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Aberrant TGFβ Responses in Post EMT cancer Cells

Abstract

Pancreatic adenocarcinoma ranks as one of the most lethal cancers in the United States, with a five-year survival rate of 6.7%. Given its tumor promoting effects in the tumor microenvironment, recent therapeutic efforts have focused on inhibiting Transforming Growth Factor Beta (TGFβ). However, while TGFβ facilitates tumor development by increasing the proliferation and matrix deposition of mesenchymal cells in the stroma, it is a potent growth inhibitory signal in benign and neoplastic epithelial cells. Therefore, prior to targeting the TGFβ pathway clinically, it is essential to identify patients in which the beneficial aspects of TGFβ are lost. Chronic exposure to a high dose of TGFβ has been demonstrated to induce epithelial cells to assume a more mesenchymal phenotype, a process known as Epithelial to Mesenchymal Transition (EMT). As mesenchymal cells proliferate in response to TGFβ, the effects of TGFβ on epithelial cells that have undergone EMT was previously unknown. To study the effect of TGFβ on mesenchymal-like epithelial cells, epithelial PanIN KC4848 cells were cultured in vitro and EMT induced with chronic TGFβ exposure. Interestingly, the mesenchymal-like PanINs proliferated in the presence of TGFβ whereas the unmodified PanIN control experienced growth-arrest. After the post-EMT PanINs were allowed to return to a normal epithelial phenotype, they exhibited a more normal response to TGFβ, again consistent with cell cycle arrest. These findings suggest divergent TGFβ signaling pathways in pre and post-EMT epithelial cells, and imply that EMT status may be relevant in predicting efficacy of therapeutic strategies targeting the TGFβ pathway.

Focusing Question

Does TGFβ elicit a more mesenchymal-like response in epithelial cells that have undergone EMT?

Introduction

While there have been several therapeutic advances in in the treatment of many solid tumors, pancreatic cancer remains remarkably virulent with a five-year survival rate of only 6.7% ("SEER Stat Fact Sheets: Pancreas Cancer," 2014), Because pancreatic cancer is typically diagnosed in advanced stages after metastasizing to the liver, large intestine, or lungs, the disease is often unresponsive to conventional therapies. Recent findings suggest that epithelial to mesenchymal transition (EMT) is implicated in aiding metastasis and conferring chemoresistance in pancreatic tumor cells (Kalluri et al., 2009). In this process, epithelial cells lose their polarity and cell-to-cell adhesion proteins like E-cadherin, allowing cellular translocation (Kalluri et al., 2009).

EMT is indispensable for development and wound healing, but is artificially induced in pancreatic cancer to coordinate tissue invasion. During embryogenesis, trophoectoderm cells undergo EMT, allowing them to translocate to the endometrium and develop the placenta, which is required to deliver nutrients to the embryo (Kalluri et al., 2009). The process of EMT is also biologically required for the epithelial migration necessary in wound healing. Inflammation near the wound causes the disassociation of epithelial cells and subsequent migration to the underlining basement membrane. There, the epithelial cells lose key markers and acquire a fibroblast-like phenotype (Lim et al., 2009). The mesenchymal-like epithelial cells then migrate to the wound, and regain their original epithelial phenotype through EMT's reverse process, mesenchymal-epithelial transition (MET). MET has been found to naturally occur in epithelial cells having undergone EMT over the course of 48 hours when TGFβ is removed from the environment (Lim et al., 2009). In the heart specifically, EMT is used to differentiate epithelial cells into fully-fledged fibroblasts. It is estimated more than 30% of fibroblasts in the human body are derived from inflammation-induced EMT, emphasizing its importance in tissue repair (Lim et al., 2009).

Although vital for development and wound healing, epithelial-mesenchymal transition is often repurposed by cancer cells to aid in the invasion of distant tissue because of the process's relative ease of initiation (Kalluri et al., 2009). Mutations in the epithelial adhesion protein E-cadherin commonly causes EMT in primary tumor epithelial cells. E-cadherin is a glycoprotein composed of five cadherin repeats that when

lost facilitates detachment from neighboring tissue and triggers additional mesenchymal differentiation (Thiery et al., 2009). E-cadherin is transcriptionally repressed by Snail and Twist, both of which are regulated by additional upstream targets (Lim et al., 2013). Similarly, Vimentin expression is crucial for EMT. Vimentin is a type three intermediate filament protein that expedites the transport of β1 integrin to the (+) end of microtubules by motor proteins, acting as the cytoskeletal backbone of mesenchymal cells (Goldman et al., 1996). Vimentin provides mesenchymal-like epithelial cells the flexible backbone needed to mobilize. Many proteins are therefore implicated in regulating E-cadherin and Vimentin, and the same is true for other EMT-inducing proteins. The high number of direct and secondary instigators make the chances of mutation-provoked EMT much greater (Goldman et al., 1996).

Transforming Growth Factor Beta (TGFβ) has been shown to induce EMT through several mechanisms including the repression of E-cadherin and upregulation of Vimentin (Principe et al., 2014). In epithelial cells, TGFβ catalyzes the phosphorylation of α-catenin and β-catenin, which destabilizes the E-cadherin adhesion complex and results in the loss of cell-to-cell adhesion (Rifkin et al., 2005). The growth factor also amplifies expression of the transcription factor Snail, which deacetylates the E-cadherin promoter, downregulating its expression. TGFβ may also increase the transcription of Vimentin through NF-kB and TAK1, though these mechanisms are poorly understood (Principe et al., 2014).

TGFβ is a globally present protein that elicits different reactions in different tissues. While canonically known as a tumor-suppressor in benign and neoplastic epithelial cells, TGFβ can initiate EMT in advanced pancreatic cancer. Composed of three isoforms, TGFβ is a driver protein that controls proliferation, cellular differentiation, and a host of other functions in cells (Kalluri et al., 2009). Inactive TGFβ is secreted as a latent complex and is bound to the extra cellular matrix. There the ligand is liberated by one of several of factors, including proteases and thrombospondin-1 (Principe et al., 2014).

The released TGFβ binds with the Type 2 TGFβ receptor (TGFBR2), which then conscripts TGFBR1. In epithelial cells, the above interaction allocates TGFBR1 facilitated phosphorylation of the receptor-regulated SMAD2 and SMAD3 proteins

(Principe et al., 2014). The activated SMAD proteins complex with SMAD4, and upregulate the transcription of the apoptosis-inducing signal p21. The presence of phosphorylated ERK (PERK), a common MAP kinase, has been shown to enable successful SMAD signaling and p21 expression (Principe et al., 2014). In fibroblasts and other mesenchymal cells, TGFβ has an opposite effect on cellular proliferation, promoting growth and cellular migration as opposed to inhibiting it in epithelial cells (Principe et al., 2014). On average, 90% of pancreatic tumors are composed a fibroblast-rich stroma (Lim et al., 2013), and many of these predominantly mesenchymal cells proliferate in response to TGFβ. Because of its strong positive association with p21 expression, PERK was used in this study as a marker of proliferation or growth-arrest in the presence of TGFβ, helping to indicate whether the growth factor elicited a more mesenchymal-like response in epithelial cells that have undergone EMT.

While TGFβ inhibits the proliferation of benign and neoplastic epithelial cells, it is unknown if these effects are retained following EMT. A number of emerging pancreatic cancer therapies are focusing on TGFβ-inhibition to target the pancreatic stroma, yet may aggravate the cancer epithelia should normal growth suppressive TGFβ still be intact. Therefore, should TGFβ-induced growth inhibition be compromised following EMT, EMT-related biomarkers such as PERK may be useful in predicting the safety and efficacy of TGFβ-targeted therapies.

Materials and Methods

To study the effects of TGFβ on mesenchymal-like epithelial cells, we utilized several experimental groups: control epithelial cells given standard media, epithelial cells that underwent TGFβ-induced EMT, post-EMT cells having allowed to revert back to a normal phenotype (Mesenchymal to epithelial transition or MET), and the latter pulsed with recombinant TGFβ. Pancreatic epithelial mouse PanIN KC4848 cells were used for all populations in the study. All cells were sub-cultured at approximately 80% confluence to prevent overgrowth. Cells were maintained at 37 degrees Celsius and 5% CO2. Standard DMEM media without TGFβ and with 10% fetal bovine serum was used and supplemented by 1% antibacterial penicillin and streptomycin. Maintenance populations had media changed every 24-72 hrs. for the duration of the study, and experimental

groups every 24 hrs. In order to induce EMT in the three experimental groups of PanINs, 10 ng/ml of recombinant TGFβ1, derived from Chinese hamster ovary cells (CHO cells), was added on five successive occasions to the media when it was changed. When EMT had been induced in the three experimental groups as indicated by morphologic analysis, one was lysed using standard cell-lysis procedure and frozen at -20°C. The two remaining experimental populations were allowed to naturally undergo MET over the course of 48 hours. One of the samples was lysed and frozen, and the final sample was serum starved for 24 hours, and then pulsed with 10 ng/ml TGFβ1. Both the control cells and final experimental group were lysed and frozen.

Standard bicinchoninic acid assay (BCA) procedure was used to determine total concentration of protein from the four samples. A western blot following standard procedure was used to evaluate for several downstream targets of TGFβ signaling, such as pSMAD2, PERK, and p21, as well as GAPDH, a protein involved in glycolysis and commonly used as a loading control because of its unchanging expression across groups.

Results

Our experiment was successful in inducing EMT in PanIN KC4848 cells after three days of dosing with TGFβ. Despite the canonical growth-inhibiting effects of TGFβ in epithelial cells, our experiment witnessed a robust pro-growth response in cells given TGFβ every day for five days (Figure 1a).

We found that in post-EMT epithelial cells, PERK expression decreased when compared to the control, indicating that TGFβ likely does not induce cell-cycle arrest in cells that have undergone EMT (Figure 1b). When the cells were allowed to naturally undergo MET and return to a more epithelial phenotype, TGFβ induced PERK was consistent with more a more normal response to TGFβ (Figure 1c).

Conclusion

As noted above, TGFβ was found to cause growth arrest in normal pancreatic epithelial cells. When the same epithelial cells underwent EMT, however, the cells proliferated in response to TGFβ. Characterization of this proliferative response to TGFβ implicates a divergent signaling mechanism in cells that have undergone EMT, though

normal signaling can be restored should these cells undergo MET. Specifically, because PERK levels decreased in mesenchymal-like epithelial cells as compared to the control, an alternate TGFβ pathway where PERK is negatively regulated (by phosphatases such as PP2a, for instance) may be at play. Additional experiments are needed to classify the divergent TGFβ signaling mentioned above.

Discussion

Mesenchymal-like epithelial cell's response to TGFβ may be clinically useful for determining the efficacy of TGFβ inhibitors in pancreatic cancer. Because post-EMT epithelial cells responded with proliferation to TGFβ, our findings support the use of clinical TGFβ inhibitors, which would help prevent EMT-founded tissue invasion, in addition to targeting the malign pancreatic stroma.

Researchers like Biswas, et al., (2006), and Markowitz, et al., (1995) have researched inhibiting TGFβ in clinical trials, and this study supports such efforts by showing TGFβ inhibition helps check tissue invasion. Additionally, our results support the therapeutic inhibition of EMT in metastatic tumors. Although EMT-induced tissue invasion typically occurs before pancreatic cancer is detected, researchers like Lu Y et al., (2014) have garnered support for the inhibition or reversal of EMT with therapeutic agents.

Additional studies could provide supplementary evidence on post-EMT epithelial cell response to TGFβ, and garnish more evidence for or against the use of TGFβ inhibitors. Forcing cells to undergo EMT through the addition of a viral vector upregulating expression of Snail and Zeb1, would remove the necessity of using TGFβ to cause EMT, and likely provide more accurate results. Extending our experiment to human epithelial cell lines would similarly deliver more relevant results.

Inquiry Process

Working at the lab has helped me not only learn useful lab techniques like western blots, protein assays, and PCR, but has helped me become a better researcher. SIR has helped teach me the value of collaboration; thanks to the help and advice of my advisors and fellow summer students, I've accomplished and learned much more than I could have done alone.

I value SIR much higher than a traditional science class. Although biology classes teach useful fundamentals, working at the lab was the first time I could apply that knowledge in means other than tests and quizzes. Most importantly, SIR gave me the opportunity to work in a cutting-edge field I would've never experienced otherwise.

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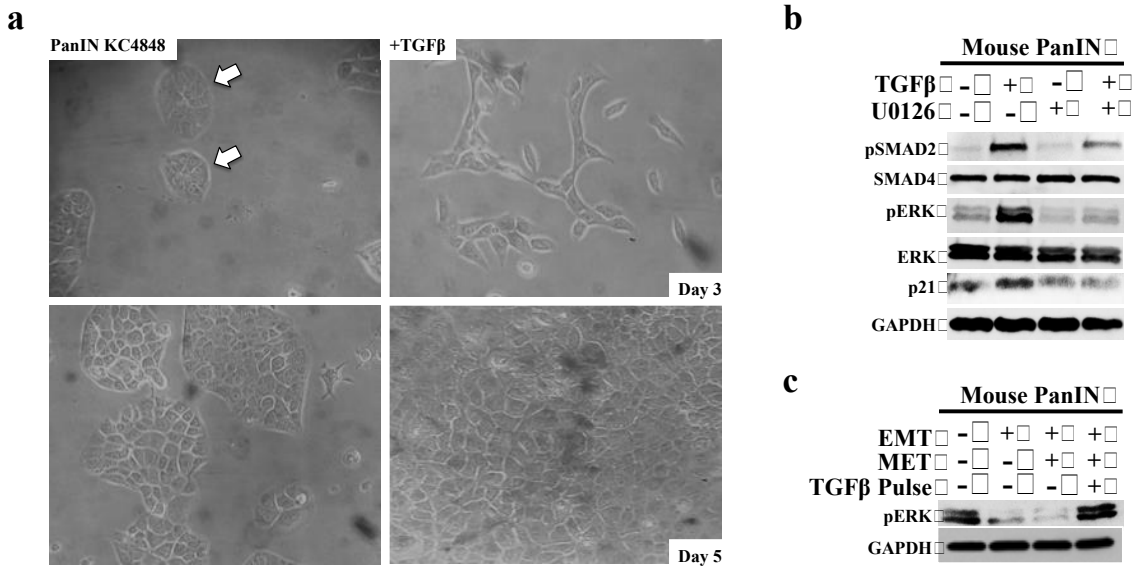
Figures and Tables

Figure 1.



(a) Three days of successive TGFβ-treatment induced EMT in PanIN KC4848. After five days, the total cell number increased significantly, indicating TGFβ is inducing proliferation in these cells. **(b)** Neoplastic epithelial cells given TGFβ show increased expression of PERK, pSMAD2, and p21, consistent with cell-cycle arrest. When ERK phosphorylation is blocked by U0126, p21 expression TGFβ fails to induce p21. These findings demonstrate the importance of PERK in cellular proliferation, directly correlating PERK with p21 production (used with permission from Principe et al., 2014; *in review*). **(c)** TGFβ failed to induce ERK phosphorylation in post-EMT epithelial cells, indicating a different response than pre-EMT controls. However, when these cells were allowed to revert back to a more normal phenotype through MET, TGFβ again activated PERK, consistent with a more normal response.