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Milky spot macrophages remodeled by gastric cancer cells promote peritoneal mesothelial cell injury

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ABSTRACT

Peritoneal dissemination (PD) is the most frequent metastatic pattern of advanced gastric cancer (GC) and the main cause of death in GC patients. Human peritoneal mesothelial cell (HPMC) injury induced by gastric cancer cells (GCCs) and GCC outgrowths supported by peritoneal milky spot macrophages (PMSMs) are the key events during gastric cancer peritoneal dissemination (GCPD). In this study, we investigated whether PMSMs remodeled by GCC can induce HPMC injury and create a favorable microenvironment for GCPD. We established a tumor-associated macrophage (TAM) model using *in vitro* cell coculture. Normal macrophages cocultured with GCCs down-regulated expression of antigen-presenting surface molecules CD80, CD86, and MHC-II, but, notably, they up-regulated expression of phagocytic scavenger receptor CD206, which is similar to the M2 macrophage phenotype. In further experiments, various experimental methods were applied to detect the injurious effect of TAMs on HPMCs in another TAM–HPMC coculture. Our results showed that GCCs can induce HPMC apoptosis by unregulated apoptosis associated with cleaved caspase3, cleaved caspase9, and p21 proteins. HPMC growth ceased, and both early- and late-stage apoptosis were observed. Additionally, GCCs can induce HPMC fibrosis via increased expression of epithelial cell marker E-cadherin and decreased expression of mesenchymal cell marker α-SMA. Our results demonstrate that, in the GCPD process, PMSMs were remodeled by GCCs, resulting in phenotypic and functional transformation. In turn, this transformation induced HPMC injury and provided a favorable microenvironment for GCC anchorage and growth. These results may provide new insight into the mechanisms of GCPD.

1. Introduction

Peritoneal dissemination (PD) is one of the most common patterns of metastases in gastric cancer (GC), and it is the main contributor to the failure of radical gastrectomy for advanced gastric carcinoma (AGC) [1,2]. Although the prognosis of patients with gastric cancer peritoneal dissemination (GCPD) seems to have improved as a result of the standardization of surgical techniques and recent advances in intraperitoneal chemotherapies [3,4], the 5-year postoperative survival rate remains low [5]. Moreover, the mechanisms of peritoneal metastasis have not yet been clearly defined.

Human peritoneal mesothelial cells (HPMCs) and peritoneal milky spot macrophages (PMSMs) constitute the first line of defense in the peritoneum. However, when this barrier is breached, the peritoneal microenvironment favors proliferation of GCCs and serves as a rich source of growth factors and chemokines known to be involved in GCPD [6–8]. The monolayer of HPMCs that covers the peritoneum plays an important role in maintaining its structure and function.

In our previous study, gastric cancer cells (GCCs) attached to the integrated HPMC monolayer, and the invasion and migration abilities of GC were effectively inhibited [9,10]. However, a longer period of GCC coculture can induce HMPC apoptosis and fibrosis both by upregulation of caspase3, caspase9, and E-cadherin and by down-regulation of B-cell lymphoma 2 (Bcl-2) and alpha smooth muscle actin (α-SMA). As a result, the HPMC layer collapses and exfoliation takes place. Naked areas of submesothelial connective tissue are thus exposed to the peritoneal cavity, and this peritoneal injury site becomes a favorable microenvironment

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for GCPD. Therefore, the structure and function of HPMCs play roles in preventing the adhesion and colonization of cancer cells [7,11,12]. HPMC injury induced by apoptotic and fibrotic cytokines is the key event in GCPD.

Peritoneal milky spots (PMSs) are widely located in mammalian peritonea, which are mainly composed of immature macrophages and natural killer cells, as well as a few antigen-presenting dendritic cells and B cells [13,14]. Tumor cells enter PMSs as they exfoliate from primary tumor lesions, colonize within them, and form micrometastases, thus inducing PD. Immature peritoneal milky spot macrophages (PMSMs) lack antigen-presenting and tumor-eradicating abilities; they cannot effectively scavenge tumor cells [15]. Conversely, PMSMs remodeled by tumor cells form tumor-associated macrophages (TAMs) with an alternative active macrophages (M2 macrophages) phenotype, which provide nutrition and support for tumor cells. Therefore, PMSM remodeling is another key event during PD [16].

Presently, the source of apoptotic and fibrotic cytokines that induce HPMC injury within the GCPD microenvironment is mainly thought to be peritoneum-free GCC [6–13]. However, in the GCPD process, many inflammatory cells are infiltrated within the PMS chemotaxis by GCCs from peripheral blood or nearby PMSs. These inflammatory cells are mainly composed of M2 PMSMs. In turn, this induces a chronic inflammatory microenvironment in which large quantities of stroma remolding factors, such as transforming growth factor beta 1 (TGF-β1) and connective tissue growth factor (CTGF), are released [17–19]. TGF-β1 is the most potent apoptotic and fibrotic stimuli released by GCCs, so we hypothesized that PMSM remodeling by GCCs induces HPMC injury and creates a microenvironment favorable to peritoneal metastasis. In this study, we investigated the interaction of TAMs and HPMCs at the early stage of GCPD, and we have formulated new explanation for HPMC injury during GCPD.

2. Materials and methods

2.1. Reagents and antibodies

RPMI-1640, Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Cell culture materials were purchased from Corning (Corning, NY, USA); diamidino-2-phenylindole (DAPI) phorbol-12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and rabbit anti-human fibronectin antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mouse anti-human CD80-fluorescein isothiocyanate (FITC), mouse anti-human CD86-FITC, mouse anti-human MHC-II-FITC, mouse anti-human CD206-FITC, mouse anti-human E-cad antibodies, and an Annexin V-PI apoptosis detection kit were purchased from Becton Dickinson (San Jose, CA, USA). A Quantikine human TGF-β1 ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Mouse anti-human CD68, mouse anti-human CD163, mouse anti-human CK-8, and rabbit anti-human α-SMA antibodies were purchased from Abcam (Cambridge, MA, USA). β-actin and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). E-caprolactone (ECL) solution was purchased from Pierce Chemical Co. (Rockford, IL, USA). Fluorescence secondary antibody was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell lines

The HPMC cell line HMR-SV5, human monocyto-macrophage line THP-1, human normal gastric glandular epithelial cell line GES-1, and the poorly differentiated GCC line SGC-7901 were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). SGC-7901 was cultured in DMEM medium containing 10% FBS. The HMR-SV5, THP-1, GES-1 cell lines were all cultured in RPMI-1640 media containing 10% FBS. All cell cultures were incubated continuously under a 5% CO_2 atmosphere at 37 °C.

2.3. Establishment of the TAM model

THP-1 cells ($\sim 1 \times 10^6$ cells/well) were inoculated with 50 μg/ml PMA; culture media were changed when THP-1 growth attached to the plastic substrates. Following attachment, a coculture insert was added, and approximately 2×10^5 GES-1 or SGC-7901 cells were plated into the upper chamber, respectively. The whole coculture system was cultured in RPMI-1640 containing 10% FBS for 72 h. THP-1 in the lower chamber was collected after coculture for cell-surface staining analysis, western blot assay, and immunofluorescence assay. THP-1 cells cocultured with GES-1 cells were referred to as the normal macrophage group (T group); THP-1 cells cocultured with SGC-7901 cells were referred to as the TAMs group (TA group).

2.4. Establishment of TAM-induced HPMC injury model

HMR-SV5 cells ($\sim 1 \times 10^6$) were inoculated into a six-well cell culture plate until 80% cell fusion, at which point a cocultured insert was added. For both the T and TA groups, approximately 2×10^5 THP-1 cells were plated into the upper chamber, then the whole coculture system was cultured in RPMI-1640 containing 10% FBS for 48 h. The cells in the upper chamber were then discarded, and the HMR–SV5 cells in the lower chamber were collected for MTT assay, western blot assay, and immunofluorescence assay. Normal untreated SV5 cells were referred to as the SV5-C group; cells cocultured with T group macrophages were referred to as the SV5-T group; and TA group macrophages and were referred to as the SV5-TA group.

2.5. Scanning electron microscopic (SEM) detection

Cell specimens were placed on a cover slip, and glutaraldehyde-sodium cacodylate fixative fluid was added to the specimens and allowed to adhere for 2–3 h. The specimens were washed twice with sodium cacodylate buffer, followed directly by gradient dehydration in ethanol. Then the specimens were transferred into 100% isopentyl acetate and dried to a critical point. Then the specimens were coated with gold in a vacuum and prepared for examination in the scanning electron microscope (JCM 5000, Nikon, Japan) .

2.6. Cell surface antigen analysis by fluorescence activated cell sorting (FACS)

Macrophages from each group were harvested using trypsin–EDTA then washed twice with ice-cold phosphate buffering solution (PBS) with 1% bovine serum albumin (BSA). Incubation was carried out, respectively, with CD80-FITC, CD86-FITC, MHC-II-FITC, CD206-FITC antibodies for 30 min at 4 °C in the dark. After washing twice with ice-cold PBS with 1% BSA, cell-surface staining was then determined by flow cytometry (FACS Caliber, Becton Dickinson).

2.7. Cytokine assay by ELISA

Cytokine production was determined using a Quantikine human TGF-β1 ELISA kit (R&D Systems) according to the manufacturer's instructions.