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Effects of digitoxin on cell migration in ovarian cancer inflammatory microenvironment

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ABSTRACT

Clinical and experimental evidence supports a role for cardiac glycosides (CGs) as potential novel anticancer drugs. However, there are no studies reporting the effect of CGs on the inflammatory tumor microenvironment (TME), which plays a central role in tumor progression and invasiveness. We investigated whether digitoxin affects a) specific pathways involved in motility and/or activation of different cell types shaping TME, and b) cancer cell growth and invasiveness in response to TME-associated factors. To test our hypothesis, conditioned media (CM) from polarized macrophages, and apoptotic or non-apoptotic ovarian cancer cells (SKOV3) were tested as chemoattractants for endothelial cells, monocytes and cancer cells.

We demonstrated that CM from M1 (LPS/IFNγ) and M2 (IL-4/IL-13) polarized macrophages, which mimic inflammatory TME, increased both HUVEC migration and tubularization. Treatment of HUVECs with digitoxin at concentrations within its plasma therapeutic range counteracted these effects. Digitoxin affected the expression of neither M1 (CD80/CD68) nor M2 (CD206/CD163) activation markers, nor the amount of cell-bound IL-1β and CCL22. Accordingly, HUVEC migration in response to CM from digitoxin-treated activated macrophages was unchanged. These data point to a direct effect of digitoxin on HUVEC signaling rather than on the modulation of the cytokine profile released from activated macrophages. At variance with what observed for HUVECs, digitoxin did not prevent monocyte migration induced by SKOV3 CM. In addition, digitoxin significantly impaired SKOV3 growth and migration in response to M1 or M2 macrophage CM. Finally, we showed that digitoxin inhibited FAK phosphorylation in SKOV3 but not PYK2 phosphorylation in monocytes, thus providing a molecular mechanism accounting for the observed differential anti-migratory effect. Overall, digitoxin counteracted salient features of the inflammatory ovarian cancer microenvironment, laying the ground for potential digitoxin repositioning as an anticancer drug.

1. Introduction

Cardiac glycosides (CGs) such as digoxin, digitoxin and ouabain are inhibitors of Na/K-ATPase, and have been used for a long time to treat congestive heart failure and cardiac arrhythmias. Epidemiological studies reported that patients on digitalis therapy appear to be more protected from some types of cancers [10,26]. Several lines of evidence suggest that CGs, with mechanisms unrelated to sodium pump inhibition [40], have a selective cytotoxic and anti-proliferative activity against tumor cells [21]. Accordingly, we recently demonstrated that ouabain induces autophagic cell death in lung cancer cells [53]. In addition, it has been demonstrated that CGs prevent cancer cell migration [17,22]. We recently observed that digitoxin at clinically relevant concentrations switches off angiogenesis by hampering growth factor-induced focal adhesion kinase (FAK) activation and endothelial cell (EC) migration and tubularization, without affecting EC proliferation and viability [54]. FAKs are non-receptor tyrosine kinases including FAK1 and proline-rich tyrosine kinase 2 (PYK2)/FAK2, and both play a central role in cell motility. Of these, FAK1 has been widely studied in the context of cancer cell migration, proliferation, and survival. Consistently, several studies have demonstrated that FAK1 promotes tumor progression and metastasis through direct effects on cancer as well as the tumor microenvironment (TME) cells [46], whereas PYK2 is a known regulator of hematopoietic cell motility [32].

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Overall, these studies point to a potential use of CGs as anticancer drugs.

The tumor microenvironment consists of various stromal cells including activated endothelial cells and inflammatory cells. The inflammatory component plays a central role in the TME and orchestrates tumor progression and invasiveness [9,43,57]. For instance, ovarian cancer (OC) is the second most common gynecological cancer and the leading cause of cancer deaths in women in the United States and Europe [58,7]. It is a highly invasive tumor characterized by a complex unique intra-tumor as well as surrounding TME, namely peritoneal fluid, the latter being responsible for the generation of ascites. In particular, besides hematogeneous dissemination, the peritoneal fluid microenvironment enables the *transcoelomic* spread of cancer cells to other pelvic and peritoneal organs. Consistently, even at a stage when the primary tumor is still confined to the ovary, cancer cells can be detected in the peritoneal lavage fluid [47,57].

Tumor associated macrophages (TAMs), which are mostly derived from peripheral blood monocytes [28,57], are a key component of the inflammatory TME and represent the most abundant immune cell type in both human ovarian tumors and ascites [48,11]. A hallmark of macrophages is their plasticity in response to microenvironmental stimuli [55]. Distinct macrophage subpopulations may adopt tumoricidal versus protumoral phenotypes engaged in different biological functions related to tumor progression, including angiogenesis. In particular, classical activation results in immune stimulatory, pro-inflammatory M1 macrophages, whereas alternatively-activated M2 macrophages comprise a wide spectrum of subtypes with functions in tissue repair and angiogenesis. TAM activation is skewed by TME factors to a spectrum of phenotypes that represent mixed forms of alternatively-activated and pro-inflammatory macrophages [31,37]. Interestingly, it has been demonstrated that ascites from patients with ovarian carcinoma contains M2 macrophages [48], and accumulation of M2-subtype TAMs correlates with ovarian cancer progression [58]. In the setting of ovarian cancer, TAMs supply the microenvironment with chemoattractant cytokines and growth factors, which in turn support various aspects of cancer growth and progression, including cell invasion, angiogenesis and metastasis [11,33,15,9,56]. Accordingly, the presence of TAMs in ovarian cancer correlates with poor prognosis [37,5]. Hence, targeting TME and particularly interfering with cell migration in the TME affects several key processes for cancer progression. No studies to date report effect of CGs on TME.

The hypothesis underlying this study is that the CG digitoxin would hinder ovarian cancer progression by affecting: a) cancer cell invasiveness in response to TME, and b) motility and/or activation of different cell types shaping TME. In particular, we sought to assess whether digitoxin at clinically relevant concentrations modulates the migration and tubularization of endothelial cells towards macrophage-conditioned media; the migration and proliferation of ovarian cancer cells in response to polarized macrophage-conditioned media; the migration of monocytes in response to specific chemoattractants or tumor cell medium; and macrophage phenotypic activation.

2. Materials and methods

2.1. Cell culture

2.1.1. Human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated from normal-term umbilical cords as previously published [1]. Cells were grown in medium M199 supplemented with 15% FBS, gentamicin (40 μg/mL), endothelial cell growth supplement (ECGS 100 μg/mL) and heparin (100 UI/mL) at 37 °C in a humidified 5% CO_2 atmosphere. HUVECs were identified by their morphology and detection of CD31-related antigen and used for experiments from passages 2 through 6.

2.1.2. Human monocytes and monocyte-derived macrophages (MDM)

Peripheral blood mononuclear cells (PBMCs) from buffy coats were isolated first by Ficoll-Histopaque density gradient centrifugation at 400*g* for 25 min followed by a second, high-density hyperosmotic Percoll gradient at 400*g* for 15 min in order to obtain purified monocytes. Freshly isolated monocytes were used for migration assay and Western blot analysis.

Macrophages were obtained by differentiation of monocytes as previously described [52]. Briefly, monocytes were seeded at the density of $1,5 \times 10^6$ in 100-mm cell culture dishes in medium RPMI 1640 (Lonza, Milan, Italy) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin in the presence of 20 nM CSF-1. The culture medium was replaced every 3 days and at day 7 macrophages were either unstimulated (M0) or activated toward M1 or M2 phenotypes for 48 h by incubation with LPS (1 μg/mL)/IFN-γ (10 ng/mL) or IL-4 (20 ng/mL)/IL-13 (5 ng/mL), respectively. M1- or M2-activated macrophages were assessed as $CD68^+/CD80^+$ or $CD163^+/CD206^+$ cells, respectively [51].

Human macrophage-conditioned media (hMCM) from M0, M1 and M2 activation phenotypes were obtained as described below.

2.1.3. Ovarian cancer cell line

The human ovarian cancer cell line SKOV3 was purchased from the American Type Culture Collection (ATCC, USA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were used from passages 9 through 25. Conditioned medium from apoptotic and non-apoptotic cells was obtained as described below.

2.1.4. Apoptotic ovarian cancer cells

To identify optimal experimental conditions to induce apoptosis, SKOV3 cells (10^4/well) were plated in 96-well plates (ViewPlate™-96, white, PerkinElmer Waltham, MA, USA) and treated with staurosporine (0.1–1 μM) for 3 h. Apoptosis was evaluated by measuring caspase-3/7 activity using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) following the manufacturer's protocol. Luminescence in each well was measured with a multilabel plate counter ($VICTOR^2$ PerkinElmer Waltham, MA, USA). In order to obtain conditioned media from apoptotic cells, 6×10^5 SKOV3 cells were plated in 100-mm dishes and challenged with staurosporine (0.5 μM) for 3 h in FBS-free medium.

2.2. Preparation of conditioned media (CM)

Conditioned media from polarized human monocyte-derived macrophages and SKOV3 were used for chemotaxis or capillary–like tube formation assays. Polarized macrophages and SKOV3 cells were kept in serum-free RPMI 1640 for 72 h or 16 h, respectively. Afterwards, human macrophage conditioned media (hMCM) or conditioned media from apoptotic and non-apoptotic SKOV3 (SKCM) were harvested, centrifuged at $4000 \times g$ for 20 min, passed through a 0.22-μm filter to remove cell debris and then stored at − 20 °C. For *in vitro* experiments, hMCM from M0, M1 and M2 macrophages as well as SKOV3 cells were concentrated 10-fold using Centriplus filters with 3 kDa cut-off (Amicon, Merck, Darmstadt, Germany).

2.3. MTT assay

SKOV3 (2.5×10^3 cells/well) were seeded in 96-well plates and incubated in complete culture medium. The next day, cells were incubated in the presence or absence of digitoxin (10–50 nM) with fresh medium containing 80% hMCM and 20% RPMI supplemented with 5% FBS for 72 h. Control cells were incubated in RPMI with 1%FBS. Four hours before the end of incubation, 10 μL of 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, 5 mg/mL in phosphate-buffered saline) was added to each well. Then, the medium was