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Exposure of fallopian tube epithelium to follicular fluid mimics carcinogenic changes in precursor lesions of serous papillary carcinoma

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HIGHLIGHTS

- An ex-vivo model of the influence of follicular fluid on fallopian tube epithelium
- FT epithelial cells present pre-neoplastic changes upon exposure to follicular fluid.

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ABSTRACT

Objectives. Ovulation-related inflammation is suspected to have a causal role in ovarian carcinogenesis, but there are no human models to study the molecular pathways. Our aim is to develop such an ex-vivo model based on human fallopian tube (FT) epithelium exposed to human follicular fluid (FF).

Methods. FT epithelium was dissociated from normal surgical specimens. FF was obtained from donors undergoing in-vitro fertilization. The cells were cultured on collagen-coated Transwells and incubated with FF for various periods of time. The transcriptomic changes resulting from FF treatment were profiled using Affymetrix expression arrays. Specific characteristics of the FT pre-cancerous lesions were studied using immunohistochemistry, immunofluorescence, RT-PCR and XTT assay.

Results. We show that FF exposure causes up-regulation of inflammatory and DNA repair pathways. Double stranded DNA breaks are induced. There is a minor increase in cell proliferation. TP53, which is the hallmark of the precursor lesion in-vivo, is accumulated. Levels of expression and secretion of Interleukin-8 are significantly increased.

Conclusions. Our model addresses the main non-genetic risk factor for ovarian cancer, namely the impact of ovulation. This study demonstrates the biological implications of in-vitro exposure of human FT epithelial cells to FF. The model replicates elements characterizing the precursor lesions of ovarian cancer, and warrants further investigation of the linkage between repeated exposure to ovulation-related damage and accumulation of neoplastic changes.

Introduction

Ovarian epithelial, fallopian tube (FT) and primary peritoneal cancers jointly represent the fifth most common cause of death from cancer among women in Western countries and the most lethal gynecological malignancy, with a mean 5-year survival rate of 44% [1]. Due to the lack of sensitive and specific screening methods, 75% of all cases are diagnosed as advanced-stage disease [2]. Factors associated with reproductive history are known risk factors, specifically nulliparity [3,4]. Fathalla and others have presented the 'incessant ovulation theory' claiming that frequent and uninterrupted ovulation increases the risk for ovarian cancer through repetitive minor trauma to the covering epithelium [5,6]. It has been postulated that the increased cellular proliferation of the ovarian surface epithelium after ovulation may form mitotic figures, crypts, papillae or cysts, consequently leading to neoplastic changes. Over time, epidemiologic data supporting this hypothesis has accumulated: Established ovulation-related risk factors include infertility, nulliparity,

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late menopause and early menarche. Protective factors are, in general, factors that reduce ovulation cycles throughout a woman's life: multiparity, use of oral contraceptive, and lactation. Hysterectomy and tubal ligation also have protective influence, with no seemingly direct effect on the ovulatory activity [7–9].

Only recently the cell-of-origin of most high-grade serous ovarian carcinomas and the precursor lesions has been identified as the fallopian tube secretory epithelial cell (FTSEC) [10,11], and not the ovarian surface epithelial cell, as previously thought. This discovery is a major breakthrough in this field, which has focused the attention on the molecular and genetic changes occurring in the fimbria of the fallopian tube (FT) early in the course of the disease. The 'incessant ovulation hypothesis' now requires adaptation to the emerging knowledge regarding the true origin of this malignancy. Taking into account the FT fimbria position in close proximity to the ovary, it is reasonable to assume that carcinogenic factors released during ovulation affect the FTSEC of the fimbria. These factors can reach the FT fimbria either via the circulation or through direct exposure to the follicular fluid (FF) and follicular cells. The repeated exposure of the epithelial cells to such active biological effectors may result in DNA damage and inflammatory changes [12,13].

Human FF is a complex body fluid that constitutes the microenvironment of the developing follicles in the ovary. FF is a product of both the transport of the circulating plasma proteins and the secretory activity of ovarian cells [14]. FF contains factors that modulate oocyte maturation and ovulation including, among others, steroid hormones, growth factors, cytokines and interleukins, reactive oxygen species (ROS), prostanoids and proteolytic enzymes [15,16]. Therefore, ovulation is considered to be comparable to an inflammatory reaction, the relation of which to cancer is well-established [9,17,18].

Studying the possible molecular pathways that mediate the link between ovulation-related inflammation and carcinogenesis requires a model of the FT epithelium. Levanon et al. reported a reproducible 'ex-vivo culture' system of primary benign FT cells that is capable of recapitulating the histology and function of the normal human FT fimbria epithelium [19]. This model is amenable to experimental manipulations including introduction of typical genetic alterations [20] and exposure to carcinogenic factors in-vitro. Currently, interest in the biology of the FF as a stimulating reagent comes from the field of assisted reproduction and fertility only and focuses on options to improve oocyte quality. This study is aimed to interrogate, model, and delineate the fundamental ovulation-induced changes and their potential contribution to the carcinogenic process. Upon understanding of these common, basic events, chemo-preventive strategies can be devised. Furthermore, it provides directions for investigation of early-detection approaches and biomarkers.

Materials and methods

Samples preparation

Fresh benign FT fimbriae were obtained from the Chaim Sheba Medical Center Institutional tissue banks upon approval of the institutional review board. Eighteen fresh fimbriae were extracted from patients with gynecological conditions other than ovarian cancer: eight with endometrial carcinoma, five with benign ovarian cysts, two with appendiceal neoplasm, and three with uterine leiomyomata. Mean age was 61.2 years (range 44–78 years). The fimbria tissues were incubated in dissociation medium (DMEM, Biological Industries, Beit Ha'emek, Israel) supplemented with 1.4 mg/ml Pronase (Roche Applied Science, Indianapolis, IN, USA) and 0.1 mg/ml DNase (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 4 °C with constant mild agitation. The dissociated epithelial cells were harvested by centrifugation and re-suspended in DMEM/Ham's F12 1:1 (Biological Industries, Beit Ha'emek, Israel) supplemented with 2% serum substitute Ultroser G (PALL Life Sciences, Cergy-Saint-Christophe, France) and 1% penicillin/streptomycin (Biological Industries, Beit Ha'emek, Israel). Cells were cultured on 24-well plates covered with collagen IV from human placenta (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C in a humidified 5% CO_2 incubator.

FF was obtained from women undergoing oocyte retrieval who provided written informed consent. Ten FF samples were used in this study as a pool. Four of the women were undergoing in-vitro fertilization treatments due to mechanical or hormonal infertility (endometriosis and polycystic ovary syndrome) and the other six for various other reasons (pre-natal genetic diagnosis, male factor or unexplained reason). The mean age of the FF donors was 36.5 (range 26–41). FF was centrifuged to remove blood cells and frozen in aliquots. Before use it was heated to 56 °C for 30 min. Serum-containing culture medium was used as control for FF, hence an equivalent concentration of 1% Ultroser G was added to the FF.

Microarray analysis

For microarray experiments we used: two commercially-available ovarian cancer cell lines (SKOV3, OVCA432); one normal human breast epithelial cell line (hMEC); 2 lines of immortalized FT epithelial cell (designated FT190 and FT194, primary cells infected with hTERT- and SV40 T-antigen-expressing retroviruses, kindly provided by the Drapkin lab, Dana-Farber Cancer Institute, Boston, MA, USA); and primary FT epithelial cells from a single donor. The cells were incubated with FF for three time periods: 4 h, 24 h, and 48 h. Total RNA was extracted using QIAzol reagent (Qiagen, Valencia, CA, USA) followed by RNeasy clean-up kit (Qiagen) according to manufacturer's protocol. It was then subjected to hybridization with Affymetrix Human Genome U133 Plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's standard protocols. Treated and control samples were compared. The comparison generated a list of "active genes" representing probe sets changed by at least 2-fold. Genes were classified into functional groups using the Ingenuity software and the GO annotation tool.

Cell proliferation assay

The effect of FF on epithelial cells' viability and proliferation was assessed using an XTT assay (Biological Industries, Beit Ha'emek, Israel). Epithelial cells were dissociated from the FT fimbria as described above, plated and treated with either FF or culture medium (as control) for 24 h followed by addition of XTT reagent to the medium. The cells were incubated for additional 3 h in the CO_2 incubator at 37 °C. Absorbance was determined using PowerWave X 340 Microplate Reader (Bio-Tek, Winooski, VT, USA) at a wavelength of 450 nm.

Immunohistochemistry

After exposure to FF, cells were trypsinized, fixed in ethanol/formaldehyde and the cell pellet was paraffin-embedded. 4 μm sections were cut and immunostained with anti-TP53 mouse monoclonal antibody (clone DO1, EMD-Millipore, Billerica, MA, USA at 1:70 dilution for 1 h at RT). To quantify the differences between FF-treated and control cells, we counted a total of >200 cells per experiment, from 3 different experiments performed on FT cells from 3 donors.

Immunofluorescence staining

Epithelial cells, pre-treated with FF for either 4 or 24 h, were fixated with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 min, permeabilized with 0.2% Triton x-100 (Sigma-Aldrich) for 20 min and blocked with 5% fetal bovine serum (Biological Industries, Israel) for 1 h at RT. Cells were incubated for 2 h at 37 °C with anti-phospho-histone H2A.X (anti-γH_2A.X, Millipore), followed by incubation with secondary antibody, Peroxidase-conjugated AffiniPure