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Research paper

Culture supernatants of cervical cancer cells induce an M2 phenotypic profile in THP-1 macrophages

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ABSTRACT

Patients with cervical cancer (CxCa) typically present an infiltrate of tumor-associated macrophages, which is associated with a poor prognosis. We found that CxCa cell lines (HeLa, SiHa, and C-33A) secreted factors involved in regulating tumor growth including IL-6, IL-4, $PDGF_{AA}$, HGF, VEGF, ANG-2, and TGF-β3. We assessed the effects of culture supernatants from these cell lines on macrophages derived from the THP-1 cell line. Macrophages treated with culture supernatants from CxCa cells developed an M2-like phenotype with expression of CD163, low nitric oxide release, and high secretion of IL-6, $PDGF_{AA}$, HGF, ANG-2, and VEGF. The macrophages continued to produce $PDGF_{AA}$, $PDGF_{BB}$, and VEGF 48 h after the CxCa cell culture supernatants were removed. The induction of M2 macrophages *in vivo* favors tumor growth, angiogenesis, tissue remodeling, and metastasis. These results demonstrated that factors secreted by CxCa cells induced a stable M2 phenotype in THP-1 macrophages.

1. Introduction

Cervical cancer (CxCa) is the second most commonly diagnosed and third leading cause of cancer death among females in developing countries [1]. The main etiological factor for the development of CxCa is persistent infection with high-risk human papillomavirus (HPV) genotypes such as HPV-16 and HPV-18 [2–4]. However, other associated co-factors including multiparity, smoking, long-term consumption of oral contraceptive pills, and immunosuppression have also been implicated in the development of CxCa [5,6].

An immunosuppressive microenvironment is produced in diverse cancers through low immunogenicity and the production of cytokines such as transforming growth factor (TGF)-β, interleukin (IL)-10, and indoleamine 2,3-dioxygenase that suppress pro-inflammatory Th1 and cytotoxic lymphocyte responses. However, in approximately 50% of patients with CxCa, a weak proliferative response of T-cells has been observed. This is associated with a phenotypic switch of tumor-infiltrated cells such as T cells, neutrophils, dendritic cells, and macrophages from tumor-suppressing to tumor-promoting behaviors in response to the tumor microenvironment [7–9]. In addition, diverse reports have described the overexpression of chemokines such as monocyte chemoattractant protein-1 in CxCa [10–12] and a high level of infiltration of macrophages into the tumor tissue [7,13].

Abbreviations: ANG, angiopoietin; CMS, culture medium supplement; CxCa, cervical cancer; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GM-CSF, granulocyte-monocyte colony-stimulating factor; HGF, hepatocyte growth factor; HLA-DR, human leukocyte antigen-antigen D related; HPV, human papillomavirus; IFN, interferon; IL, interleukin; LPS, lipopolysaccharides; M-CSF, macrophage-colony stimulating factor; MFI, mean fluorescence intensity; NO, nitric oxide; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; RPMI, Roswell Park Memorial Institute medium; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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Macrophages that infiltrate into the tumor are generally called tumor-associated macrophages and mainly exhibit two contrasting phenotypes. First, classically activated or M1 macrophages that are activated by interferon (IFN)-γ, lipopolysaccharides (LPS) through Toll-like receptors, and granulocyte-monocyte colony-stimulating factor (GM-CSF). Macrophages of the M1 phenotype are characterized by a high expression of major histocompatibility complex class II and co-stimulatory molecules such as CD86/CD80, as well as a high production of IL-12, IL-23, tumor necrosis factor (TNF)-α, reactive oxygen species, and reactive nitrogen species such as nitric oxide (NO). The main functions of M1 macrophages are to kill intracellular pathogens, destroy tumors, and promote the Th1 immune response. Second, alternatively activated or M2 macrophages that are activated by IL-4, IL-13, IL-10, IL-33, and IL-21 and are characterized by the expression of CD163 and CD206. Macrophages of the M2 phenotype participate in parasite clearance, tissue remodeling, wound healing, immunoregulation, promoting the Th2 immune response, and tumor promotion through the expression of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, indoleamine 2,3-dioxygenase, and programmed death ligand 1 expression. Tumor-associated macrophages have a high plasticity and frequently share features of both the M1 and M2 phenotypes. In diverse tumors, the presence of M2 macrophages is associated with a poor prognosis [14–16].

Several reports have described the secretion of various factors including IL-6, IL-13, TGF-β, VEGF, and prostaglandin E_2 by CxCa cells [11,17–19]. However, it has not yet been clearly established whether factors secreted by CxCa cells perform an important role in the induction or maintenance of the M2 macrophage phenotype.

Therefore, in this work, we first characterized the profile of cytokines, growth factors, and NO present in the CxCa cell culture supernatants. Then, we investigated whether those supernatants could induce the M2 phenotype in THP-1 macrophages. Finally, we evaluated whether the M2 phenotype was maintained after removing the CxCa cell culture supernatants.

2. Methods and materials

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute-1640 medium (RPMI-1640), heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin antibiotics solution, and phosphate-buffered saline (PBS) were purchased from Life Technologies (Carlsbad, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-4, IL-10, and IFN-γ were purchased from BioLegend (San Diego, CA, USA). For flow cytometry assays, the following anti-human monoclonal antibodies were employed: CD14-PerCP, CD80-FITC, CD86-PE, CD163-FITC, and CD206-PECy7 (BioLegend), and human leukocyte antigen-antigen D related (HLA-DR)-APC, STAT1-PE, pSTAT1-PE, STAT6-PE, pSTAT6-PE, and NF-κB p65-PE (BD Biosciences, San Jose, CA, USA). For the determination of cytokines and growth factors in the cell culture supernatants, the Human Th1/Th2/Th17 CBA kit (BD Biosciences) and Human Growth Factor Panel Multi-analyte Flow Assay kit (BioLegend) were utilized. For the determination of TGF-β, the Bio-Plex® Pro TGF-β-3-plex Assay (Bio-Rad Laboratories, Hercules, CA, USA) was employed. NO production was measured using the Total Nitric Oxide Nitrate/Nitrite Colorimetric Assay (R&D Systems, Minneapolis, MN, USA).

2.2. Cell culture

HeLa (HPV-18$^+$), SiHa (HPV-16$^+$), and C-33A (HPV$^-$) CxCa cell lines were kindly provided by Dr. Boukamp (DKFZ, Heidelberg, Germany) and were propagated *in vitro* by culture in DMEM containing culture medium supplement (CMS; 10% [v/v] heat-inactivated FBS, 2 mM L-glutamine, and 1 × streptomycin/penicillin solution). THP-1 monocytes (cat. no. TIB-202) were purchased from American Type Culture Collection (Manassas, VA, USA) and were propagated *in vitro* by culture in RPMI-1640 containing CMS. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Collection of culture medium supernatants from CxCa cell lines

CxCa cell lines were independently seeded in 6-well culture plates at 1×10^5 cells per 3 mL of DMEM containing CMS per well. At day 5 of cell culture, the culture medium supernatants were collected [17] and stored at −80 °C until they were employed for the assessment of M2 macrophage induction and the release of cytokines, growth factors, and NO.

2.4. THP-1 cell line differentiation and treatment with CxCa cell culture supernatants

To differentiate the THP-1 cell line into macrophages, the undifferentiated THP-1 cells were seeded onto 12-well culture plates at 1×10^6 cells per 2 mL of culture medium (RPMI containing CMS and 200 nM PMA) and cultured for 3 d. Subsequently, the cells were washed 3 times with PBS and treated with CxCa cell culture supernatants (50% of the total cell culture volume) for either 1 h or 3 d. In the THP-1 macrophages treated for 1 h with CxCa cell culture supernatants, we assessed the phosphorylation of STAT1, STAT6, and NF-κB (subunit p65) transcription factors. In the THP-1 macrophages treated for 3 d with CxCa cell culture supernatants, we evaluated the expression of membrane receptors (CD14, CD80, CD86, HLA-DR, CD163, and CD206) and the concentrations of cytokines, NO, TGF-β family proteins, and growth factors in the macrophage culture medium. In another group of THP-1 macrophages, after treatment with CxCa cell culture supernatants for 3 d, we replaced the culture medium with fresh medium, cultivated the cells for an additional 48 h, and then evaluated the concentrations of cytokines, NO, TGF-β family proteins, and growth factors in the culture medium.

As an experimental control, we activated THP-1 macrophages with 20 ng/mL of IFN-γ plus 100 ng/mL of LPS or 20 ng/mL of IL-4 plus 20 ng/mL of IL-10 to induce the M1 and M2 phenotypes, respectively. The cells were incubated with these cytokines during the final 18 h of treatment with 200 nM PMA. Untreated Mθ macrophages (baseline condition without phenotypic induction) were maintained in RPMI containing CMS during the assays, and M1 and M2 macrophages were also maintained in RPMI containing CMS after activation.

2.5. Assessment of CD14, CD80, CD86, CD163, CD206, and HLA-DR expression, and STAT1, p65, and STAT6 phosphorylation in THP-1 macrophages by flow cytometry

To evaluate the expression of CD14, CD80, CD86, CD163, CD206, and HLA-DR in THP-1 macrophages treated for 3 d with the CxCa cell culture supernatants, we harvested the cells in all experimental groups, washed them twice with PBS, and stained them to discriminate live and dead cells with the LIVE/DEAD® Fixable Near-IR Dead Cell Stain kit (Life Technologies) according to the manufacturer's protocol. The harvested cells were then washed twice with PBS, and human Fc receptors were blocked using Fc Receptor Blocking Solution (BioLegend). We then incubated the cells with antibodies for human CD163-FITC, CD80-FITC, CD86-PE, CD14-PerCP, CD206-PECy7, or HLA-DR-APC for 30 min at room temperature. Subsequently, we washed the cells twice with PBS,