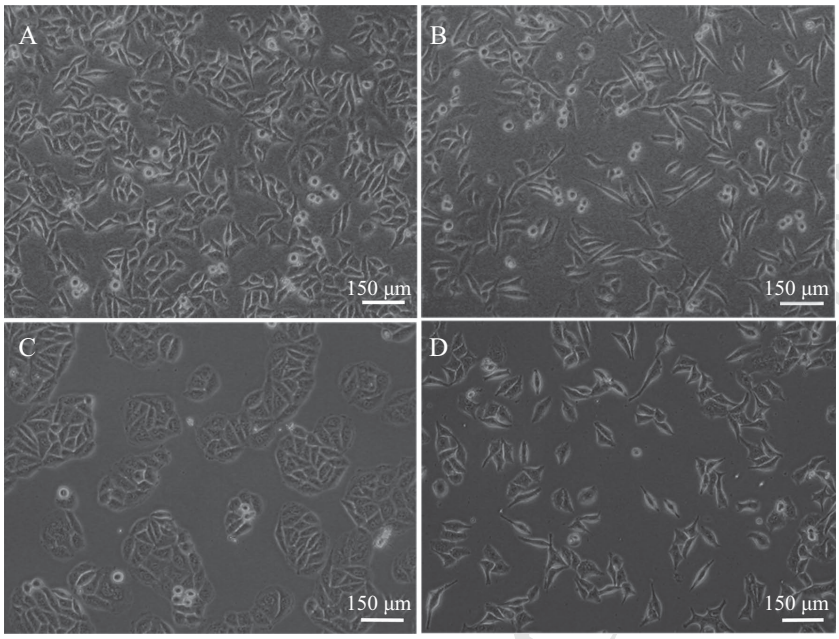
1.7 统计学分析

实验每组设3个复孔, 均重复3次。所有数据以均数±标准差(mean±S.D.)表示, 采用SPSS 17.0统计软件处理, 多样本比较采用单因素方差分析(One-Way ANOVA), 两样本比较采用*t*检验, $P<0.05$表示差异具有统计学意义。

2 结果

2.1 HSC-CM培养肝癌细胞的形态变化

肝癌细胞用肝星形细胞条件培养基(HSC-CM)培养48 h后, 肝癌细胞形态发生改变。HepG2细胞由上皮样三角形、多边形转化成为一种梭形细长的间质细胞形态; SMMC-7721由鹅卵石样上皮样细胞表型转化为梭形成纤维细胞样表型。常规培养的肝癌细胞生长呈岛形结构、细胞间连接紧密, 而在HSC-CM条件培养基中, 细胞连接疏松、呈散在生长、细

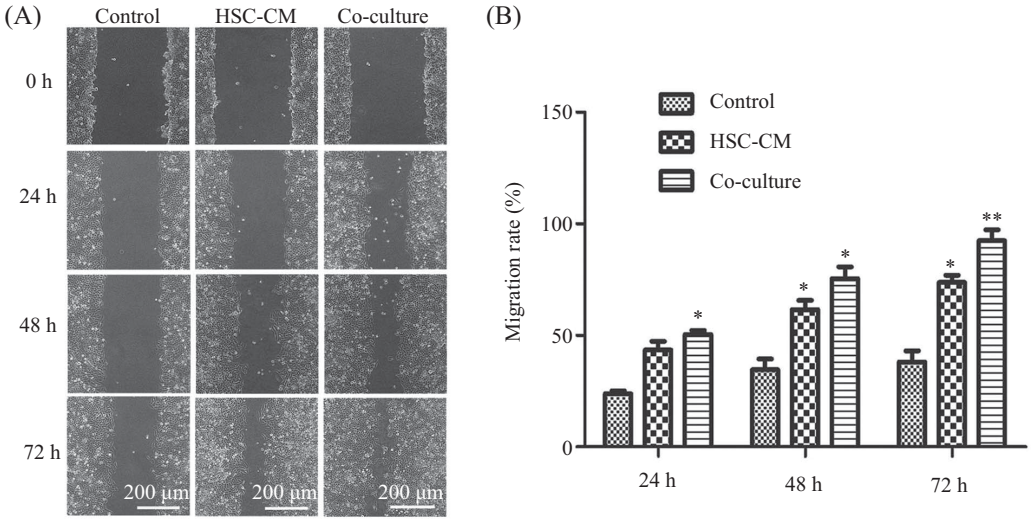


A: 无血清培养基常规培养48 h后, HepG2细胞的形态; B: HSC-CM条件培养48 h后, HepG2细胞的形态; C: 无血清培养基常规培养48 h后, SMMC-7721细胞的形态; D: HSC-CM条件培养48 h后, SMMC-7721细胞的形态。

A: morphology of HepG2 cells with regular culture in serum-free medium for 48 h; B: morphology of HepG2 cells with HSC-CM conditional culture for 48 h; C: morphology of SMMC-7721 cells with regular culture in serum-free medium for 48 h; D: morphology of SMMC-7721 cells with HSC-CM conditional culture for 48 h.

图1 HSC-CM培养HepG2、SMMC-7721细胞48 h后的形态

Fig.1 Morphology of HepG2 and SMMC-7721 cells cultured in HSC-CM for 48 h



A: HepG2细胞的迁移; B: HepG2细胞的迁移率。*P<0.05, **P<0.01, 与对照组比较。

A: migration of HepG2 cells; B: migration rate of HepG2 cells. *P<0.05, **P<0.01 compared with the control group.

图2 不同培养条件下肝癌细胞HepG2的迁移能力

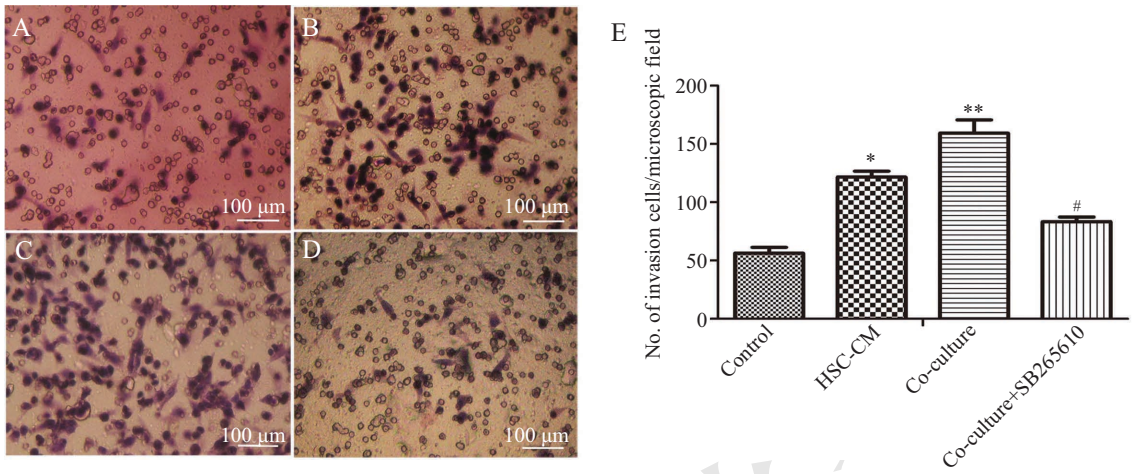
Fig.2 Migration of HepG2 cells in different culture conditions

胞失去接触联系、形态发生改变, 出现丝状伪足, 转为能动表型(图1)。

2.2 肝星形细胞促进肝癌细胞的迁移和侵袭

为研究肝星形细胞对肝癌细胞迁移的影响, 分别对肝癌细胞的常规培养、HSC-CM培养和共培养进行划痕实验。结果表明, 与常规培养比较, HSC-CM培养的肝癌细胞迁移增加、共培养的肝癌细胞迁移能力更加显著, 差异有统计学意义(图2)。

将肝癌细胞HepG2按上述设计的三种不同条件分别培养3 d后, 细胞消化计数, 铺入Transwell上层小室内, 孵育24~28 h, 细胞固定, 染色, 对穿越Transwell小室的细胞计数。结果表明, HSC-CM



A: 对照组; B: HSC-CM组; C: 共培养组; D: co-culture+SB265610组; E: Transwell细胞侵袭实验相对定量结果。*P<0.05, **P<0.01, 与对照组比较; #P<0.05, 与共培养组比较。

A: control group; B: HSC-CM group; C: co-culture group; D: co-culture+SB265610 group; E: Transwell cell invasion assays were scanned and quantified. *P<0.05, **P<0.01 *vs* control group; #P<0.05 *vs* the co-culture group.

图3 不同培养条件下肝癌细胞HepG2的侵袭能力

Fig.3 Invasion of HepG2 cells in different culture conditions

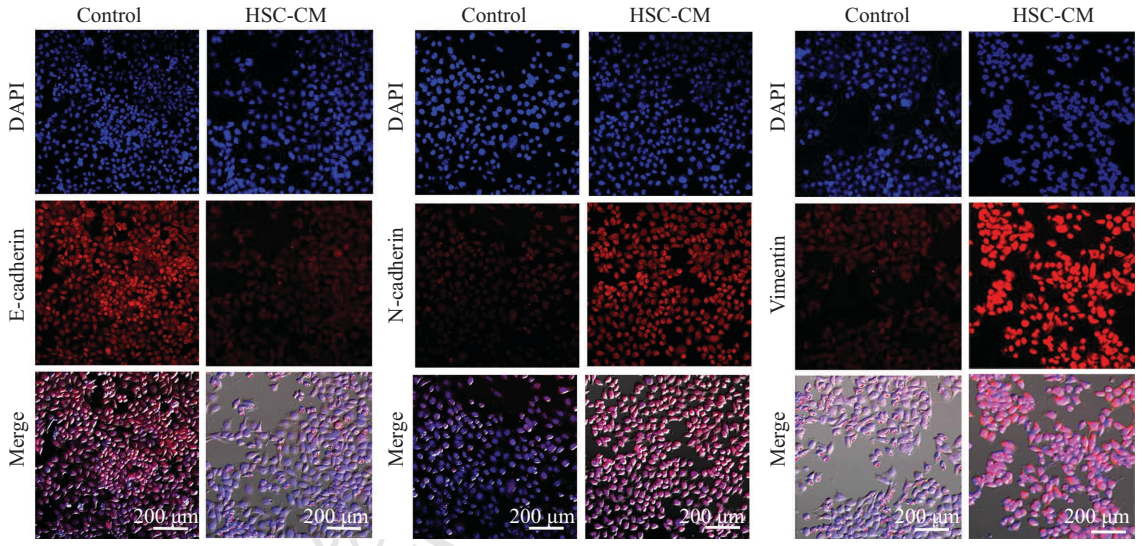


图4 上皮标志物E-cadherin、间质标志物N-cadherin和Vimentin在HepG2细胞中的表达情况

Fig.4 The expression of epithelial markers E-cadherin, mesenchymal markers N-cadherin and Vimentin in HepG2 cells

培养下HepG2细胞的侵袭增强、共培养组HepG2细胞的侵袭更为明显, 而添加CXCR2受体抑制剂SB265610(1 μmol/L)后, 细胞侵袭明显减弱, 与添加前比较差异有统计学意义(图3)。

2.3 肝星形细胞诱导肝癌细胞上皮–间质转化

将常规培养和HSC-CM培养的细胞分别消化计数, 铺入激光共聚焦扫描显微镜专用的玻底皿中, 放入温箱孵育待细胞贴壁伸展后进行免疫荧光染色。结果显示, HSC-CM培养的HepG2细胞中上皮标志物E-cadherin表达下调, 间质标志物N-cadherin和Vimentin表达上调, 表明肝星形细胞诱导肝癌细胞发生EMT(图4)。

2.4 趋化因子CXCL1激活PI3K/AKT信号通路诱导肝癌细胞EMT

为探讨肝星形细胞是如何诱导肝癌细胞发生EMT的, 我们收集了HSC-CM条件培养基并进行浓缩。Western blot分析显示, HSC-CM中存在CXCL1, 而条件培养液中的CXCL1只能来源于HSC。因此, 我们同样对肝星形细胞进行蛋白免疫印迹, 结果证明, 肝星形细胞确实高表达CXCL1, 而在肝癌细胞

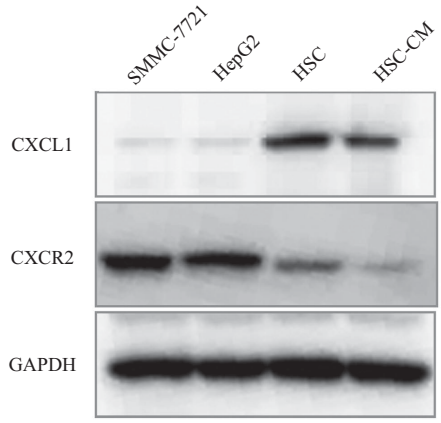


图5 SMMC-7721、HepG2和HSC细胞及HSC-CM中*CXCL1*和*CXCR2*基因的表达

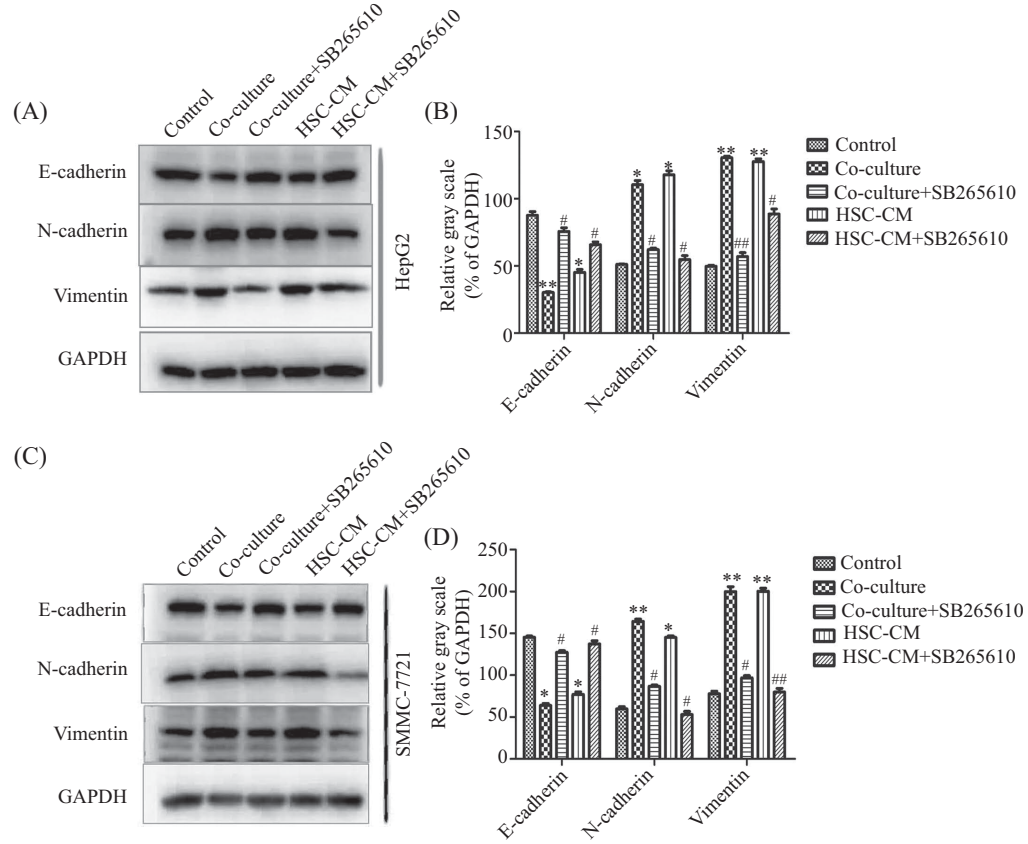
Fig.5 The expression of *CXCL1*, *CXCR2* in SMMC-7721, HepG2, HSC cells and HSC-CM

HepG2和SMMC-7721中基本不表达。与之相对应的是, CXCL1的受体CXCR2在肝癌细胞中呈高表达, 而在肝星形细胞和HSC-CM中微量表达(图5)。

与常规培养比较, 在条件培养下的肝癌细胞上皮标志物E-cadherin表达下调, 间质标志物N-cadherin和Vimentin表达上调, 差异有统计学意义。条件培养中加入CXCR2受体特异性抑制剂SB265610(1 μmol/L)后发现, EMT的三个标志蛋白的表达出现逆转, 即上皮标志物E-cadherin表达较添加前上调, 间质标志物N-cadherin和Vimentin表达较添加前下调, 差异有统计学意义(图6)。

条件培养的肝癌细胞, 与常规培养下相比, 细胞内p-PI3K和p-AKT表达上升, 差异有统计学意义, 而添加CXCR2特异性受体抑制剂后, p-PI3K和p-AKT表达下调, 与添加前比较, 差异有统计学意义(图7)。

另外, 条件培养下的肝癌细胞HepG2, 细胞内p-GSK-3β和Snail表达上调, 与常规培养对比, 差异有统计学意义; 而添加CXCR2受体抑制剂后, p-GSK-3β和Snail表达较添加前下调, 差异有统计学意义。结果表明, PI3K/Akt/GSK-3β/Snail信号通路可能参与肝星形细胞诱导肝癌细胞上皮间质转化过程(图8)。

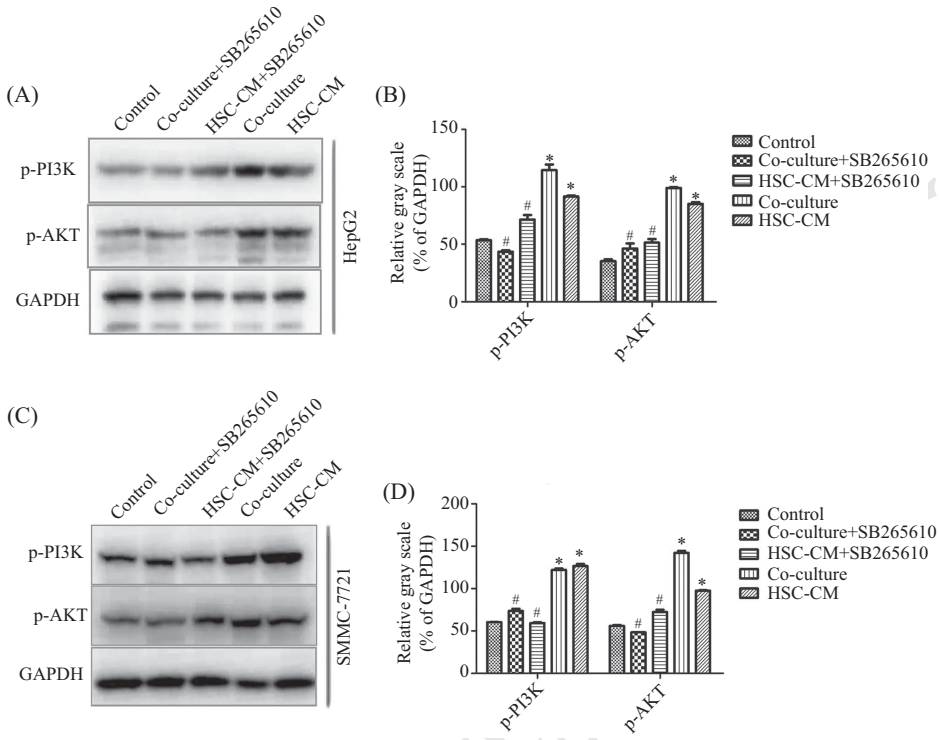


A、C: Western blot检测HepG2和SMMC-7721细胞中E-cadherin、N-cadherin和Vimentin的蛋白水平; B、D: 分别是HepG2和SMMC-7721细胞的相对灰度值。**P*<0.05, ***P*<0.01, 与对照组比较; #*P*<0.05, ##*P*<0.01, 与添加抑制剂前对应组比较。

A,C: expressions of E-cadherin, N-cadherin and Vimentin determined by Western blot in HepG2 and SMMC-7721 cells; B,D: relative gray scale of HepG2 and SMMC-7721 cells, respectively; **P*<0.05, ***P*<0.01 *vs* control group; #*P*<0.05, ##*P*<0.01 *vs* the group that before treated by inhibitor.

图6 肝星形细胞共培养、条件培养液和CXCR2抑制剂对肝癌细胞EMT的影响

Fig.6 The impact of co-culture with HSC, HSC-CM and CXCR2 inhibitor on EMT in hepatocellular carcinoma cells

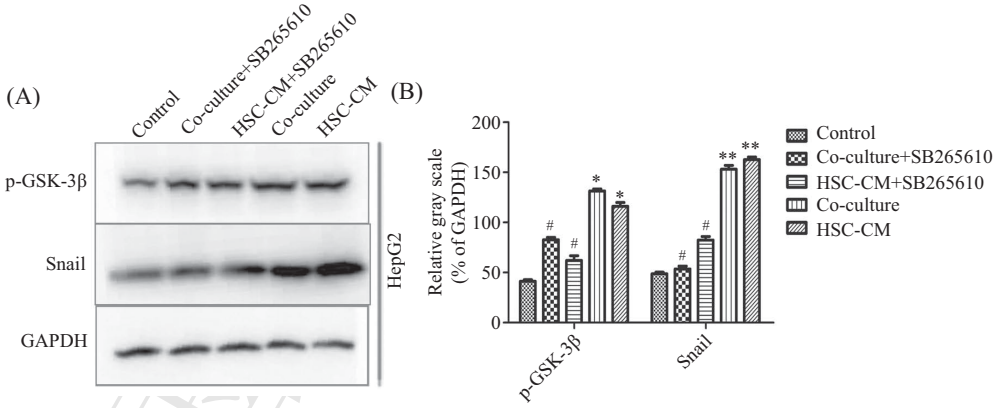


A、C: Western blot检测HepG2和SMMC-7721细胞中p-PI3K和p-AKT的蛋白水平; B、D: 分别是HepG2和SMMC-7721细胞的相对灰度值。*P<0.05, 与对照组比较; #P<0.05, 与添加抑制剂前对应组比较。

A,C: expressions of p-PI3K and p-AKT determined by Western blot in HepG2 and SMMC-7721 cells; B,D: relative gray scale of HepG2 and SMMC-7721 cells, respectively; *P<0.05 *vs* control group, #P<0.05 *vs* the group that before treated by inhibitor.

图7 肝星形细胞共培养、条件培养液和CXCR2抑制剂对肝癌细胞PI3K/AKT磷酸化的影响

Fig.7 The impact of co-culture with HSC, HSC-CM and CXCR2 inhibitor on phospho-PI3K/AKT in hepatocellular carcinoma cells



A: Western blot检测HepG2细胞内p-GSK-3β和Snail的蛋白水平; B: HepG2细胞的相对灰度值。*P<0.05, **P<0.01, 与对照组比较; #P<0.05, 与添加抑制剂前对应组比较。

A: expressions of p-GSK-3β and Snail determined by Western blot in HepG2 cells; B: relative gray scale of HepG2 cells. *P<0.05, **P<0.01 *vs* control group, #P<0.05 *vs* the group that before treated by inhibitor.

图8 肝星形细胞共培养、条件培养液和CXCR2抑制剂对HepG2细胞p-GSK-3β和Snail的影响

Fig.8 The impact of co-culture with HSC, HSC-CM and CXCR2 inhibitor on phospho-GSK-3β and Snail in HepG2 cells

3 讨论

恶性肿瘤发生发展和侵袭转移与肿瘤细胞所处的内外环境有密切的关系, 肝癌细胞的微环境包括了肝星形细胞(HSC)、成纤维细胞(fibroblasts)、免疫细胞、各种生长因子、各种蛋白酶或炎症因子等, 其中肝星形细胞是肝癌微环境中最重要的基质细胞。有文献报道, 活化的HSC可通过分泌各种细胞生长因子、细胞外基质和金属蛋白酶等促进肝癌细胞的转移, 而肝癌细胞又能促进HSC活化和增殖, 最终加速肝癌的进程[15]。

本研究通过条件培养肝癌细胞发现, 其形态发生改变, 细胞之间连接疏松, 失去上皮极性, 呈运动表型并伸出伪足。划痕和Transwelll实验也证实活化的HSC使肝癌细胞迁移和侵袭能力增强。免疫荧光检测肝癌细胞EMT的上皮标志物E-cadherin表达下调、间质标志物N-cadherin和Vimentin表达上调。此外, Western blot检测条件培养的肝癌细胞EMT的三个标志蛋白变化与免疫荧光一致, 更重要的是调控EMT核转录因子表达明显上调, 本研究表明肝癌细胞发生了EMT。

在体外, HSC已被证明促进肝癌细胞的迁移和侵袭, 可能是因为分泌了一些细胞外基质蛋白和生长因子, 导致信号通路激活[15]。本研究发现, HSC可表达和分泌大量的CXCL1。早期研究证明, CXCL1在很多肿瘤的发生、发展、增殖、迁移、侵袭、血管新生、淋巴管新生、淋巴转移、转化等过程中发挥关键作用[16]。另外, 本研究证实肝癌细胞高表达CXCL1的受体CXCR2。条件培养的肝癌细胞, 其EMT的三个标志蛋白中E-cadherin表达明显下调, N-cadherin和Vimentin表达明显上调, 即发生EMT转型; 加入CXCR2受体抑制剂SB265610, EMT现象明显逆转。这些结果证明, 活化的肝星形细胞分泌的CXCL1与肝癌细胞中CXCR2受体结合, 促进了肝癌细胞的上皮–间质转化, HSC活化后可能是通过CXCL1/CXCR2轴调节肿瘤的转移过程。本研究还发现, 条件培养的肝癌细胞p-PI3K、p-AKT及下游p-GSK-3β和调控EMT发生的重要核转录因子Snail表达上调; 加入CXCR2受体抑制剂后, p-PI3K和p-AKT表达很微弱, p-GSK-3β和Snail水平下调。结果表明, 活化的HSC可能是通过CXCL1/CXCR2轴激活PI3K/Akt/GSK-3β/Snail信号通路诱导肝癌细胞发生上皮–间质转化。另一方面, 我们不能排除肝星形细胞通过其他可能的机制诱导肝癌细胞EMT。Mikula等[17]研究证明, 活化的HSC自分泌并诱导TGF-β加速肝癌的进程, 本研究中活化PI3K/Akt通路是否存在TGF-β信号通路的非依赖Smad信号途径有待进一步证实。总之, 探讨趋化因子CXCL1及其信号通路的调控并进行干预, 将为研究肝癌的转移及复发提供实验基础。

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