学 位 論 文 の 要 旨

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学 位 論 文 名　Attenuation of Reactive Oxygen Species by Antioxidants Suppresses Hypoxia-Induced Epithelial-Mesenchymal Transition and Metastasis of Pancreatic Cancer Cells

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論 文 内 容 の 要 旨

INTRODUCTION

Pancreatic cancer is a disease with a poor prognosis. It is well known that pancreatic cancers are poorly vascularized and thus very hypoxic. Tumor hypoxia is a micro-environmental stimulus that plays a key role in angiogenesis, chemoradiotherapy resistance, invasion, and metastasis.

The main regulator of the hypoxic response of these cells is hypoxia-inducible factors (HIFs), which are a heterodimer of HIF-α and -β subunits. HIF-1 targets genes include those responsible for glucose metabolism such as glucose transporter-1 (*Glut-1*) angiogenesis such as vascular endothelial growth factor (*VEGF*), survival and metastasis. Hypoxia is also known to stimulate the production of reactive oxygen species (ROS), which are thought to be generated at the site of mitochondrial complex Ⅲ. Mitochondrial ROS have been shown to stabilize HIF-1 α and activate the transcription factor nuclear factor-κB (NF-κB) under hypoxic conditions.

Hypoxia has been shown to potently induce epithelial-mesenchymal transition (EMT) that is considered critical event in the induction of morphological changes and cell motility during metastasis. However, a causal link between ROS generation and EMT in hypoxia is poorly understood.

N-Acetylcysteine (NAC) is one of the most popular antioxidants. It has been shown to prevent chemical carcinogenesis, angiogenesis, and TGF-β1-induced EMT in rat epithelial cells. However, there are no studies demonstrating that NAC is able to inhibit hypoxia-induced EMT in tumor cells *in vitro*, or *in vivo*.

In the present study, we investigated the role of ROS in hypoxia-induced EMT and whether attenuation of ROS by NAC suppresses hypoxia-induced EMT and metastasis of human pancreatic cancer cells in a xenografted nude mouse model.

MATERIALS AND METHODS

Human pancreatic cancer cell lines, PANC-1 and MiaPaCa-2, were used. The cells were cultured under normoxic or hypoxic (1% O_2, 94% N_2, and 5%CO_2) conditions. Intracellular ROS production was measured by a fluorometer (wavelength; excitation 485 nm, emission 538 nm) after incubating the cells with 5 μM 2', 7'–dichorodihydrofluorescein diacetate (H_2DCFDA) for 10 min. Cell viability was examined by both trypan blue dye exclusion test and clonogenic assay. Cell motility and invasiveness were evaluated by scratch wound assay and Matrigel invasion assay, respectively. Immunofluorescent staining and Western blotting were performed for evaluation of the expression of E-cadherin, ZO-1, fibronectin, SLUG, HIF-1 α, NF-κB at the protein level. Semi-quantitative RT-PCR analysis was used to examine the expression of EMT regulators, *SLUG*, *SNAI1* and *TWIST*, and HIF-1 target genes. To examine the *in vivo* growth and spontaneous metastasis of PANC-1 or MiaPaCa-2 cells, they were implanted into the pancreas of nude mice. The mice were given drinking water containing NAC *ad libitum*. Hepatic metastasis was evaluated by counting the number of the cytokeratin AE1/AE3 positive micrometastases on paraffin sections. To detect hypoxic areas in tumors, Hypoxyprobe-1 (pimonidazole HCl) was injected intraperitoneally into mice bearing orthotopic tumors. The expression of EMT regulators in hypoxic areas of primary tumors was examined by double immunofluorescent staining using anti-Hypoxyprobe-1 monoclonal antibody and polyclonal antibody specific for each EMT regulator.

RESULTS AND DISCUSSION

PANC-1 and MiaPaCa-2 cells exposed to hypoxia showed increased ROS generation and characteristic changes of EMT such as morphological changes, down-regulation of epithelial marker proteins, E-cadherin and ZO-1, up-regulation of mesenchymal marker protein, fibronectin, and enhanced motility and invasiveness.

Treatment of the cells with NAC resulted in a significant suppression of all of these hypoxia-induced changes. The fact that another antioxidant ebselen also showed similar effects indicated the importance of ROS in hypoxia-induced EMT.

Hypoxia up-regulated the EMT regulators, *SLUG*, *SNAI1* and *TWIST*, in both PANC-1 and MiaPaCa-2 cells. NAC significantly suppressed the expression of these EMT regulators. Among the regulators, the level of *SLUG* remained high for a longer time than those of *SNAI1* and *TWIST* in hypoxia, suggesting that *SLUG* is regulated by the mechanism that is different from those of *SNAI1* and *TWIST*. By using pharmacological and RNA interference techniques, we found that SLUG expression was mediated by HIF-1 α and NF-κB activation in hypoxia. NAC suppressed the expression of SLUG through inhibition of both hypoxia-dependent HIF-1α and NF-κB activation. It may be possible that a ROS/HIF-1/NF-κB axis exists in the regulation of SLUG expression and EMT program under hypoxic conditions.

The expression of SLUG, TWIST and SNAI1 in normoxic and hypoxic areas of orthotopic PANC-1 tumors was examined by immunofluorescent staining. The results showed that the expression of the EMT regulators was up-regulated in hypoxic areas compared to normoxic areas. Administration of NAC to the mice resulted in a significant decrease in the expression of the regulators in hypoxic areas as well as normoxic areas. This result suggested that NAC is capable of suppressing the expression of EMT regulators *in vivo*. Based on the result, we investigated whether NAC is able to inhibit hepatic metastasis in orthotopic tumor model of PANC-1 and MiaPaCa-2 cells. Comparison of the number of the cytokeratin AE1/AE3 positive micrometastases between the control and the NAC-administered groups revealed that NAC significantly inhibited hepatic metastasis of PANC-1 and MiaPaCa-2 cells ($P < 0.01$). In these experiment, NAC administration did not lead to any adverse side effects such as body weight loss or diarrhea suggesting that NAC is well tolerated when administered orally.

CONCLUSION

Our results provide compelling evidence that hypoxia induces EMT in PANC-1 and MiaPaCa-2 cells through generation of ROS. Of particular importance is that attenuation of ROS by NAC suppresses hypoxia-induced EMT and metastasis. The use of antioxidants to inhibit EMT and metastasis might therefore be of therapeutic benefit in patients with pancreatic cancers.