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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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

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 $13\,000 \times g$ 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 $13\,000 \times g$ 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 $13\,000 \times g$ 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.

Equal amounts of proteins were resolved by SDS–polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Schleicher & Schuell BioSciences, Inc., Boston, MA, USA). The membranes were blocked for 1 h at room temperature with Tris-buffered saline (TBS; 150 mM NaCl in 50 mM Tris, pH 8.2) containing 1% BSA and 0.1% Tween 20, then incubated overnight at 4 °C with primary antibodies. After washing with 0.1% Tween 20 in TBS, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated secondary antibodies, and bound antibody visualized by incubation with a substrate solution containing H_2O_2, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The density of the bands was quantified by densitometry using GelPro 3.1 (Media Cybernetics, Silver Spring, MD, USA).

2.6. Statistical analysis

All results are expressed as the means ± S.D. Statistical differences between means were assessed using Student's *t* test, with a *P* value less than 0.05 being considered significant.

3. Results

3.1. Macrophage activation alters the morphology of HepG2 cells

To investigate the effects of activated macrophages on tumor cell morphology, we cultured HepG2 cells in RAW/AMCM or RAW/CCM. PMA was used to trigger macrophage activation, since it has been shown to induce activation of macrophages from several sources [24,25]. In this culture system, changes in HepG2 cell morphology are dependent on factors secreted from macrophages, but not on a direct interaction with macrophages. This strategy allowed us to mimic indirect interactions between cancer cells and macrophages through chemical crosstalk, rather than physical interaction between the two cell types [2].

In the presence of RAW/CCM, HepG2 cells formed aggregated islet-like clusters, with a typical polarized epithelial phenotype (Fig. 1A, CCM). This epithelial morphology was also seen when HepG2 cells were cultured in growth medium, showing that conditioned medium from non-activated macrophages did not affect the morphology of HepG2 cells. However, HepG2 cells cultured in RAW/AMCM showed a striking morphological change. These cells showed loss of epithelial morphology, became dissociated from the epithelial clusters, and acquired a mesenchymal phenotype, a process called the EMT (Fig. 1A, AMCM). To investigate whether this phenomenon was associated with increased cell proliferation, we measured the growth of cells maintained in RAW/CCM and RAW/AMCM over time and found no significant difference between the two sets of cells over a 72 h incubation period (Fig. 1B). Thus, the greater surface area covered by the RAW/AMCM-treated cells was due to altered biological activities of the HepG2 cells and not to an increased growth rate.

3.2. Macrophage activation enhances the invasive characteristics of HepG2 cells

The morphological change described above implied weakening of cell adhesion in the RAW/AMCM-treated cultures. To determine whether this weakened adherence led to a higher mobility, we performed migration and invasion assays, both of which are widely used to examine the metastatic characters of tumor cells.

After 20 h of culture, cells in RAW/AMCM showed a greater than 10-fold increase in the number of migrating cells com-
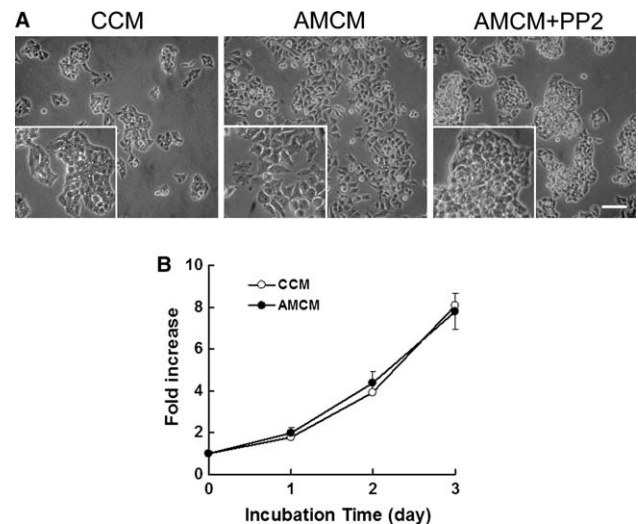

Fig. 1. Effect of macrophage conditioned media on HepG2 cell morphology. (A) Phase microscopy of HepG2 cells cultured in RAW/CCM, RAW/AMCM, or RAW/AMCM + 10 μM PP2. HepG2 cells were plated in growth medium for 1 day, then the medium was changed to the indicated medium. The images shown are representative of cells after 24 h of culture in the indicated medium. Bar = 100 μm. Inset: Higher magnification. (B) Growth curves of HepG2 cells cultured in RAW/CCM or RAW/AMCM for the indicated time. At each time-point, the cells were trypsinized and counted and the cell number used to calculate the fold increase. The data shown are the means ± S.D. of triplicate experiments.

pared to RAW/CCM-treated cultures (Fig. 2A and B). This suggested that RAW/AMCM treatment increased the migratory ability of HepG2 cells. This was confirmed by another migration assay, commonly referred to as the wound-healing assay. In accordance with their higher migration ability, cells maintained in RAW/AMCM closed the wound cleft 2.5 times faster than those in RAW/CCM (Fig. 2C; 15.6% closure during the first 24 h in RAW/CCM versus 38.7% in RAW/AMCM).

For the invasion assay, the procedures were similar to those for the Transwell migration assay, except that a thick layer of Matrigel was applied to the top surface of the Transwell membrane; this assay therefore measures not only cell mobility, but also the ability of the cells to digest the extracellular matrix, a prerequisite for metastatic tumor cells. As shown in Fig. 3, RAW/AMCM-treated cultures showed a 3-fold increase in the number of invasive cells passing through the Matrigel compared to RAW/CCM-treated cells. Together, these results clearly indicated that conditioned media from activated macrophages enhanced both the migratory and invasive abilities of HepG2 cells.

3.3. Macrophage activation downregulates the E-cadherin/β-catenin complex

We hypothesized that the observed increase in the migration and invasiveness of HepG2 cells caused by RAW/AMCM treatment might be attributable to altered cellular adhesion. The involvement of the cadherin/catenin complex at cellular junctions is well characterized in terms of the migratory and invasive activities of tumor cells [26]. To determine whether this cadherin/catenin system played a role in the RAW/AMCM-induced biological changes in HepG2 cells, we exam-
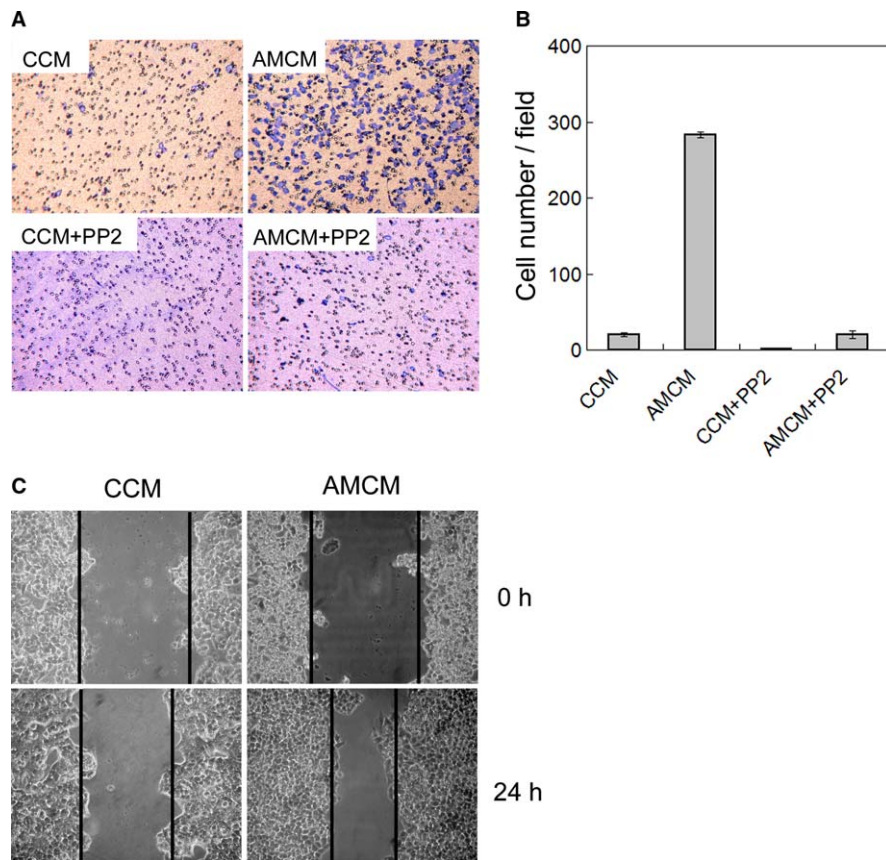
Fig. 2. Conditioned medium from activated macrophages induces migration of HepG2 cells. (A) Migration assay using a Transwell apparatus. HepG2 cells were plated in the upper chamber and RAW/CCM or RAW/AMCM in the presence or absence of 10 μM PP2 was added to the lower chamber. After 20 h, the membranes were stained with Coomassie Blue and photographed. (B) Quantification of the data. Three fields were randomly selected at 40× magnification and the number of stained cells counted. The data are the means ± S.D. for three independent experiments. (C) Wound closure by HepG2 cells induced by activated macrophages. The wounds were made on a confluent monolayer of cells cultured in growth medium, then the medium was changed to RAW/CCM or RAW/AMCM for the indicated time.
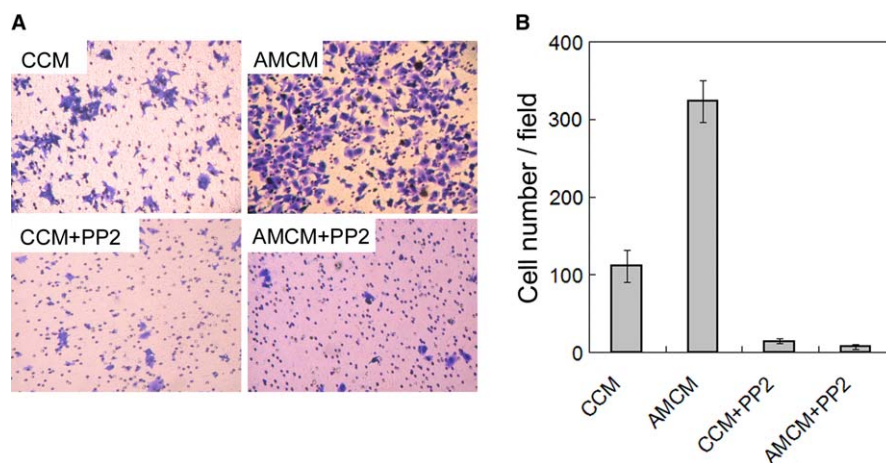

Fig. 3. Conditioned medium from activated macrophages induces invasiveness of HepG2 cells. (A) Invasion assay using a Transwell apparatus coated with a layer of Matrigel. HepG2 cells were plated on the Matrigel layer in growth medium and RAW/CCM or RAW/AMCM in the presence or absence of 10 μM PP2 was added to the lower chamber. After 72 h, invading cells were visualized by Coomassie Blue staining. (B) Quantification of the data. Three fields were randomly selected under 40× magnification and the number of stained cell counted. The data shown are the means ± S.D. for three independent experiments.

ined the expression of β-catenin and E-cadherin in these cells by immunofluorescence. As shown in Fig. 4A, in RAW/CCM-treated cells, the staining pattern for both β-catenin and E-cadherin was continuous and linear along the cell border, whereas, in RAW/AMCM-treated cells, staining was discontinuous, with a dotted appearance at the cell–cell
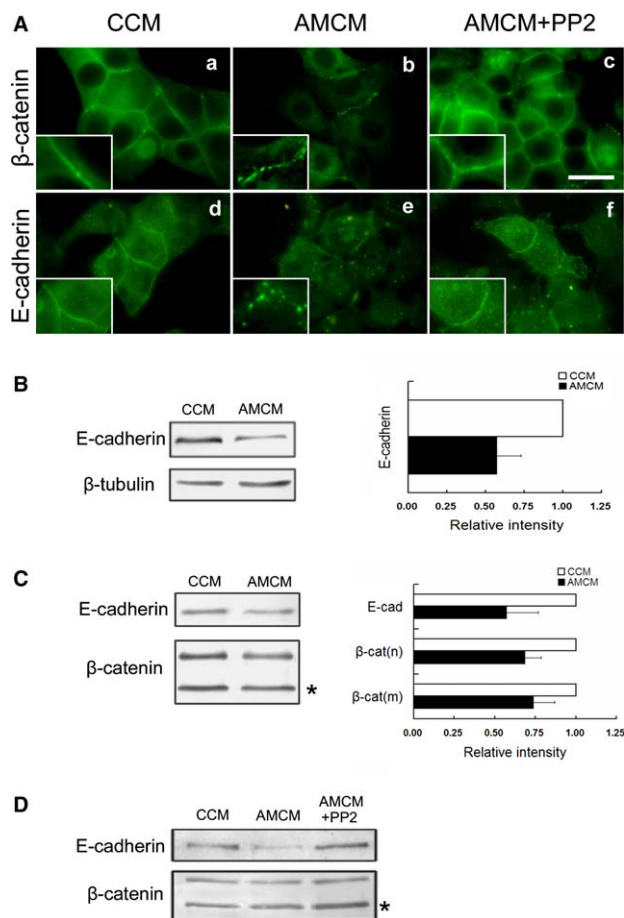
Fig. 4. Conditioned medium from activated macrophages decreases the expression of E-cadherin and β-catenin in HepG2 cells. (A) Distribution of E-cadherin and β-catenin in HepG2 cells treated with RAW/CCM or RAW/AMCM in the presence or absence of 10 μM PP2 for 24 h. The cells were fixed, permeabilized, and immunostained for β-catenin and E-cadherin. Bar = 20 μm. Insets are representative views of higher magnification showing the localization of these proteins at adherens junctions. (B) Western blot analysis of E-cadherin in HepG2 cells. Cells were cultured for 24 h in RAW/CCM or RAW/AMCM, then whole cell lysates were analyzed for E-cadherin or β-tubulin (loading control). The data shown are representative of the results for three independent experiments. For the densitometric data (means ± S.D. of three experiments), the intensity of the E-cadherin band is expressed relative to that for the RAW/CCM-treated cells. (C) E-cadherin and β-catenin in the membrane fraction of HepG2 cells. After 24 h incubation in different conditioned media, the membrane fraction was prepared and analyzed. Densitometric data showing the means ± S.D. of the results for three independent experiments. E-cad: E-cadherin. β-cat (n): native form of β-catenin. β-cat (m): mutant form of β-catenin (∗). (D) β-Catenin-associated E-cadherin. Whole cell lysates of cells treated for 24 h with RAW/CCM, RAW/AMCM, or RAW/AMCM + 10 μM PP2 were immunoprecipitated using monoclonal anti-β-catenin antibody and the immunoprecipitates analyzed for E-cadherin and β-catenin. $n = 2$.

junction. This finding was confirmed by Western blot analyses, which showed that E-cadherin expression in whole cell lysates of RAW/AMCM-treated cells was approximately half of that in RAW/CCM-treated cells (Fig. 4B). β-catenin exists as cytosolic and membrane-bound pools. Analysis of the membrane fractions revealed that levels of both the native form and the truncated form of β-catenin, the latter characteristic of HepG2

cells [27], were substantially decreased by RAW/AMCM treatment (Fig. 4C).

Recently, a Src family kinase inhibitor, PP2, was shown to upregulate cadherin/catenin expression in human cancer cell lines [21]. We therefore examined whether it was able to block the effects of RAW/AMCM on cadherin/catenin expression in HepG2 cells. Interestingly, pretreatment of RAW/AMCM-treated cultures with PP2 resulted in cluster formation of HepG2 cells (Fig. 1A, AMCM + PP2), similar to that seen in the RAW/CCM-treated group (Fig. 1A, CCM), and completely restored the continuous distribution of E-cadherin and β-catenin at the cellular junctions (Fig. 4A, AMCM + PP2). Further evidence for weakening of the adherens junction was provided by the observation that the amount of E-cadherin detectable in β-catenin immunoprecipitates was reduced after 24 h of RAW/AMCM treatment (Fig. 4D). Furthermore, inactivation of Src family kinases by PP2 prevented the decrease in the amount of E-cadherin associated with β-catenin caused by RAW/AMCM (Fig. 4D). This observation is consistent with the recent finding that PP2 decreases the invasive activity of cancer cells by restoration of cadherin/catenin expression [21].

We then examined whether prevention of the decrease in cadherin/catenin expression by PP2 prevented the migration and invasiveness of HepG2 cells induced by activated macrophages. Pretreatment with 10 μM PP2 completely blocked the increase in the migratory (Fig. 2A and B) and invasive (Fig. 3) activities of HepG2 cells induced by RAW/AMCM, resulting in basal levels of activity or even lower. We therefore conclude that the increase in invasiveness of HepG2 cells caused by RAW/AMCM is mediated, at least in part, by activation of Src family kinase.

3.4. Macrophage activation increases tyrosine phosphorylation of β-catenin

The above data clearly showed that RAW/AMCM treatment decreased the expression of β-catenins and E-cadherin at cell junctions (Fig. 4). Tyrosine phosphorylation of β-catenins by Src family kinases has been shown to destabilize junctional β-catenin [20]. To investigate whether β-catenin phosphorylation by Src family kinases was also responsible for the RAW/AMCM-mediated decrease in junctional β-catenin, we examined levels of tyrosine phosphorylated β-catenin after RAW/CCM or RAW/AMCM treatment by immunoprecipitating β-catenin from treated cell lysates and performing immunoblotting with anti-phosphotyrosine and anti-β-catenin antibodies. Treatment with RAW/AMCM for 1 h significantly increased the levels of tyrosine-phosphorylated β-catenin compared to RAW/CCM-treated cultures, whereas total β-catenin levels were unchanged (Fig. 5A). Since our data suggested that Src family kinases played a role in the regulation of β-catenin stabilization (Fig. 4A), we next examined the levels of active c-Src, which can be detected using a specific antibody against Tyr416-phosphorylated c-Src [23,28], and found that c-Src was rapidly phosphorylated within 15 min by RAW/AMCM treatment and that levels of phosphorylated c-Src remained high (on average, 1.5-fold higher than basal levels) for at least 60 min (Fig. 5C and D). In contrast, RAW/CCM-treated cells showed only a minor increase in the first 5 min (Fig. 5C and D). To confirm this result, we performed immunoprecipitation of lysates of HepG2 cells treated for 15 min with RAW/
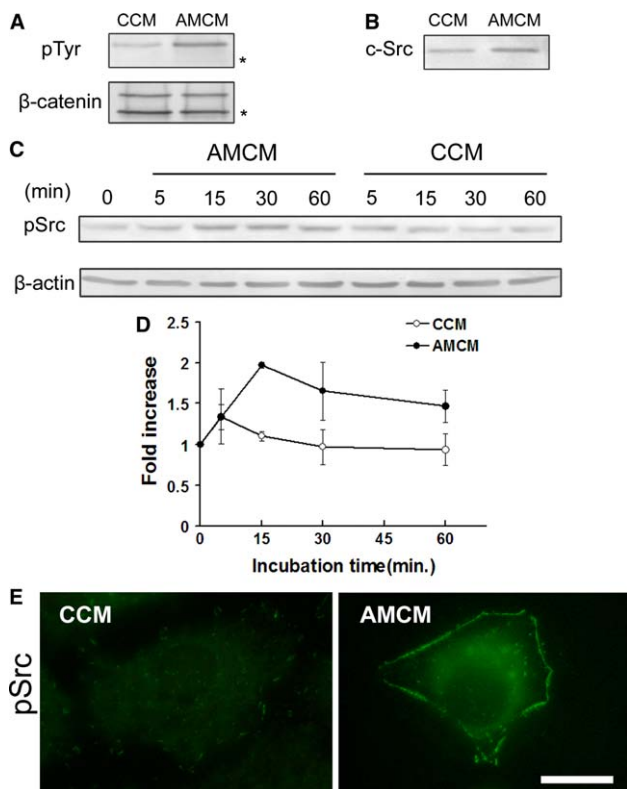
Fig. 5. Conditioned medium from activated macrophages increases tyrosine phosphorylation of β-catenin by activation of c-Src. (A) Immunoprecipitation of tyrosine phosphorylated β-catenin. Whole cell lysates of cells treated for 1 h with RAW/CCM or RAW/AMCM were immunoprecipitated with monoclonal β-catenin antibodies, and analyzed for phosphotyrosine (pTyr) and β-catenin. The asterisks indicate the mutant form of β-catenin. (B) Increase in c-Src phosphorylation caused by RAW/AMCM treatment. HepG2 cells were incubated for 15 min in RAW/AMCM or RAW/CCM, then the whole cell lysate was precipitated with anti-phospho-c-Src antibodies and subjected to immunoblotting with anti-c-Src antibodies. (C) Time-course study of c-Src activation by RAW/AMCM. HepG2 cells were treated with RAW/CCM or RAW/AMCM for the indicated time period, then whole cell lysates were subjected to electrophoresis and immunoblotting with antibodies against phospho-c-Src (upper panel) or β-actin as a loading control (lower panel). (D) Densitometric data derived from three independent experiments performed as in (C) and are expressed as the density of the band relative to the time zero result. The white circles and black circles represent, respectively, RAW/CCM-treated and RAW/AMCM-treated cells. (E) Membrane translocation of phosphorylated c-Src kinase by RAW/AMCM. The cells were treated with RAW/CCM or RAW/AMCM for 15 min, and processed for immunostaining for phosphorylated c-Src. Bar = 20 μm.

AMCM or RAW/CCM using an excess of anti-Tyr416-phospho-c-Src antibody followed by immunoblotting of the immunoprecipitated proteins using anti-c-Src antibody (Upstate, clone GD11). This experiment confirmed that levels of phosphorylated c-Src in the immunoprecipitate increased by 50–60% after 15 min incubation (Fig. 5B). In parallel with the biochemical data, immunostaining also showed membrane translocation of phosphorylated c-Src after 15 min of RAW/AMCM treatment (Fig. 5E, AMCM), whereas in RAW/CCM-treated cells phosphorylated c-Src was localized mainly on focal adhesions (Fig. 5E, CCM). Taken together with the previous data, these results suggest that activated macrophages cause disruption of the E-cadherin/β-catenin complex in HepG2 cells by activating c-Src.

3.5. Cytokines IL-4, IL-6, and IL-13 are not responsible for the macrophage-induced loss of adherens junction and the morphological changes

To identify the factors responsible for the above described effects, we incubated HepG2 cells for 24 h in medium containing 2% FBS and 1, 10, or 100 ng/ml of IL-4, IL-6, or IL-13. Phase-contrast microscopy and immunostaining showed that none of these cytokines induced the EMT or caused downregulation of β-catenin at the adherens junctions (data not shown).

3.6. An EGFR antagonist partially blocks the RAW/AMCM-induced phenotypic change

In order to examine whether EGFR was involved in the downregulation of E-cadherin/β-catenin at the adherens junctions, we used the EGFR inhibitor, gefitinib, to block the EGFR pathway. Pretreatment with 15 μM gefitinib partially prevented RAW/AMCM-induced downregulation of E-cadherin and β-catenin at the adherens junctions (Fig. 6A) and decreased the stimulatory effect of RAW/AMCM on the migratory ability of HepG2 cells (Fig. 6B).

3.7. The effects of activated macrophages are not species-specific and are not dependent on the method used to activate the macrophages

To determine whether the effects of AMCM were restricted to RAW 264.7 macrophages stimulated by PMA, we prepared conditioned media from RAW macrophages or macrophages from other sources (human THP-1 monocyte cell line and mouse peritoneal macrophages) treated with PMA, LPS or interferon γ. Conditioned medium from interferon γ-treated RAW macrophages did not affect the behavior of HepG2 cells (data not shown). After 24 h incubation, AMCMs from THP-1 cells activated with PMA, RAW cells activated with LPS, and mouse peritoneal macrophages activated with PMA decreased
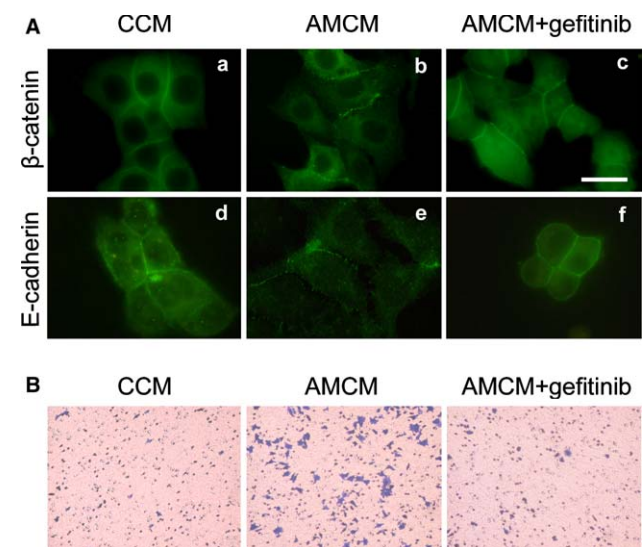

Fig. 6. An EGFR inhibitor blocks the RAW/AMCM-induced migration and changes in E-cadherin and β-catenin at the adherens junctions. HepG2 cells were treated for 24 h with RAW/CCM, RAW/AMCM, or RAW/AMCM + 15 μM gefitinib, then tested for (A) E-cadherin and β-catenin immunostaining or (B) migration. Bar = 20 μm.
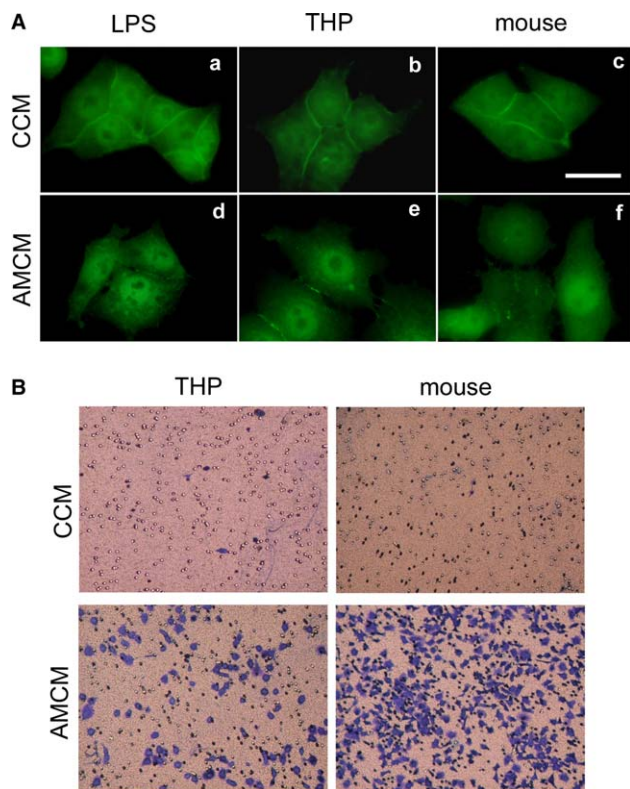
Fig. 7. Conditioned media from macrophages from various species activated by different methods alter the β-catenin distribution and migration of HepG2 cells. (A) Immunostaining for β-catenin. HepG2 cells were cultured in CCM or AMCM obtained from PMA-treated human THP cells (THP), LPS-treated RAW (LPS), or PMA-treated mouse peritoneal macrophages (mouse) for 24 h, then immunostained for β-catenin. (B) Migration assay. HepG2 cells were plated in the upper chamber and THP/AMCM, mouse/AMCM, or the respective control medium added to the lower chamber. After 20 h, the membranes were stained with Coomassie Blue and photographed.

β-catenin expression at the adherens junctions (Fig. 7A). Fig. 7B shows the increased migration of HepG2 cells caused by THP/AMCM or mouse/AMCM. These results clearly indicate that the functional components responsible for the disruption of the adherens junctions and the migration are secreted by human THP-1 cells, RAW macrophages, and mouse peritoneal macrophages activated by PMA or LPS, but not by interferon.

4. Discussion

In this study, we investigated the effect of activated macrophages on the metastatic behavior of HepG2 human hepatocellular carcinoma cells. We showed that conditioned media from macrophages from various sources activated in different ways significantly increased the migration and invasiveness of HepG2 cells. In accordance with this finding, levels of E-cadherin and β-catenin in the membrane fraction and the association between them were substantially decreased by treatment with AMCM. In addition, tyrosine-phosphorylation of β-catenin and c-Src was increased by the same treatment. Furthermore, both the Src family kinase inhibitor, PP2, and

the EGFR inhibitor, gefitinib, abrogated the AMCM-induced downregulation of E-cadherin and β-catenin at the adherens junctions, providing a mechanistic explanation for the AMCM-induced events.

As mentioned earlier, the role of TAMs in tumor progression has not yet been clearly defined. It was previously shown that co-culture of hepatocarcinoma cells with macrophages, resulting in macrophage activation, increases the invasive capacity of the tumor cells and that this effect is cell contact-dependent [29]. In our study, conditioned medium from activated macrophages was able to increase the invasive ability of the HepG2 cells, whereas conditioned medium from non-activated macrophages did not, showing that factors secreted by activated macrophages were responsible for the behavioral changes of HepG2 cells. It has been known for a long time that cancer is frequently associated with inflammation and that constant inflammation may aggravate the transformation of precancerous tissues [1]. A wide variety of chemical substances, such as cytokines and chemokines, are released by activated macrophages [24,30]. Several lines of evidence show that TAMs can play a dual role in the transformation of neoplasms [31]. For example, cyclooxygenase-2 secreted by activated macrophages has been shown to induce tumorigenic progression by downregulating E-cadherin in normal intestinal cells [32], while reactive nitrogen intermediates released by activated macrophages have been shown to have anti-tumor activity [33]. TAMs play an important role in tumor angiogenesis [24], but their role in tumor progression remains controversial. However, no correlation has been found between macrophage activation and the weakening of the adherens junctions in liver cancer cells. Macrophage conditioned medium has been shown to decrease E-cadherin expression at the adherens junctions in colon cancer cells [32]. A body of evidence supports the roles of Src and EGF in the downregulation of β-catenin at the adherens junctions [20,21,34], but their contribution to the macrophage-mediated deregulation of E-cadherin or β-catenin awaits elucidation. In the present study, we demonstrated that the c-Src- and EGFR-driven modulation of the adherens junctions may contribute to an enhanced aggressive phenotype of HepG2 cells in the microenvironment of activated macrophages. Although several tumor-progressive and tumor-regressive substances can be released by activated macrophages [24], our data suggest that, for liver cancer cells, the overall effects of activated macrophages tend to be tumor-progressive.

In this study, we ruled out the possibility that IL-4, IL-6, or IL-13 was involved in mediating the effects of AMCM. Our results agree with a previous finding that IL-6 does not affect cell adhesion in HepG2 cells [35]. IL-4 and IL-13 have been shown to regulate the expression and distribution of E-cadherin and β-catenin in a keratinocyte model [36]. In contrast, the present study showed that neither IL-4 nor IL-13 caused changes in cell morphology and β-catenin immunostaining patterns in HepG2 cells. The conflicting effects of IL-4 and IL-13 in these studies might be due to tissue specificity. One of the candidates in AMCM might be EGF, since inhibition of the EGFR by gefitinib partially blocked the AMCM-induced disassembly of the adherens junctions and the increase in migration. The identification of other potential tumor-progressive factors secreted by activated macrophages would be helpful for clinical cancer therapy.

The Src kinase is overexpressed in hepatocellular carcinoma cells [37]. Activation of c-Src kinase is linked to tumor-progres-

sive activities, such as mobility, invasion, and adhesion, by increasing focal adhesion kinase (FAK) activity and weakening adherens junctions [38]. In this study, pretreatment with PP2 effectively blocked the AMCM-induced mobility and invasiveness of HepG2 cells. However, the action of PP2 can be mediated by other pathways, such as the phosphatidylinositol-3 kinase-Akt cascade, as reported in a bladder cancer cell invasion model [39]. Thus, we cannot exclude possible involvement of the Akt pathway in the inhibitory effect of PP2 on cell invasion. In addition, Src kinase phosphorylates E-cadherin, which is then ubiquitinylated and degraded [40]. We noted translocation of active Src kinase to the cell membrane in AMCM-treated cells, which might favor the interaction between Src kinase and E-cadherin/β-catenin. Consistent with the above observations, some of the E-cadherin appeared as punctuate staining in the cytosol (Fig. 4A), which might represent internalized E-cadherin. This degradation may account for the Src kinase-dependent decrease in E-cadherin seen at adherens junctions in the present study.

NF-κB and JNK play important roles in macrophage-induced tumor-progression [41–43], and this pathway is considered a potential target for cancer treatment [44]. A recent study indicates that c-Src activation leads to IKK/NF-κB activation in PC-12 cells [45]. Thus, it is possible that, in addition to phosphorylating β-catenin, c-Src might exert its tumor progression effects via cross-talk with the IKK/NF-κB signaling pathway.

Our data showing that gefitinib partially blocked the AMCM-induced downregulation of β-catenin at the adherens junctions point out the involvement of the EGFR in the process of tumor progression. c-Src is involved in the transactivation of EGFR [46,47]. It is also known that phosphorylation of β-catenin Tyr654 is modified by EGFR-related proteins [21]. It is plausible that direct modification of β-catenin by the EGFR and/or transactivation of the EGFR by c-Src may mediate the AMCM-induced downregulation of β-catenin at the adherens junction.

Taken together, our results clearly demonstrate that that tyrosine phosphorylation of β-catenin plays an important role in activated macrophage-dependent regulation of the E-cadherin/β-catenin complex and that conditioned medium from activated macrophages have a tumor-progressive effect on hepatocellular carcinomas. Whether these effects are limited to HepG2 cells or certain types of cancers awaits further investigation.

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