Cumulus Cells Are Potential Candidates for Cell Therapy

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Abstract. *Background/Aim:* Cumulus cells (CCs) originate from the membrane granulosa cells and surround oocytes during follicle maturation. CCs produce high levels of hyaluronan that targets CD44, which is a major tumorigenic marker. This study aimed to investigate whether CCs have a role in cell therapy by targeting CD44 in pancreatic cancer cells. *Materials and Methods:* CCs were isolated from the oocytes and incubated in a hypoxic environment. BxPC-3 pancreatic cancer cells were treated with CC conditioned media for three days. *Results:* Conditioned media of CC cells incubated in hypoxic conditions caused a 25% reduction in the viability of BxPC-3 cells. Expression of anti-apoptotic genes was down-regulated, while that of pro-apoptotic genes was upregulated. An increased number of BxPC-3 cells exhibited increased levels of reactive oxygen species and arrested in the synthesis (S) phase of the cell cycle. *Conclusion:* CCs conditioned medium induced apoptosis of pancreatic cancer cells.

Cumulus cells (CCs) are a specialized group of granulosa cells, which are in intimate contact with oocytes (1, 2) both in the ovarian follicle and after ovulation. CCs are removed in routine human intra-cytoplasmic sperm injection (ICSI) practices for better capturing of the polar body. CCs play an essential role in regulating oocyte maturation (3, 4); they surround the mature oocyte and give rise to a cumulus mass

of several millimeters (5). Several studies on CCs have focused on their role in oocyte maturation and fertilization and on their use as novel non-invasive diagnostic biomarkers to determine oocyte quality (6).

Many types of cancers are characterized by elevated levels of reactive oxygen species (ROS), which play an essential role in cell proliferation, differentiation, and cell survival (7). CCs have a protective effect against ROS, but only to a certain extent. The process of CC expansion requires the production of hyaluronic acid (8, 9), which is the primary CD44 binding molecule overexpressed in many solid tumors, such as pancreatic cancer (10). CD44 is a non-kinase transmembrane glycoprotein, which has been shown to be overexpressed in a variety of cells such as cancer stem cells and mutations in this gene are thought to play a role in tumorigenesis (11). Targeting this protein through CCs might have an anti-carcinogenic effect on pancreatic cancer cells.

Materials and Methods

Tissue collection and sample preparation. The tissue of origin of CCs was obtained from the IVF lab. Tissues were minced in phosphate buffered saline (PBS) by pipetting vigorously and transferred into T25 flasks coated with Type I Collagen (STEMCELL Technologies, Kent, WA, USA). CCs were cultured in RPMI-1640 (Life Technologies, Grand Island, NY, USA), supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin/amphotericin (PSA) Life Technologies), and incubated in hypoxic conditions (2% O₂). Floating tissues and cells flask were discarded and the medium was replaced with fresh medium and left in the incubator for three to four days. Conditioned medium was collected and filtered using a 0.22 µm filter and kept in -80°C until used.

Ethical approval. This study is involved human participants and ethical approval was obtained. Informed consent was obtained from all individual participants included in the study.

Characterization of CCs by flow cytometry and gene expression analyses. Cells were dissociated from the flask using 0.25%

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Figure 1. Morphology and gene expression of CCs. a) Cumulus cell under light microscope b) Gene expression analysis of CCs samples show expression of COX2, PTX3, CX43 and HAS2 genes. CCs: Cumulus cells, S: samples.

Trypsin-EDTA (Life Technologies), re-suspended in PBS, and incubated with phycoerythrin-conjugated (PE-conjugated) anti-CD44 antibody (MEM-263, Abcam, USA) for further analysis by flow cytometry using BD FACS Calibur (Becton Dickinson, San Jose, CA, USA). Human CCs are characterized by the expression of pentraxin 3 (PTX3) (12), hyaluronic acid synthase 2 (HAS2) (13), Cyclooxygenase 2 (COX2) (14), and Connexin 43 (15). Total RNA was isolated by NucleoSpin® RNA kit (740955.50, MACHEREY-NAGEL®, Duren, Germany) according to the manufacturer's protocols. QuantiTect Reverse Transcription Kit (Qiagen®, Germantown, MD, USA) was used to synthesize cDNA that was used as a template for conventional PCR using Q5® Hot Start High-Fidelity 2X Master Mix (NEW ENGLAND BIOLAB®, Beverly, MA, USA). The PCR products were analyzed by agarose electrophoresis and the bands were further visualized under UV light.

Indirect co-culture. BxPC-3 cells (CRL-1687, ATCC, USA), a pancreatic adenocarcinoma cell line, were grown in complete medium (RPMI1460 + 10% heat-inactivated fetal bovine serum + 1% penicillin-streptomycin/amphotericin). Cells were seeded in two 6-well plates at a density of 2×10^5 cells per well and incubated under normoxia (18% O₂) with various concentrations of CCs conditioned medium (previously incubated in hypoxic conditions, collected, and filtered) including 100%, 80%, 60%, and 50%. Conditioned media from a pancreatic epithelial healthy cell line (hTERT-HPNE) was separately used to assess the specificity of the detrimental effect of CC conditioned media.

Apoptotic assays via annexin V, and multi-caspase assays. Early apoptotic cells were confirmed by using the Annexin V Kit (MCH100105, Merck, Darmstadt, Germany) and Multi-Caspase Assay Kit (MCH100108, Merck) according to manufacturer's protocol. Cellular samples were incubated with the Muse™ Annexin V & Dead Cell reagent (MCH100102, Merck) containing 1% FBS and apoptotic/dead cells were analyzed by Muse® Cell Analyzer.

Quantification of apoptotic genes. Quantitative real-time PCR was performed using TaqMan probe qPCR master mix (Promega®, USA). Exon spanning primer probes (all purchased from Thermo scientific, USA) targeting *Bak* (Hs00832876_g1), *Bax* (Hs99999001_m1), *Bcl2* (Hs01048932_g1), *Casp3* (Hs00234387_m1), *TNF* (Hs00174128_m1), *TP53* (Hs01034249_m1) and the housekeeping gene *GAPDH* (Hs02786624_g1) were used. CFX96® real-time PCR system (Bio-Rad) was used and data analysis was performed using CFX manager software.

Reactive oxygen species (ROS) measurement. Reactive oxygen species (ROS) were quantitated by Muse® Oxidative Stress Kit (MCH100111, Merck) according to the manufacturer's protocol. Briefly, cell samples prepared at a density of 1×10^6 to 1×10^7 cells/ml, were incubated with the Muse® Oxidative Stress working solution, and analyzed by Muse® Cell Analyzer.

Cell cycle analysis. The effect of CC conditioned media on the cell cycle of BxPC-3 cells was determined by Muse™ Cell Cycle Kit (Merck) according to the manufacturer's protocol. Briefly, ice cold 70% ethanol was added slowly, for at least 3 h, to cells while mixing and were then mixed with Muse™ Cell Cycle Reagent. Cells were incubated for 30 min at room temperature in the dark and analyzed by Muse® Cell Analyzer.

Cell viability assay for combination therapy. BxPC-3 cells were treated with gemcitabine (BioVision, Milpitas, CA, USA) combined with CC conditioned media and their synergistic effect on cell viability was assessed by the MTS assay using CellTiter 96® Aqueous MTS Reagent Powder (Promega®, Mannheim, Germany). The cells were seeded in 96-well plates and incubated with different concentrations of gemcitabine and CC conditioned media for 72 h. A colorimetric viability assay to assess the mitochondrial activity of cells was performed where the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced to formazan crystals which were dissolved in DMSO. Optical density was measured

Figure 2. CCs conditioned media induced apoptosis of BxPC-3 cells. Early and late apoptotic BxPC-3 cells treated with various concentrations of CCs or hTERT-HPNEs CM. CM: Conditioned media, hTERT-HPNEs: Immortalized healthy human pancreatic epithelial cells, $p < 0.0001$.

at 490 nm using a microplate reader (Biotek Instruments ELx800 Absorbance Micro-plate Reader).

Direct co-culture. BxPC-3 cells were transfected with GFP plasmid using FuGENE® HD Transfection Reagent (Promega, Mannheim, Germany). GFP-tagged BxPC-3 cells were directly co-cultured with PKH26 Red (Merck) fluorescent labeled Human Cumulus Cells for 72 h. Live images were captured by Olympus Imaging System.

Results

Isolation and characterization of cumulus cells. CCs were successfully isolated as illustrated in Figure 1a. The majority of cells expressed high levels of CD44 (data not shown). The cells express COX2, PTX3, CX43, and HAS2 (Figure 1b).

Apoptotic assays. Incubation of BxPC-3 cells with various concentrations of CCs conditioned medium (previously incubated and collected from hypoxia) caused up to 25% cell death as shown in Figure 2. Treatment of BxPC-3 cells with hTERT-HPNE conditioned medium did not induce apoptosis, suggesting that cell death was specifically due to the effects of the conditioned media of CCs (Figure 2).

Multi-caspase assay. Incubation of BxPC-3 cells with various concentrations of CCs conditioned medium induced an increase in the percentage of caspase activity by 34% (Figure 3).

Figure 3. CCs conditioned media increased the activity of caspases. BxPC-3 cells treated with various concentrations of CCs CM, $p < 0.0001$.

qRT-PCR. Upon incubation of BxPC-3 cells with CCs conditioned medium the expression of *BAX* and *BCL2* genes was downregulated and that of *BAK* and *TNF* genes was upregulated, while expression of *CASP3* and *TP53* did not change significantly (Figure 4).

Figure 4. Expression of apoptosis-related genes in BxPC-3 cells treated with CCs conditioned media. Apoptotic gene expression of BxPC-3 cells treated with various concentrations of CCs CM. NC: Negative control; CM: conditioned medium, $p < 0.0001$.

Figure 5. CCs conditioned media increased the levels of ROS a) ROS levels of BxPC-3 cells treated with various concentrations of CCs CM in b) Cell cycle phases of BxPC-3 cells treated with various concentrations of CCs CM, $p < 0.0001$.

Figure 6. Combination of gemcitabine and CCs conditioned media reduced cell viability. Cell viability assay for combination therapy. NC: Negative control; CM: conditioned medium, $p < 0.0001$.

Reactive oxygen species (ROS) measurement. As shown in Figure 5a, there is an increase in the percentage of BxPC-3 cells exhibiting increased levels of reactive oxygen species upon incubation with different concentrations of the CCs conditioned medium in relation to untreated cells.

Cell cycle analysis. Upon incubation of BxPC-3 cells with different concentration of CCs conditioned medium the percentage of cells in the S phase increased and those in the G₂/M phase decreased (Figure 5b).

Combination therapy. Treatment of BxPC-3 cells with different doses of gemcitabine caused a reduction of cell viability (Figure 6). Higher levels of cell death were observed upon treatment of cells with the combination of CCs conditioned medium and 0.5 µM or 1 µM of gemcitabine compared to cells treated with 0.5 µM or 1 µM of gemcitabine alone (Figure 6).

Direct co-culture. As shown in Figure 7, after co-culture of GFP-tagged BxPC-3 cells with PKH26 Red fluorescent labelled Human CCs for 72 h, the number of GFP-tagged BxPC-3 cells decreased, indicating that BxPC-3 cells were undergoing apoptosis.

Discussion

The process of CCs expansion upon Luteinizing hormone (LH) stimulation requires the production of hyaluronic acid (HA) that accumulates in the extracellular space (8, 9). CCs express the surface receptor CD44, which binds to HA and allows the

Figure 7. Direct co-culture of GFP tagged BxPC-3 cells with PKH26 Red fluorescent labelled Human Cumulus cells induced cell death. (a) 0 h, (b) 24 h, (c) 48 h, (d) 72 h.

formation of the extracellular matrix (ECM) between CCs (16). Overexpression of certain surface receptors have been used to distinguish malignant cells from benign ones (17). Among them, CD44 plays a critical role in metastasis (18) and is associated with bad prognosis (19). We examined the effect of conditioned medium of CCs on the growth and viability of pancreatic tumor cells. CCs conditioned medium of varying concentrations induced apoptosis, as assessed by various assays. Pancreatic cancer cells are very resistant to apoptosis (20), however, co-culturing with different concentrations of CCs conditioned medium (100%, 80%, 60%, and 50%) induced approximately 25%, 24%, 20%, and 17% cell death, respectively. There was also a parallel increase in caspase activity, suggesting the detrimental effect of CCs conditioned medium over cancer cells. Downregulation of BCL2 and slight upregulation in caspase 3 (casp3) and Tp53 genes along with non-significant changes in Bax, Bak and TNF genes suggests that cell death is independent on BAX, and probably BAK, and might proceed through the intrinsic mitochondrial apoptosis pathway (21).

Our data are consistent with a published study showing the anti-proliferative and apoptotic effect of human umbilical cord mesenchymal stem cells (hUCMSCs) conditioned medium (22). An increase in the percentage of BxPC-3 cells exhibiting increased levels of ROS upon incubation with CCs conditioned medium was observed as suggested by others (23, 24). In addition, CCs conditioned medium influenced cell cycle and caused an arrest in the S phase, accumulation of cells in which DNA replication is blocked, and ultimately apoptosis (25).

We further examined the synergistic effect of CCs conditioned medium and gemcitabine, the first line of conventional chemotherapy, for pancreatic ductal adenocarcinoma cells. As has been demonstrated in a similar study performed by Morisaki *et al.*, the combination of CCs conditioned medium and a low dose of gemcitabine (as low as 1 μ M) increases the percentage of growth inhibition (26). This synergistic effect enabled us to decrease the dose of gemcitabine, which will decrease the side-effects improving the quality of life of cancer patients.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

Concept, design (CAE, MEE), definition of intellectual content (CAE, MEE, YS), literature search (MEE, YS, CAE, FC, SF, NB), experimental studies (MEE, AAO), data acquisition (CAE, MEE), data analysis (MEE, CAE, YS, SF), manuscript preparation (MEE, CAE), manuscript editing (CAE, MEE, YS, SF), manuscript review (CAE, MEE, SF, NB).

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