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Cell Markers Present in the TGF-β-Activated Transdifferentiation of Normal Fibroblasts to Cancer-Associated Fibroblast in Endometrial Cancer

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Abstract

Introduction: Cancer-associated fibroblasts (CAFs) are an essential component of carcinogenesis. The biological origins of CAFs in humans depend on the histotype of the tumour and the region where it first originated, and thus CAFs could be derived from many different cell types. Normal fibroblasts (NFs) are abundant in the endometrium and are highly susceptible to transdifferentiation to CAFs through TGF-β activation. This study aims to identify the cell markers present in the TGF-β signalling pathways for the transition of NFs to CAFs in endometrial cancer (EC).

Methods: EC will be chemically induced in ICR (Institute of Cancer Research) mice with N-methyl-N-nitrosourea (MNU) and a 17β-estradiol (E2) diet. Cancer progression will be monitored using magnetic resonance imaging (MRI) at a field of 4.7 T. CAFs will then be isolated from the TME using PDGFRα as the cell marker. Immunohistochemistry (IHC) staining will be used on EC tumour cells to identity the presence the location of cell markers phosphorylated Smad2/3 (pSmad2/3), ERK1/2, and PI3K.

Anticipated Results: CAF cells are expected to test positive for markers expressed in PDGFRα mediated signalling pathways. Presence of pSmad2/3 is expected to increase over time as usage of the canonical pathway increases in CAF establishment and cancer progression. Non-canonical pathway activation would show levels of ERK2/3 and PI3K.

Discussion: pSmad2/3 levels will be examined to determine the usage of the canonical pathway in CAF expansion. Detection of pSmad 2/3 or PI3K/ERK2/3 allows for targeted therapy on the appropriate TGF-ß pathway to block CAF production, thus stopping tumour progression. Suppression of the pathways by targeting specific biomarkers such as PTEN to inhibit mTOR or CAV-1 inhibitors could normalize an upregulated or downregulated TGF-ß pathway.

Conclusion: Identifying the key cell markers in the transdifferentiation of NFs allows for the targeting of specific proteins that play a role in the signalling pathways. Standardizing identification of significant cell markers in CAF establishment improves individualized treatment to the cancer patient. Treatment(s) would target the cell markers involved to prevent further CAF proliferation and tumour development

Keywords: cancer; endometrial cancer; TGF-ß pathway; cancer-associated fibroblasts; CAFs; genomics; PI3K; ERK; tumour microenvironment

Introduction

The endometrium plays a crucial role in uterine activity, proliferating and shedding under the regulation of different hormones, and changes according to the uterine cycle. The shifting hormonal levels determine the thickness and shedding of the lining [30]. Endometrial cancer (EC) is the most common gynecological cancer resulting from abnormal cell growth in the endometrium. EC grows slowly if left untreated, however, it can metastasize to surrounding areas and organs at later stages, such as the bladder, rectum, reproductive organs, and distant organs [31]. EC is composed of two types of endometrial cells: Type I endometrial cells typically arise from atypical hyperplasia due to excessive estrogen exposure, whereas Type II endometrial cells are associated with endometrial intraepithelial carcinoma as a precursor, rising from atrophic endometrium [1].

The tumour microenvironment (TME) consists of various components, including blood vessels, stromal cells, and immune cells. The TME is notably important in EC progression due to the roles of the stromal cells at different EC stages [28]. A particular cell of interest are cancer-associated fibroblasts (CAFs) which play a significant role in the growth and maintenance of cancers primarily through paracrine signalling and modification of the extracellular matrix [2]. In the EC TME, CAFs promote tumour proliferation, migration and invasion into neighbouring cells, tumorigenesis, and development of drug resistance [3]. Crosstalk between the tumour cells and CAFs initiates and sustains different functions of the endometrial tumour

cell, including influencing the proliferation of the tumour cell through multiple signalling pathways [3], such as the TGF-β signalling pathway.

TGF-β is a secreted growth and differentiation factor with various functions, depending on the cell and tissue. Its large transduction network controls numerous critical cell behaviours, including the activation of fibroblast proliferation [4]. Due to this cytokine's significant involvement, overproduction is regularly observed in many pathological conditions [4]. TGF-β is highly expressed in the endometrium, mainly to prepare for implantation and during pregnancy. However, in EC, TGF-beta plays a role in supporting tumour proliferation. TGF-β's role in fibroblast differentiation is associated with the transdifferentiation of CAFs; they can arise from resident tissue fibroblasts from the secretion of TGF-β, resulting in an autocrine signalling loop. This maintains fibroblast differentiation, which can continue regardless of TGF-β release from other sources. [5].

Many transdifferentiation routes to CAFs are TGF-β dependent due to their prominent role in cell proliferation and differentiation [5]. This study aims to quantify the expression of components of the TGF-β cascading pathways as a means of identifying CAFs undergoing transdifferentiation and the mechanisms which maintain the active CAF state.

Methods

Chemical Induction of Endometrial Cancer in ICR Mice

Endometrial cancer was chemically induced in ICR (Institute of Cancer Research) mice because of their high reproductive performance, low cost, and rapid growth rate. ICR mice are a common mouse stock chosen because of their good reproductive performance and rapid growth rate [11]. The chemical induction of endometrial carcinoma in mice was done one a week by intravaginal instillation of N-methyl-N-nitrosourea (MNU) solution at a concentration of 1mg for 100g of mice body weight for three weeks. The mice are also fed a diet of 5 ppm 17β-estradiol (E2) for 20 weeks, as per the rapid endometrial carcinoma induction method from Niwa et al. [9]. MNU is a strong carcinogen, resulting in a high incidence rate of endometrial adenocarcinoma and preneoplastic endometrial lesions with similar histology to those in humans, which is useful in examining the pathogenesis of endometrial carcinoma in humans.

Monitoring Endometrial Cancer Progression

Development and growth of the EC tumour were monitored for twenty weeks after the initial three weeks of MNU instillation. A total of 40 female ICR mice were used in the experiment. Tumour volume and development was monitored using magnetic resonance imaging (MRI) at a field of 4.7 T. Four mice with the largest tumour volumes were selected biweekly for sacrifice.

CAF Isolation From Tumour Microenvironment

The CAFs were isolated from the TME following the CAF isolation protocol from Sharon et al. [6]. Tissue digestion single-cell suspension technique in the CAF isolation protocol was substituted with the tissue digestion procedure from the relevant protocol by Chen and Roan [7]. Two independent protocols were used for the digestion and isolation to account for differences in tissue structure which required a different processing step. Cells were first labelled with PDGFRα was used as a surface marker to isolate and collect highly pure populations of CAFs from the endometrium samples, which were analyzed soon after retrieval. The cells were labelled with PDGFRα monoclonal antibody conjugated to PE (purchased from ThermoFisher (16A1))) for FACs sorting. PE was used as PDGFRα is expressed at high levels in abnormal endometrium tissue, as well as in uterine and ovarian cancers [32] [33], and PE is used for molecules requiring high sensitivity. Healthy endometrial cells were also dissected and digested from control mice without any endometrial disorders.

Immunohistochemistry Staining for Cell Markers

Immunohistochemistry (IHC) staining was used on the EC tumour cells and control endometrial cells to identify the presence and location of present cell markers throughout the CAF development expressed in the TME of the tissue samples. Chromogen 3-3'-Diaminobenzidine (DAB) staining was used, following the immunohistochemistry protocol from Jurukovski et al. [10]. The assays detect either the active form of TGF-βR or a phosphorylated form of Smad using the TGF-β1/1.2 antibody (purchased from R&D systems) antibody developed against pSmad2/3. pSmad2/3 levels indicate TGF-β receptor activity. Anti-ERK 1/2 and anti-PI3K antibodies will be used to stain for the presence of the respective kinases to determine the main TGF-β-mediated transdifferentiation pathway. ELISA assays detecting mouse TGF-β isoforms are used to measure TGF-β receptor levels using anti-TGF-β antibody, following the R&D System's Mouse TGF-β Quantikine ELISA immunoassay. The sandwich assay method was used; the layer coating the wells was the anti-TGF-β antibody to capture the TGF-β from the samples. The PE conjugate already bound to the antibody was the second layer, and the isolated endometrial cells was the third layer.

Hormonal Assay

Development of endometrial tumours were confirmed using an E2 Coated Tube RIA kit (purchased from fisherscientific) and a Progesterone ELISA kit (purchased from Cayman Chemical) to estimate E2 plasma values.

Results

High Plasma 17β-estradiol to Progesterone Ratio in EC Chemically Induced Mice

Administration of both MNU solution and E2 diet is expected to result in the development of adenocarcinomas in the uterine corpus. It is expected that all treated mice will generate tumours as per Niwa et al.'s protocol, where all mice with the administered induction developed preneoplastic and neoplastic lesions in the uterine cervix. Furthermore, atypical hyperplasia and adenomatous hyperplasia is also expected to develop in the endometrium at a high incidence rate. A hormonal assay would be used to measure plasma E2 and progesterone concentrations, as mice administered MNU and E2 would exhibit higher E2/progesterone ratio, indicating development of endometrial adenocarcinoma and adenomatous hyperplasia due to the chemical induction [9].

Bioluminescence Imaging Displaying High RLU in Uterine Region

BLI would be used to follow the growth of the tumour cells by measuring the luminescence activity detected from the endometrial adenocarcinoma. Luminescence, measured in Relative Light Units (RLU) increases over time as cell number increases. Monitoring of bioluminescence activity would be done weekly to follow tumour progression and is expected to demonstrate increasing and expanding intensity in the uterine region of the mice (Figure 1). Mice would be weighed and an increase in weight would be expected in mice with atypical hyperplasia development and tumour growth.

FACS Sorting Displaying CAF Purity

Single cell suspensions of the endometrial cells stained with PDGFRα antibodies would be sorted using fluorescence-activated cell sorting (FACS). The FACS sorting plot is expected to display high levels of fluorescence in the PDGFRα+ cell gate, as illustrated in Sharon's CAF isolation protocol.

Smad2/3 Levels Increasing as Tumour Develops

The IHC staining would detect the presence of phosphorylated Smad2/3 in the CAFs at varying levels, indicative of the activation of the TGF-β/Activin/Nodal-Smads pathway [34]. TGF-β/Activin/Nodal binding prompts the phosphorylation of Smad2/3. pSmad2/3 levels are expected to be high in the early stages of EC as the cancer cell secrete high levels of TGF-β to mediate the transformation of NFs to CAFs. As cancer progresses, pSmad2/3 levels are expected to increase as the usage of the canonical pathway increases to drive other CAF maintenance mechanisms, such as cancer invasion [12].

Increasing TGF-βI/II Receptor Levels Alongside Cancer Cell Growth

Higher levels of ERK1/2 and PI3K than in normal tissue are expected to be detected in IHC staining, indicating the activation of the non-canonical pathways for CAF proliferation, energy metabolism, and apoptosis. The increase in kinase levels would be elevated as there is an addition from regular levels due to the pathway activation. The non-canonical pathway represents TGF-βI/II receptor activation but the Smad2/3 pathway is expected to remain inactive [12]. Activation of the non-canonical pathway causes the activation of the PI3K/AKT/mTOR signalling pathway and the MEK/ERK1/2 signalling pathway [12].

ELISA Assays Measure Increasing TGF-β Levels as EC Progresses

TGF-β is released by tumour cells through paracrine signalling, activating the non-canonical and canonical TGF-β pathways. ELISA assays are expected to measure consistently increasing levels of TGF-β in the CAFs as the canonical pathway would be dominant in the early stages of cancer cell growth but would be balanced by the increased activation of the non-canonical pathway as the EC progresses. Since TGF-β is used in both pathways, measurement of the levels of the growth factor alone will not determine which pathways are occurring. ELISA used alongside with pSmad detection to measure activation levels of canonical pathways is expected to decrease in TGF-β as pSmad2/3 levels decrease. ELISA used in conjunction with ERK and PI3K detection is expected to have an increase in TGF-β as kinases' presence increases.

Discussion

Canonical TGF-β Signalling Pathway and the Mechanisms Driving CAF Establishment, Maintenance, and Cancer Proliferation

The TGF-β signalling pathway is crucial in the transdifferentiation of normal fibroblasts to CAFs in EC and maintenance of the CAF active state [5]. The cell markers for the main pathways (canonical and non-canonical) would be examined to provide a deeper insight on the potential treatments against EC. The presence of phosphorylated Smad2/3 (pSmad2/3) increasing over time would indicate the use of the canonical pathway in increasing DNMT3B methylation, upregulation of Snail and Twist genes, and inducing the Reverse Warburg Effect state [12]. pSmad2/3 absence along with the presence of PI3K and ERK1/2 would indicate the use of the non-canonical pathway to induce the PI3K/AKT/mTOR and MEK/ERK1/2 pathways. These complex pathways involve an extensive number of components with potential for future therapeutic interventions.

The presence of phosphorylated Smad2/3 in the isolated CAFs would indicate activation of the TGF-β/Activin/Nodal ligands. TGF-βII phosphorylates Smad2/3, resulting in translocation to the nucleus and regulation of

TGF-β target genes, such as α-SMA and FAP expression, established markers for CAF activation. DNMT3B methylates miR-200s promoters in normal fibroblasts (NFs) and leads to CAF activation [13]. Treatment focusing on the suppression of DNMT3B methylation could significantly reduce EC cell growth as CAF establishment and maintenance would be diminished. This is already observed in breast cancer treatment where DNMT3B knockdown or administration of 5-aza-2'-deoxycytidine (5'-AZA), a DNA methylation inhibitor, attenuated breast cancer cell growth without other additional treatment [13].

Our research suggests that Smad1/2 levels are expected to increase over time through NF transdifferentiation, CAF migration, and induce the transcriptional regulation of Snail and Twist genes. Snail1 and Twist1 genes increase CAF contractility and ECM remodelling, both mechanisms are essential for cancer cell invasion into neighbouring tissues and organs [12]. Treatments targeting the clearance of Snail1 and Twist1 genes would significantly decrease CAF ability in maintaining tumour growth, which has been observed in a study through the knockdown of Twist1 in CAFs [25]. The canonical TGF-β pathway also enhances CAF migration by driving the overexpression of occludin, which is important in stabilizing intercellular tight junctions [12]. Occludin that have adopted the CpG island methylator phenotype (CIMP) has been observed to enhance the tumorigenic and metastatic properties of cancer cells [29]. Therefore, therapeutically targeting occludin may help in reducing metastatic potential.

Another major key mechanism induced by the canonical pathway is the Reverse Warburg effect, the reprogramming of the cell's metabolic processes. TGF-β downregulates Caveolin-1 (CAV-1) protein and isocitrate dehydrogenase-3α (IDH3α) activity, programming a switch from oxidative phosphorylation to aerobic glycolysis, decreasing mitochondrial activity [12]. Decreased CAV-1 reduces glucose uptake [35] while downregulated IDF3α in CAFs provides metabolites necessary for aerobic glycolysis in tumours [36]. The purpose of glycolysis in CAFs is different than in tumour cells. Here, glycolysis in CAFs plays a large role in accelerating tumour cell proliferation and growth, demonstrating the adaptivity of cancer [15]. IHD3α or CAV-1 are potential anti-tumour therapeutic candidates as dysregulated expression of these biomarkers are key in inducing NF transdifferentiation, cancer maintenance and progression [12].

Non-Canonical TGF-β Signalling Pathway

The non-canonical pathway is characterized by TGF-β binding to the TGF-βI/II receptors without activating the Smad pathway. Instead, kinases PI3K and ERK1/2 would be activated by the PI3K/AKT/mTOR and MEK/ERK1/2 pathways, both of which promotes CAF transdifferentiation and cancer progression.

The binding of the TGF-β and TGF-βI/II receptors activate a signalling pathway which activates PI3K. In the CAF, the resulting mammalian target of rapamycin (mTOR) is transferred into the nuclei to regulate the transcription of many genes involved in metabolic and biogenesis, such as adipogenesis autophagy [16]. mTOR overexpression through deregulation of upstream PI3K/AKT signalling in CAFs plays a role in regulating gene expression, including that of miRNAs and lncRNAs associated with CAF motility and transdifferentiation of various cell types into CAFs, such as pericytes and NFs [12]. For example, PI3K/AKT/mTOR pathway suppresses miR-143 expression, leading to an increase in KRAS protein which regulates EMT [12]. An overactive PI3K/AKT/mTOR pathway is frequently observed in EC and is considered crucial to EC development [26]. Pathway inhibitors for the metastatic stages of EC, such as ridaforolimus (mTOR inhibitor), have shown to be a promising treatment target [26]. Targeting the PI3K/AKT/mTOR pathway can be done by inhibiting PI3K/AKT signalling cascade or inhibiting mTOR, such as using PTEN (tumour suppressor) as treatment [17].

The MEK/ERK1/2 signalling pathway is key for the development and progression of cancer [18]. ERK1/2 phosphorylation upstream leads to translocation into the nucleus and regulation of gene expression [12]. Increased ERK1/2 activity is associated with expression of CAF migratory phenotypes [20], metabolism of fatty acids in CAFs [12], glycolysis in CAFs [21], and other oncogenic mechanisms. In EC, ERK1/2 prolongs cell survival when activated by E2. Treatment of EC with PD98059 MEK/ERK pathway inhibitor reported lower ERK1/2 levels, however, the effects on CAF activity remained the same despite changed kinase levels [27], suggesting the CAF maintenance may be a result of the synergistic effects of ERK1/2 with another signalling pathway to maintain the CAF active state. Thus, the ERK1/2 pathway holds strong potential as a chemotherapeutic target due to its diverse role in cancer progression.

Conclusions

In conclusion, investigating the TGF-β pathway in CAFs can identify and delineate the important influencers of CAF transdifferentiation and activation. The canonical and non-canonical pathways both induce various mechanisms crucial in establishing CAFs through NF transdifferentiation and maintaining CAF establishment. The expression of key cell markers pSmad1/2, PI3K, and ERK1/2 from the TGF-β signalling pathway provides insight into key activated pathways. Thus, these are strong candidates for potential therapeutic interventions in EC and can be translated to other cancers with extensive stroma.

List of Abbreviations Used

5'-AZA: 3-aza-2'-deoxycytidine
Akt: protein kinase B
BLI: bioluminescence imaging
CAV-1: caveolin-1

DNMT3B: DNA-methyltransferase 3 beta
E2: 17b-estradiol
EC: endometrial cancer
ELISA: enzyme-linked immunosorbent assay
ERK: extracellular signal-regulated kinases
CAF: cancer-associated fibroblasts
ICR: Institute of Cancer Research
IDH3: isocitrate dehydrogenase-3
IHC: immunohistochemistry staining
lncRNA: long non-coding RNA
MEK: mitogen-activated protein kinase
miRNA: microRNA
MNU: N-methyl-N-nitrosourea
mTOR: mammalian target of rapamycin
NF: normal fibroblasts
PDGFR: platelet-derived growth factor receptor
PI3K: phosphoinositide 3-kinase
pSmad: phosphorylated Smad
TGF: transforming growth factor

Conflicts of Interest
The author declare that they have no conflict of interest.

Ethics Approval and/or Participant Consent
The study would be reviewed through the Animal Use Protocol (AUP) Review process by the Animal Care Committee (ACC) at the University of Western Ontario. ACC follows the Canadian Council on Animal Care (CCAC) guidelines, which has previously approved similar protocols for tumour growth of mice.

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