International Journal of Biochemistry and Cell Biology 92 (2017) 6–13

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Research paper

Malignant ascites determine the transmesothelial invasion of ovarian cancer cells

Justyna Mikuła-Pietrasik[a], Paweł Uruski[a], Sebastian Szubert[b], Dariusz Szpurek[b], Stefan Sajdak[b], Andrzej Tykarski[a], Krzysztof Książek[a,*]

[a] Department of Hypertensiology, Angiology and Internal Medicine, Poznań University of Medical Sciences, Długa 1/2 Str., 61-848 Poznań, Poland
[b] Division of Gynecological Surgery, Poznań University of Medical Sciences, Polna 33 Str, 60-535 Poznań, Poland

ARTICLE INFO

Keywords:
Intercellular junctions
Invasion
Malignant ascites
Ovarian cancer
Peritoneal mesothelium

ABSTRACT

The exact role of malignant ascites in the development of intraperitoneal ovarian cancer metastases remains unclear. In this report we sought to establish if ascites can determine the efficiency of transmesothelial invasion of ovarian cancer cells, and, if so, whether the fluid generated by highly aggressive serous and undifferentiated tumors will promote the invasion more effectively than ascites from less aggressive clear cell and endometrioid cancers. The study showed that the invasion of ovarian cancer cells (SKOV-3 and primary cancer cells) across monolayered peritoneal mesothelial cells was elevated upon mesothelial cell exposure to fluid produced by serous and undifferentiated cancers, as compared with cells subjected to ascites from clear cell and endometrioid tumors. This effect coincided with decreased mesothelial expression of junctional proteins: connexin 43, E-cadherin, occludin, and desmoglein. Moreover, it was accompanied by transforming growth factor β1-dependent overproduction of reactive oxygen species by these cells. The activity of ascites from serous and undifferentiated tumors was mediated by p38 mitogen-activated protein kinase and nuclear factor κB. When the mesothelial cells were protected against oxidative stress, both deterioration of junctional proteins and intensification of cancer cell invasion in response to ascites from serous and undifferentiated tumors were effectively prevented. In conclusion, our findings indicate that the high aggressiveness of some histotypes of ovarian cancer may be related to the ability of malignant ascites generated by these cells to oxidative stress-dependent impairment of mesothelial cell integrity and the resulting increase in their transmesothelial invasion.

1. Introduction

Epithelial ovarian cancer (EOC) is a heterogenous disease with respect to its origin, genetics, and aggressiveness, and hence it is categorized into type I and type II tumors. Type I is represented by low-grade serous, endometrioid, mucinous, malignant Brenner and clear cell cancers which originate from the ovaries, display high genetic stability, and develop slowly. Type II includes high-grade serous, carcinosarcoma, and undifferentiated cancer, it comes from outside the ovaries and is characterized by a high progression rate and mortality (Shih and Kurman, 2004). As per the genetic background, type I cancers bear mutations in the *KRAS*, *BRAF*, *PTEN*, and *PI3 K* genes, while type II tumors have mutated *p53* and mutated or dysfunctional *BRCA1/2* genes (Romero and Bast, 2012). At the same time, although clinical and genetic differences between type I and type II tumors are well defined, cellular pathomechanisms responsible for the high aggressiveness of the latter type remain to be explored.

In this study we verified the hypothesis that the diverse aggressiveness of ovarian cancer histotypes may be associated with the activity of malignant ascites, which is a pathological fluid that accumulates in the peritoneum in a significant group of patients (Cvetkovic, 2003). In particular, we examined whether the transmesothelial invasion of cancer cells, which is one of the most critical elements of the formation of intraperitoneal metastasis (Steinkamp et al., 2013), proceeding in the presence of fluid from serous and undifferentiated ovarian cancers (type II tumors) would be more intense than ascites from clear cell and endometrioid cancers (type I tumors). Mechanistically, we addressed the ascites' effect on the expression of various junctional proteins that provide the integrity of the peritoneal mesothelium and thus restrict the efficiency at which cancer cells move towards the tissue stroma (Defamie et al., 2014). Oxidative stress and related signaling pathways were analyzed to delineate factors regulating the ascites-dependent deterioration of intercellular junctions and those contributing to an increased transmesothelial invasion of ovarian cancer cells.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and plastics were from Sigma

* Corresponding author.
E-mail addresses: jmikula@ump.edu.pl (J. Mikuła-Pietrasik), puruski@ump.edu.pl (P. Uruski), szuberts@o2.pl (S. Szubert), dszpurek@gmail.com (D. Szpurek), ssajdak@ump.edu.pl (S. Sajdak), tykarski@o2.pl (A. Tykarski), kksiazek@ump.edu.pl (K. Książek).

http://dx.doi.org/10.1016/j.biocel.2017.09.002
Received 8 May 2017; Received in revised form 3 September 2017; Accepted 5 September 2017
Available online 06 September 2017
1357-2725/

(St. Louis, MO, USA). MG132 (the inhibitor of NF-κB), API-1 (the inhibitor of AKT), and SP600125 (the inhibitor of JNK) were purchased from Tocris Bioscience (Ellisville, MO, USA), whereas SB202190 (the inhibitor of p38 MAPK) was from Cell Signaling Technology (Danvers, MA, USA). Exogenous, recombinant human transforming growth factor β1 (TGF-β1) was purchased from R & D Systems (Abingdon, UK).

2.2. Cell cultures

Human peritoneal mesothelial cells (HPMCs) were isolated from pieces of omentum obtained from 8 patients (28–32 years old) undergoing elective abdominal surgery (institutional consent number 187/14), as described in detail elsewhere (Ksiazek, 2013). The reasons for the surgery included aortic aneurysm (4), hernia (3), and bowel obstruction (1). The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were identified as pure mesothelial by their typical cobblestone appearance at confluency and uniform positive staining for cytokeratins and HBME-1 antigen. Primary cultures of HPMCs obtained during the 1st passage (corresponding to ~5% of their replicative lifespan) without any contamination with stromal cells were used in the experiments.

Ovarian cancer cells, SKOV-3, were purchased from the ECCC (Porton Down, UK) and propagated in RPMI 1640 medium with L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 g/ml), and 10% FBS. Primary epithelial ovarian cancer cells (EOCs) were isolated from a tumor excised during cytoreductive surgery from a patient with serous ovarian carcinoma (stage III according to FIGO). Briefly, the tumor was divided with a scalpel into ten pieces of equal weight and then placed in a solution of 0.05% trypsin and 0.02% EDTA for 20 min at 37 °C with gentle shaking. After resuspension in RPMI1640 containing 20% FBS, the cells were probed with an antibody directed against the epithelial-related antigen (MOC-31; Abcam, Cambridge, UK) to confirm their cancerous nature. Finally, ovarian cancer cells were cultured in RPMI 1640 supplemented with L-glutamine (2 mM) and 20% FBS.

2.3. Malignant and benign ascites

Malignant ascites were obtained at the time of cytoreductive surgery from patients with high-grade serous, undifferentiated, clear cell and endometrioid ovarian carcinoma at stage IIIC and IV (n = 8 per group). The histopathology, grade, and stage of the tumors were assigned in accordance with the criteria of the International Federation of Gynecology and Obstetrics. Patients received no chemotherapy prior to surgery. Control, benign fluids were obtained from 8 age-matched patients undergoing abdominal surgery due to the presence of non-cancerous lesions – cystadenoma mucinosum multiloculare. Upon collection in sterile conditions, the fluids were centrifuged at 2500 rpm for 5 min and then cell-free supernatants were stored in aliquots at −20 °C until required. The study was approved by an institutional ethics committee (consent number 543/14).

2.4. Cancer cell invasion assay

Analysis of cancer cell invasion was performed using Cultrex 96 Well BME Cell Invasion Assay (Trevigen Inc. Gaithersburg, MD, USA) as per manufacturer's instructions. In brief, HPMCs (1×10^5 cells per well) was seeded on the basement membrane extract (BME) to form a monolayer. Afterwards, the cells were subjected to 10% malignant and benign ascites (50 µl per well) for 72 h. After the incubation, ovarian cancer cells (1×10^4 cells per well) were placed into the upper chamber of the system, that is on top of HPMCs. The cancer cells invaded through the HPMCs lying on the BME towards a chemotactic gradient generated by 1% FBS. The intensity of fluorescence emitted by the cancer cells was recorded using a Synergy™ 2 spectrofluorometer (BioTek Instruments, Winooski, VT, USA) at 435 nm excitation and 535 nm emission wavelengths, respectively. In some experiments, cancer cell invasion was examined in the presence of HPMCs subjected to malignant and benign ascites (10%, for 72 h) upon their pre-incubation (for 4 h) with the ROS spin-trap scavenger, *N*-tert-butyl-alpha-phenylnitrone (PBN, Sigma; 800 µM).

2.5. Analysis of intercellular junctions

In order to examine expression of junctional proteins, HPMCs were seeded into semi-transparent 96-well plates (1×10^5 cells per well), and then they were allowed to form a monolayer for next 24 h. Upon reaching the confluency, the plates were carefully washed to remove all non-adherent cells and debris, and then the cells were subjected to 10% malignant and benign ascites (50 µl per well) for 72 h. After the incubation, the cells were fixed in paraformaldehyde, washed and treated with antibodies against connexin 43 (cat # ab11370, Abcam, 1:100, overnight), E-cadherin (cat # ab15148, Abcam, 1:100, overnight), occludin (cat # NBP1-87402, Novus Biologicals, Littleton, CO, USA, 1:100, overnight), and desmoglein (cat # ab12077, Abcam, 1:10, overnight). Then the cells were extensively washed with phosphate-buffered saline (PBS) and incubated with DyLight 488 antibody (cat # ab96899, Abcam; 1:500, 1 h) to quantify connexin 43, E-cadherin and occludin, and with Alexa Fluor 488 antibody (cat # ab150113, Abcam, 1:500, 1 h) to quantify desmoglein. Finally, the cells were washed three times with PBS and fluorescence emitted was recorded using a Synergy™ 2 spectrofluorometer (BioTek Instruments, Winooski, VT, USA). Representative pictures of immunoreactions were taken using an Axio Vert.A1 microscope (Carl-Zeiss, Jena, Germany).

In some experiments, the expression of junctional proteins was measured in HPMCs exposed to both malignant and benign ascites (10%, for 72 h) upon their pre-incubation (12 h) with MG132 (10 µM) and SB202190 (10 µM) for 12 h. In other experiments the proteins were analyzed upon HPMC pre-treatment (for 4 h) with PBN (800 µM).

Additionally, the expression of the junctional proteins was quantified in cell homogenates using specific, colorimetric ELISA-based kits purchased from Abbexa Ltd (Cambridge, UK), as per manufacturer's instructions. In order to collect appropriate amounts of cellular protein, the experiments were performed with HPMCs seeded in 24-well plates (5×10^5 cells per well) and allowed to form the monolayer for 24 h. Upon the exposure to 10% malignant and benign ascites (250 µl per well) for 72 h or the exposure to MG132 and SB202190 for 12 h, the cells were homogenized by sonication. The homogenates were centrifuged at 5000 × *g* for 5 min and the supernatants collected were stored at −80 °C until assayed.

2.6. Production of reactive oxygen species (ROS)

In order to examine the production of ROS, HPMCs were seeded into semi-transparent 96-well plates (1×10^5 cells per well), and then they were allowed to form a monolayer for next 24 h. Upon reaching the confluency, the plates were carefully washed to remove all non-adherent cells and debris, and then the cells were subjected to 10% malignant and benign ascites (50 µl per well) for 72 h. The ROS release by HPMCs was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), essentially as described in (Mikula-Pietrasik et al., 2012). In some experiments, ROS production was monitored in cells exposed to exogenous, recombinant TGF-β1 used at concentrations of 100–400 pg/ml for 72 h. In another group of experiments, ROS were measured in HPMCs subjected to malignant and benign ascites (10%, for 72 h) upon their pre-incubation with MG132 (10 µM), API-1 (50 µM), SP600125 (10 µM), and SB202190 (10 µM) for 12 h.

2.7. Statistics

Statistical analysis was conducted with GraphPad Prism™ 5.00 software (GraphPad Software, San Diego, USA). The means were compared using repeated measures analysis of variance (ANOVA) with the Newman-Keuls test as a post-hoc test. When appropriate, the Wilcoxon matched pairs test was used. The results were expressed as means ± SD. Differences with a P value < 0.05 were considered to be statistically significant.