RESEARCH ARTICLE

Cytokines Released from Activated Human Macrophages Induce Epithelial Mesenchymal Transition Markers of Cholangiocarcinoma Cells

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Abstract

Stromal-epithelial interactions are important for carcinogenesis. Once cancerous lesions develop, a chronically inflamed tumor microenvironment promotes migration and invasion of tumor cells. Multiple immune cell populations are involved in inflammatory processes, including tumor-associated macrophages (TAMs) which have been proposed as major contributors to tumor progression. The epithelial-mesenchymal transition (EMT) is a process in which epithelial cells trans-differentiate and acquire an invasive mesenchymal phenotype. As EMT represents a crucial step in disease progression, it is important to investigate the mechanisms regulating this step. We aimed to identify the profiles of cytokines produced by activated human macrophages and to demonstrate effects on the expression of EMT-related genes in human cholangiocarcinoma (CCA) cell lines. Our results showed that LPS-activated macrophages produced and secreted IL4, IL6, IL10, TNF-α and TGF-β1. After addition of macrophage conditioning media to CCA cells, expression of epithelial markers E-cadherin and CK-19 was significantly reduced, whereas the expression of mesenchymal markers, S100A4 and MMP9 was strongly induced. Taken together, various cytokines secreted by activated macrophages could induce EMT by altering the expression of EMT-related genes in CCA.

Keywords: Macrophages - macrophage-derived cytokines - epithelial mesenchymal transition - cholangiocarcinoma

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Introduction

The inflammatory microenvironment plays a key role in the progression of solid malignant tumors (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Multiple immune cell populations are involved in the inflammatory process. Among them, the macrophage appears markedly important in chronic inflammation (Torisu et al., 2000). Indeed, tumor-associated macrophages (TAMs) have been extensively studied and proposed as a major contributor to tumor progression (Bingle et al., 2002). TAMs can release a vast diversity of cytokines, proteolytic enzymes, growth factors and inflammatory mediators that may directly influence the behavior of tumor cells (Jedinak et al., 2010). An increased number of TAMs is associated with a better prognosis in lung cancer (Kerr et al., 1998) but with a poor prognosis in breast cancer and cholangiocarcinoma (CCA) (Leek et al., 1996; Subimerb et al., 2010). These opposite effects might be explained by tissue-type specificity. However, the interaction between TAMs and cancer cells is extremely complicated and has not been clearly elucidated.

An important step in tumor progression is the acquisition of invasive properties by tumor cells. Epithelial mesenchymal transition (EMT) is a well characterized mechanism through which epithelial cells trans-differentiate and acquire an invasive mesenchymal phenotype (Nawshad et al., 2005; Thiery et al., 2009). EMT has recently been recognized for its involvement in tumor progression and the mechanisms have been linked to fibrosis and metastasis (Yang and Weinberg, 2008; Wu et al., 2009; Yilmaz and Christofori, 2009). As EMT represents a crucial step in tumor progression, it is of interest to identify and characterize the mechanisms regulating this step. TAMs have likewise been shown to induce EMT mainly through TNF-α mediated Snail, a key mediator and marker of EMT (Bates and Mercurio, 2003; Wu et al., 2009). Interestingly, TAMs induction of EMT in tumor cells correlates with metastasis in a murine breast cancer model which underscores the importance of both EMT and the macrophage in disease progression (Wu et al., 2009).

In this study, we sought to identify the production of cytokines and pro-inflammatory mediators in the activated macrophage and investigated the effects of the activated macrophage conditioned media (AMCM) on

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the regulation of EMT-related genes in CCA in order to reveal whether activated macrophages can be regulators of EMT in CCA.

Materials and Methods

Cell culture and preparation of macrophage-conditioned media

The M214 human CCA cell line, and the human U937 macrophage cell line (American Type Culture Collection; ATCC) were maintained in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum in a humidified atmosphere containing 5% CO2. All other chemicals used were of analytical grade.

Human U937 macrophage cell lines were treated with 2 ug/ml of lipopolysaccharide (LPS, Sigma. St. Louis, MO) for 48 h. Then the medium was collected and centrifuged to remove debris. The supernatant from the LPS-treated U937 macrophages was designated as activated macrophage-conditioned media (AMCM) and that from the untreated control cells as non- activated macrophage-conditioned media (NAMCM).

Treatments

CCA cells were seeded at $1x10^5$ cells in 6-well culture plates prior to treatment. Cells were 1) left untreated, or 2) treated with 2 ug/ml LPS 3) AMCM 4) NAMCM for 48 hr. After incubation, cells were harvested for further analysis. All treatments were done in duplicates.

Cytokine measurements

The release of cytokines and pro-inflammatory mediators in AMCM and NAMCM were determined by commercial ELISA kits (#MEH003A; QIAGEN, Valencia, CA) for IL2, IL4, IL5, IL6, IL10, IL12, IL13, IL17A, IFN-γ, TNF-α, G-CSF and TGF-β1 according to the manufacturer's instructions.

Real time quantitative RT-PCR for EMT-related genes

Total RNA was extracted and purified from CCA cell lines of treated and non-treated groups using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. cDNA was synthesized using the MMLV-RT system (Promega, Madison, USA) for RT-PCR. Quantitative real-time RT-PCR of EMT-related genes (E-cadherin, CK-19, S100A4 and MMP9) determinations were carried out on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using master SYBR green kits (Roche Diagnostics, Brandford, CT). The PCR conditions were an initial denaturation step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles consisting of 15 s at 95°C, and a 1 min at 55°C. β-actin was used as an internal control.

Western blot analysis

The whole cell lysates were electrophoresed and transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membranes were incubated with anti-E-cadherin antibody at 4°C overnight and secondary antibody at room temperature for 1 h. Peroxidase activity was detected by western lighting chemiluminescence reagents (Perkin Elmer, Boston, MA). β-actin was used as the internal control.

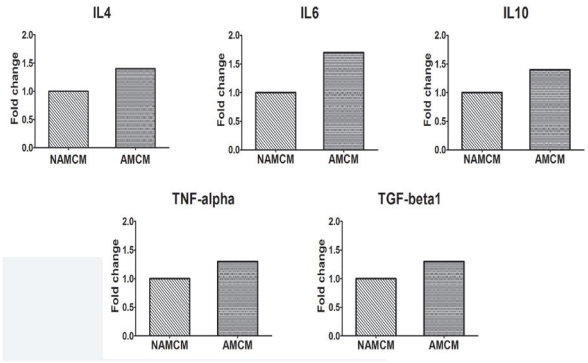

Figure 1. Production of Cytokines and Proinflammatory Mediators in Activated Macrophage Conditioned Media (AMCM) Compared with Non- Activated Macrophage-Conditioned Media (NAMCM). Cytokines levels were determined by ELISA as described. The data shown are representative of fold changes between AMCM as compared to NAMCM

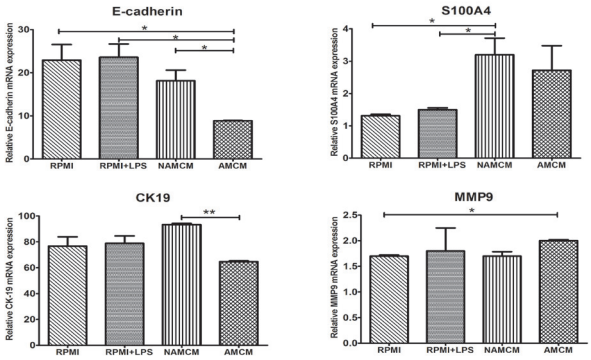

Figure 2. Quantitative RT-PCR Assay for EMT-Related Genes Epithelial markers, E-cadherin and CK-19 and mesenchymal markers, S100A4 and MMP9 were asssessed in activated macrophage conditioned media (AMCM) and non-activated macrophage-conditioned media (NAMCM) compared with RPMI (media alone) and Llpopolysacharide (LPS) in Combination with RPMI (RPMI+LPS). Data are shown as mean ± SD **P*-value <0.05; ***P*-value <0.01

Statistical analysis

Data were expressed as mean ± Standard deviation, SD and statistically analyzed using the Student's t-test. A *P*-value <0.05 was considered statistically significant.

Results

Production of cytokines and proinflammatory mediators in activated macrophages

In order to confirm that LPS induces secretion of cytokines and proinflammatory mediators from macrophages, U937 macrophage cells were untreated (NAMCM) or treated with LPS (AMCM) and the secretion of those molecules were determined as described above. Our results showed that the stimulation of macrophage with LPS increased the level of IL4, IL6, IL10, TNF-α and TGF-β1 in AMCM as compared with unstimulated macrophages, NAMCM. As shown in Figure.1, AMCM

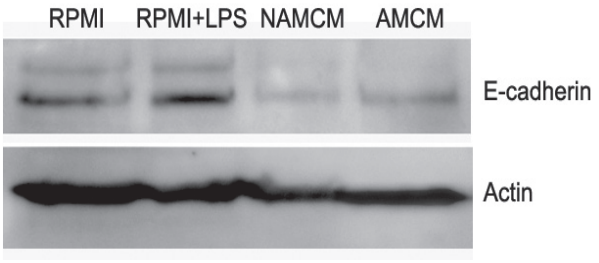

Figure 3. Western Blot Analysis of E-cadherin. Protein expression was assessed upon stimulation with activated Macrophages Conditioned Media (AMCM) and Non- Activated Macrophage-Conditioned Media (NAMCM) compared with RPMI (media alone), or Lypopolysacharide (LPS) in Combination with RPMI (RPMI+LPS)

showed increase in the levels of IL4, IL6 and IL10 at 1.4, 1.7 and 1.4 fold respectively when compared with NAMCM. In addition, both TNF-α and TGF-β1 increased to 1.4 fold over NAMCM. In contrast, no different levels of IL5, IL12, IL13, IL17A, IFN-γ and G-CSF were seen.

Activated macrophage induces EMT-related genes in CCA

We evaluated whether inflammatory mediators released by macrophages altered the expression of EMT-related genes in CCA cells. As shown in Figure. 2, conditioned media from activated macrophages (AMCM) caused a significant reduction of E-cadherin mRNA expression when compared with RPMI, RPMI+LPS-treated and non-activated macrophage-conditioned media (NAMCM) (P=0.03, 0.02 and 0.03, respectively). In addition, CK-19, the other epithelial marker, was significantly down-regulated in AMCM (P=0.001) when compared with NAMCM. Moreover, up-regulation of S100A4, the mesenchymal marker, was found in NAMCM and AMCM groups. Increasing the level of MMP9, the other mesenchymal marker, was found in the AMCM group. No different levels between LPS-treated and untreated control were found in all EMT-related markers. In addition, loss of E-cadherin expression is generally accepted as a hallmark of the EMT process, therefore we sought to further investigate whether activated macrophages altered the protein level of E-cadherin. Our results showed that E-cadherin protein was completely reduced in both NAMCM and AMCM groups when compared with LPS-treated and untreated controls as shown in Figure. 3.

Discussion

This is the first report demonstrating paracrine stimulation of CCA cells via soluble mediators produced by activated macrophages. The major findings in this study are: the supernatant of LPS-activated macrophage contains a number of cytokines and proinflammatory mediators including IL4, IL6, IL10, TNF-α and TGF-β1. Moreover, addition of conditioned media from activated macrophages altered the expression of EMT-related genes, especially a reduction of E-cadherin expression which is considered as a hallmark of the EMT process in both mRNA and protein level. Our results provide information about the role of macrophages in tumor inflammatory microenvironment as a regulator for the EMT process.

Stromal–epithelial interactions are important in carcinogenesis. Once cancerous lesions develop, a chronically inflamed tumor microenvironment stimulates aberrant angiogenesis and promotes migration and invasion of tumor cells. The tumor inflammatory microenvironments are infiltrated with multiple subsets of immune cells, including macrophages, neutrophils, eosinophils, dendritic cells, mast cells and lymphocytes (Coussens and Werb, 2002). In particular, we focused on macrophages because they are a major component of the immune infiltrates present in the tumor microenvironment and play one of the key roles in chronic inflammation (Torisu et al., 2000). Furthermore, tumor-associated macrophages (TAMs) are involved in promoting tumor progression and metastasis (Pollard, 2004).

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 (Mukai et al., 1987). Moreover, macrophages have been shown to regulate EMT at the invasive front through paracrine TNF-α signaling and Snail stabilization, linking tumor inflammation to EMT and metastasis (Bates and Mercurio, 2003). In our study, conditioned media from activated macrophages was able to alter the expression of EMT-related genes by which decrease the mRNA expression of epithelial markers, E-cadherin and CK-19 occurred whereas mesenchymal markers including S100A4 and MMP9 were slightly increased. In addition, our results showed that E-cadherin protein was completely reduced in both NAMCM and AMCM groups when compared with LPS-treated and untreated controls, showing that factors secreted by both activated and non-activated macrophages were responsible for the behavioral changes of CCA cells via the EMT process. This is similar to Ko et.al, who demonstrated that macrophage-conditioned medium has been shown to decrease E-cadherin expression at the adherens junctions in colon cancer cells (Ko et al., 2002). Our study highlights the interaction between the inflammatory response and EMT-related CCA development and suggests that CCA cells can play a direct role in communication with a local inflammatory response.

A wide variety of chemical substances, such as cytokines and chemokines, are released by activated macrophages (Chen et al., 2005). Those factors were responsible for several cancer progressions including EMT process. In the present study, activated macrophages released a higher number of cytokines including IL4, IL6, IL10, TNF-α and TGF-β1 upon LPS stimulation. IL4 has been shown to regulate the expression and distribution of E-cadherin, the EMT marker in the keratinocyte model (Fujii-Maeda et al., 2004). Recently, the inflammatory cytokine IL-6 has been reported as a potent inducer of EMT in breast, head and neck cancer cells (Sullivan et al., 2009; Yadav et al., 2011). Moreover, TGF- β induces EMT through activation of various intrinsic pathways (Bakin et al., 2000; Nawshad et al., 2005; Gal et al., 2008; Vincent et al., 2009). It stimulates the production of MMPs which are important stroma-derived inducers of tumor cell EMT (Radisky et al., 2005). Additionally, it has

recently been shown that EMT phenotypes are acquired in CCA by TGF- β1 (Sato et al., 2010). TNF-α can also promotes EMT of various cancer cells including CCA (Tanimura et al., 2005; Chuang et al., 2008; Techasen et al., 2012). The activation of signaling pathways results in the activation of transcriptional regulators which regulate the changes in gene expression patterns underlying EMT. However, no report is focused on the relationship between IL10 and EMT process. Taken together, various cytokines secreted by activated macrophages can induce the EMT phenotype in cancer cells. In our model, it implies that those factors released from activated macrophages may contribute to EMT phenotype and alter the expression of EMT-related genes in CCA. Our data provide information about the role of macrophages in the tumor inflammatory microenvironment that can be a regulator for the EMT process through their secreted cytokines.

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