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Macrophage activation increases the invasive properties of hepatoma cells by destabilization of the adherens junction☆

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Abstract **Tumor-associated macrophages play an important role in tumor progression, but whether they exert a tumor-progressive effect remains controversial. Here, we demonstrated that activated macrophage-conditioned medium (AMCM) obtained from RAW macrophages (RAW/AMCM) induced epithelial-mesenchymal transition (EMT) and stimulated the migratory and invasive activities of HepG2 cells, whereas control conditioned media had no effect. Epithelial-cadherin (E-cadherin) and β-catenin staining patterns were altered at the adherens junctions by RAW/AMCM treatment, with an approximately 50% decrease in E-cadherin and β-catenin in the cell membrane. Importantly, levels of β-catenin-associated E-cadherin were also decreased. Following RAW/AMCM treatment, enhanced activation of c-Src was seen prior to increased tyrosine phosphorylation of β-catenin, and this led to the destabilization of adherens junctions. Pretreatment of HepG2 cells with the Src kinase inhibitor, PP2, completely abolished the effects of RAW/AMCM on the EMT, migration, invasion, and expression and association of E-cadherin and β-catenin. AMCMs obtained from human THP-1 monocytes and mouse peritoneal macrophages also caused disassembly of the adherens junctions and migration of HepG2 cells. Furthermore, inhibition of the epidermal growth factor receptor (EGFR) with gefitinib partially prevented the downregulation of E-cadherin and β-catenin at the adherens junctions and migration behavior induced by RAW/AMCM. Our results suggest that activated macrophages have a tumor-progressive effect on HepG2 cells which involves the c-Src- and EGFR-dependent signaling cascades.**
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Keywords: β-Catenin; Epithelial-cadherin; Tyrosine phosphorylation of β-catenin; c-Src kinase; Epidermal growth factor receptor; Macrophages; Metastasis

1. Introduction

The inflammatory microenvironment plays a key role in the progression of solid malignant tumors [1,2]. In the past decade, tumor-associated macrophages (TAMs) have been extensively studied and proposed as a major contributor to tumor progression [3]. However, the interaction between TAMs and cancer cells is extremely complicated and has not been clearly elucidated. Most importantly, whether TAMs increase tumor-progression remains a subject of controversy. For instance, an increased number of TAMs is associated with a better prognosis in lung cancer [4], but with a poor prognosis in breast cancer [5]. These opposite effects might be explained by tissue-type specificity. However, even in the same tumor type, such as prostate cancers, conflicting results have been obtained [6,7].

Cell adhesion molecules are essential for cell–cell and cell–matrix interactions in both physiological and pathological conditions. Of these molecules, epithelial-cadherin (E-cadherin), a transmembrane glycoprotein, has been shown to maintain normal epithelial morphology through a Ca^{2+}-dependent homotypic interaction [8,9]. Loss of E-cadherin in cancer cells decreases adhesiveness and releases these cells from the primary locus into distant sites [10], a process called tumor metastasis. Because of this adhesive function, E-cadherin has been suggested as an invasion suppressor molecule [10]. Since E-cadherin expression is inversely correlated with the degree of invasiveness in hepatocellular carcinoma [11], E-cadherin levels are considered as a potential biomarker of these tumors [12]. The epithelial-mesenchymal transition (EMT), a process in which epithelial cells acquire mesenchyme-like properties, is characterized by loss of E-cadherin and is associated with development and tumor progression. Several recent studies have shown that Src kinase is involved in the EMT [13,14].

β-Catenin is an important factor regulating both cellular signaling and adhesion. While cytosolic β-catenin is involved in the Wnt-signaling pathway, membrane-bound β-catenin anchors E-cadherin to actin filaments by binding to the intracellular domain of E-cadherin [9,15]. The existence of two different localized pools of β-catenin may indicate crosstalk between the cellular adhesion and signal transduction machineries [16].

The structural integrity of the E-cadherin/β-catenin complex is determined by the phosphorylation status of β-catenin [17]. Tyrosine kinases, including Fer, Fyn, Yes, Src, and the epidermal growth factor receptor (EGFR), have been shown

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Abbreviations: AMCM, activated macrophage-conditioned media; CCM, control macrophage-conditioned media; DMSO, dimethyl sulfoxide; E-cadherin, epithelial-cadherin; EGFR, epidermal growth factor receptor; EMT; epithelial-mesenchymal transition; FBS, fetal bovine serum; IL, interleukin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; TAM, tumor-associated macrophage; TBS, Tris-buffered saline

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to phosphorylate specific tyrosine residues in β-catenin, leading to dissociation of the E-cadherin/β-catenin complex [18–21]. Conversely, inhibition of Src family kinases restores E-cadherin-mediated cell adhesion in a wide variety of cancer cells [22].

In this study, we investigated the effects of macrophage activation on adherent junctions of human hepatocellular carcinoma cells. Our results showed that factors secreted by activated macrophages promoted the migration and invasiveness of these tumor cells by activation of c-Src and tyrosine phosphorylation of β-catenin, leading to disruption of the adherens junctions. We also showed that inhibition of Src family kinase and EGFR signaling prevented the effects of activated macrophage-conditioned media (AMCM) on the properties of tumor cells. These results showing that activated macrophages increase the mobility and invasiveness of hepatocarcinoma cells provide the basis for the tumor-progressive effect of activated macrophages on cancer formation.

2. Materials and methods

2.1. Cell culture and preparation of macrophage-conditioned media

The HepG2 human hepatocellular carcinoma cell line, RAW264.7 murine macrophage cell line, and THP-1 human monocyte cell line (American Type Culture Collection, Manassas, VA, USA) were maintained in growth medium [Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin; all from Gibco, Grand Island, NY, USA] in a humidified atmosphere at 37 °C in 5% CO_2.

To obtain mouse peritoneal macrophages, BALB/c mice were anesthetized with ether, then sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) was injected into the peritoneal cavity, and the abdomen massaged. After drainage of the peritoneal fluid, a mixture of macrophages and other cells was collected by centrifugation and plated on culture dishes. The macrophages were separated from other blood cells by differential attachment; macrophages adhered to the dishes within 2 h and the non-adherent blood cells were washed off.

For the preparation of conditioned media from activated or non-activated macrophages, macrophage cell lines or primary culture of macrophages were treated for 24 h with 500 nM phorbol myristate acetate (PMA) (Biomol, Plymouth Meeting, PA, USA) in dimethyl sulfoxide (DMSO), 1 mg/ml of lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA) in ethanol, 500 units/ml of interferon-γ (PeproTech EC, London, UK) in growth medium, or with vehicle alone, washed once with PBS, and cultured in serum-free growth medium for 4 h to eliminate residual stimulants. Except for the LPS-stimulated group, which were left in serum-free growth medium, the cells were then briefly washed with PBS and cultured in serum containing growth medium for another 24 h, then the medium was collected and filtered to remove debris. The filtrate from the PMA-treated RAW macrophages was designated as RAW/AMCM and that from the vehicle-treated control cells as RAW/control macrophage-conditioned media (CCM). AMCM and CCM obtained from human THP-1 monocytes and primary mouse macrophages are referred to as THP/AMCM, THP/CCM, mouse/AMCM, and mouse/CCM. For cytokine studies, interleukin (IL)-4, IL-6, and IL-13 were purchased from PeproTech EC (London, UK) and used at 1, 10, or 100 ng/ml in growth media containing 2% FBS. PP2 was obtained from Biomol and gefitinib from AstraZeneca (Macclesfield, UK).

2.2. Antibodies

The mouse primary antibodies used were anti-E-cadherin (Transduction Labs, Franklin Lakes, NJ, USA), anti-β-catenin (Transduction Labs), anti-β-tubulin (Sigma), anti-β-actin (Sigma), anti-c-Src (clone GD11, Upstate, Lake Placid, NY, USA), anti-Tyr416-phospho-c-Src kinase (Cell Signaling, Beverly, MA, USA) [23], and anti-phosphotyrosine (clone PY7E1 and PY20, Zymed, Carlsbad, CA, USA). The secondary antibodies were fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) and alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI, USA).

2.3. Immunofluorescence

HepG2 cells plated on glass coverslips were washed twice with PBS and fixed with 10% formalin for 10 min at room temperature, then were permeabilized for 5 min at room temperature with PBS containing 0.1% Triton X-100. After blocking with PBS containing 5% skim milk (blocking buffer), the cells were incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. After three washes with PBS, the cells were incubated for 1 h at room temperature with FITC-conjugated secondary antibody, then the coverslips were washed three times with PBS and mounted on glass slides. Images were acquired using a Zeiss fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan).

2.4. Migration, wound healing, and invasion assays

For the migration assay, appropriate numbers of HepG2 cells were seeded in the upper chamber of a Transwell apparatus with an 8 μm pore size membrane (Costar, Acton, MA, USA). After attachment, 0.5 ml of CCM or AMCM was added to the lower well. After 20 h, the polycarbonate membranes were fixed in 10% formalin for 10 min and stained with Coomassie Brilliant Blue G250 (Sigma) for 5 min, then the number of cells that had migrated to the reverse surface of the membrane was counted in three randomly selected fields under light microscopy. For the wound healing assay, HepG2 cells were grown on glass coverslips in growth medium. After formation of a confluent monolayer, straight wounds were created using a sterile pipette tip and the medium replaced with CCM or AMCM. Microscopic photographs were taken at 0 and 24 h.

For the invasion assay, the same procedures were carried out as described above except for addition of a layer of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) on top of the upper chamber membrane. The HepG2 cells were then placed on the Matrigel layer and RAW/CCM or RAW/AMCM in the lower chamber. After 72 h, the membranes were fixed and stained as described above.

2.5. Western blot analysis

To prepare whole cell lysates, the cells were collected, ultrasonicated in lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml of pepstatin A, 1 μg/ml of leupeptin, 1 mM NaF, 1 mM Na_3VO_4), and centrifuged at $13000 \times g$ for 30 min at 4 °C. The protein concentration of the lysate was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), then equal amounts of the proteins were boiled in Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris–HCl, pH 6.8).

To prepare the membrane fraction, the cells were harvested in RIPA buffer (150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 mM Na_3VO_4, 1 mM NaF in 50 mM Tris–HCl, pH 7.4) containing 1% Triton X-100. The cell suspensions were briefly sonicated and centrifuged at $13000 \times g$ for 10 min at 4 °C, then the supernatants were discarded, and the pellets solubilized in RIPA buffer containing 1% NP-40, the protein concentration measured, and a sample treated with sample buffer as above.

For immunoprecipitation, HepG2 cells were treated with RAW/CCM or RAW/AMCM for different intervals before incubation at 4 °C for 30 min with immunoprecipitation buffer (1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 μg/ml of pepstatin A, 1 μg/ml of leupeptin in 50 mM Tris–HCl, pH 7.5). The cell suspensions were briefly ultrasonicated and centrifuged at $13000 \times g$ for 30 min, then the supernatants were collected and their protein concentrations determined. A sample containing 800 μg of protein was mixed with 2 μg of anti-β-catenin antibodies or 5 μl of anti-Tyr416-phospho-c-Src kinase antibodies (Cell Signaling) and the mixture incubated at 4 °C for 3 h before addition of 100 μl of protein G–Sepharose beads (Pharmacia, Uppsala, Sweden), followed by incubation at 4 °C for 30 min. The Sepharose beads were extensively washed with immunoprecipitation buffer, boiled in Laemmli sample buffer, and spun down, and the released proteins used for Western blotting.

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