In Vitro Studies on the Impact of Ergothioneine on Idiopathic and Bleomycin-induced Pulmonary Fibrosis Markers in Human Lung Epithelial Cells

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Introduction

Idiopathic pulmonary fibrosis (IPF) can be characterised as chronic, progressive and irreversible lung scarring. The IPF pathology remains unknown, but tobacco smoking is considered a risk factor. Tobacco smoke-induced oxidative stress can initiate/worsen epithelial-mesenchymal transition (EMT), for which TGF-β1 is a chief inducer, in lung epithelial cells, leading to IPF.[1] On the other hand, bleomycin (BLM), an anticancer agent, can induce EMT in lung epithelial cells by the TGF-β/Smad signalling pathway, leading to pulmonary fibrosis.[2] Ergothioneine (ET) is an antioxidant amino acid available exclusively from the diet. The organic cation transporter novel 1 (OCTN1/*SLC22A4*) is its physiological transporter, which is essential for cellular uptake, distribution and accumulation of ET.[3] Nonetheless, the physiological functions of ET are not fully understood. Interestingly, genetic variants of *SLC22A4* have been associated, *inter alia*, with chronic obstructive pulmonary disease (COPD), a disease with fibrotic phenotype.

We aimed to investigate the hypothesis that ET protects human lung epithelial cells *in vitro* from factors implicated with EMT. Our objectives were to evaluate the impact of ET on 1) CSE-attenuated cell proliferation rate, TGF-β1- and BLM-induced 2) cell migration and 3) EMT in A549 and NCl-H441 cells.

Materials and Methods

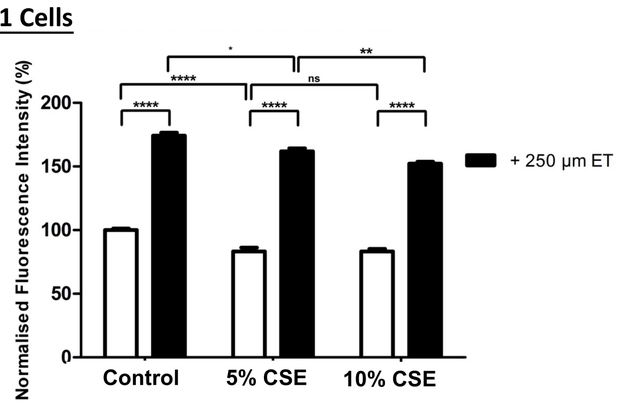
NCl-H441 and A549 cells were cultured in RPMI-1640 medium supplemented with 5% FBS and 1% sodium pyruvate, at 37°C and in 5% CO_2 atmosphere. In some experiments, the medium was supplemented with 250 µM ET. To assess the impact of tobacco smoke, cells were cultured in the presence of 5 or 10% freshly prepared cigarette smoke extract (CSE) for 24 h. CSE was generated by bubbling one Kentucky 2R4F research cigarette through 10 mL of medium. The CSE was filtered and diluted before use. To initiate EMT, cells were exposed to either 5 ng/mL TGF-β1 or 25 IU and 100 IU BLM in serum-free medium for 3 days in the respective experiments.

Cell proliferation rates were determined by the CyQUANT® NF assay. Scratch wound assays were carried out to measure cell migration in wound closure. Before the assay, cells were subjected to serum deprivation for 24 h, followed by 24 h of 5% CSE or 25 and 100 IU BLM treatment. Cell monolayers were wounded with a pipette tip, followed by 24 h serum-free culture. Cell migration in the wound areas was quantified by phase-contrast microscopy using the Cell^A or ImageJ software. EMT cell morphological changes were measured by phase-contrast microscopy and ImageJ software.

Results and Discussion

Impact of ET on CSE-attenuated Cell Proliferation Rate

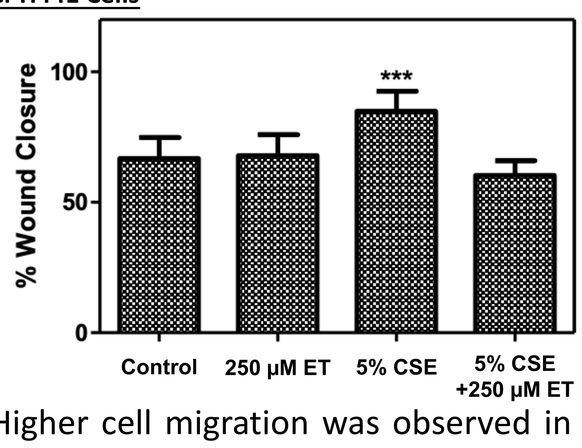
NCl-H441 Cells



ET increased the cell proliferation rate of NCl-H441 cells significantly (****, p<0.0001). Furthermore, CSE-attenuated cell proliferation rate was ameliorated by ET significantly (****, p<0.0001). However, the rapid proliferation rate of A549 cells compromised the sensitivity of the CyQUANT® NF assay (data not shown).

Impact of ET on CSE-induced Cell Migration

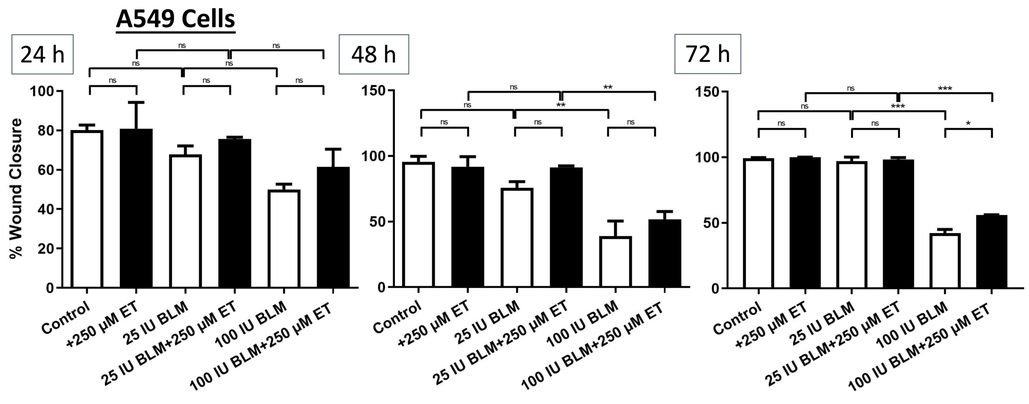
NCl-H441 Cells



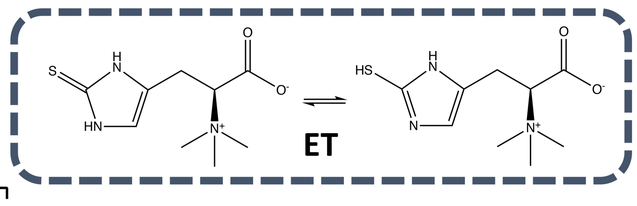
Higher cell migration was observed in 5% CSE-treated NCl-H441 cells and the effect was diminished after ET treatment (***, p<0.001). This experiment demonstrated that ET decreases CSE-induced cell migration.

Impact of ET on BLM-induced Cell Migration

A549 Cells

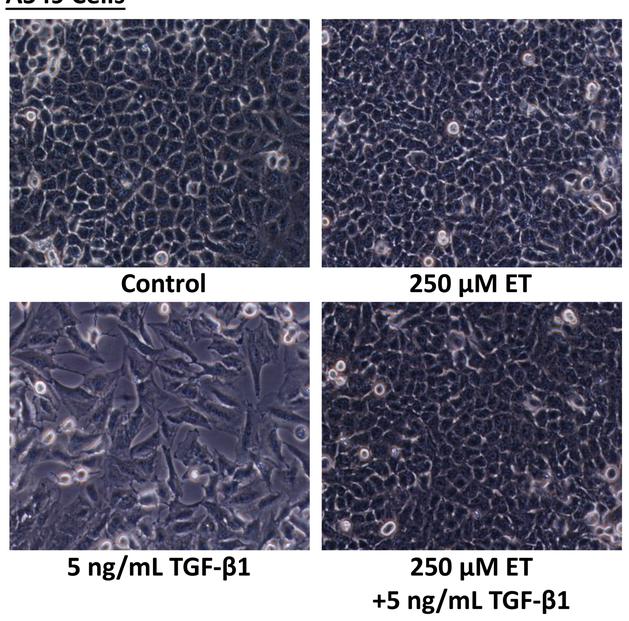


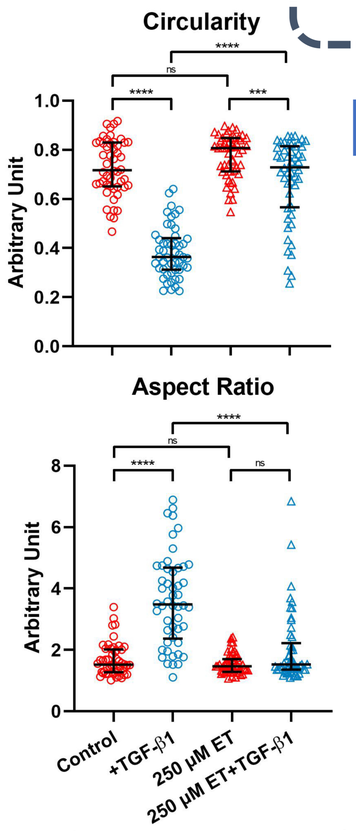
Cell migration was decreased by BLM in a concentration-dependent manner up to 100 IU. This effect was not diminished by ET except in 100 IU BLM-treated cells at 72 h (*, p<0.01). Wound healing in all but 100 IU BLM-treated groups, where wound closure was absent up to 72 h, was time-dependent. This might be because the high BLM concentration impaired the wound healing process, perhaps through inducing cell necrosis or apoptosis.



Impact of ET on TGF-β1-induced EMT

A549 Cells

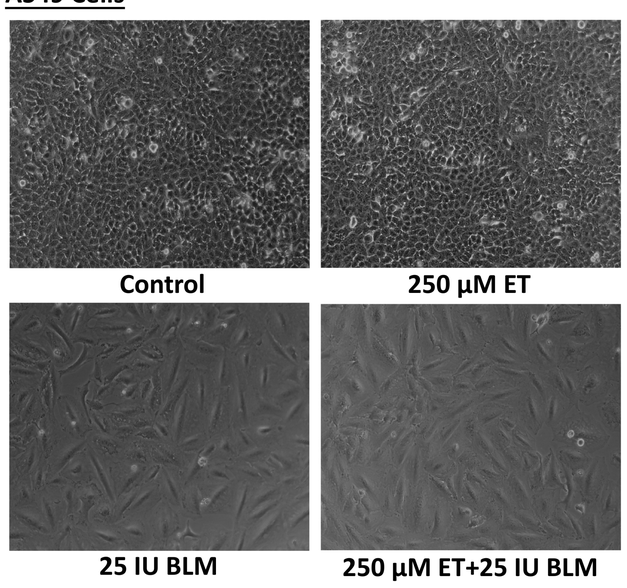


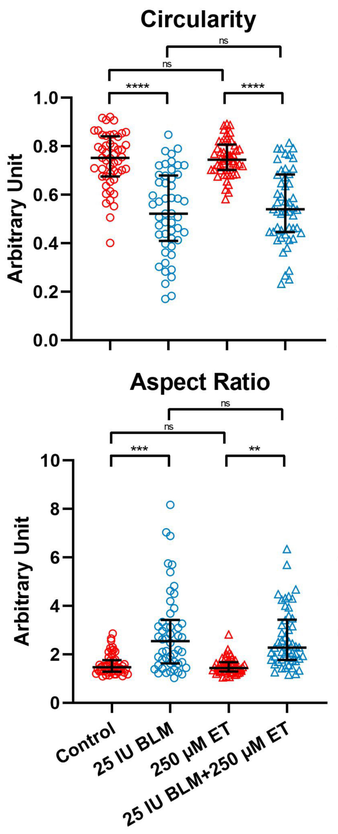


Decreased circularity and increased aspect ratio were measured after TGF-β1 treatment (****, p<0.0001). These effects were significantly reduced when cultured in the presence of 250 µM ET (****, p<0.0001). Treatment with 500 µM ET did not increase the protective effects (data not shown). Of note, TGF-β1 failed to establish morphological changes in NCl-H441 cells (data not shown).

Impact of ET on BLM-induced EMT

A549 Cells





BLM decreased circularity (****, p<0.0001) and increased aspect ratio (***, p<0.001) of A549 cells at both 25 IU and 100 IU (data not shown). However, 250 µM ET treatment did not prevent the EMT morphological changes (ns). This could be explained by BLM being able to induce other pathways implicated with EMT. Again, NCl-H441 cells did not demonstrate EMT morphological changes despite BLM treatment (data not shown).

Conclusions

ET not only protects human lung epithelial cells from CSE-induced cell death *in vitro*, but also prevents CSE-induced cell migration, thereby ameliorating EMT. Thus, it can be inferred that ET shows protective effects against IPF. However, ET has not been shown to be protective against BLM-induced pulmonary fibrosis *in vitro*. Future research will investigate the impact of ET on EMT molecular markers such as α-SMA, vimentin and collagen.

References

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3. Cheah, IK & Halliwell B. *Biochim Biophys Acta* 2012;*1822*(5):784-93.

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