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Tumor endothelial cells survive in lactic acidosis via the activity of pH regulators

（乳酸アシドーシス環境における pH 調節機構を介した腫瘍血管内皮細胞の生存）

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学位論文の要約

博士の専攻分野の名称　博士（医学）　氏名　DORCAS AKUBA-MUHYIA ANNAN

学位論文題名

Tumor endothelial cells survive in lactic acidosis via the activity of pH regulators （乳酸アシドーシス環境における pH 調節機構を介した腫瘍血管内皮細胞の生存）

Tumor survival and establishment largely depend on the process of angiogenesis. The tumor blood vessels formed from pre-existing ones provide nutrients for the growth of the tumor cells and also serve as portals for waste disposal and metastasis. Lining the walls of the tumor blood vessels are the tumor endothelial cells (TECs). TECs have various alterations which make them different from normal endothelial cells (NECs). A number of the changes in TECs arose from epigenetic modifications, influences from tumor-derived factors, tumor hypoxia, and reactive oxygen species. In addition to these factors products of tumor metabolism may also influence the function of endothelial cells in the tumor microenvironment. Tumor cells undergo glycolysis in the presence of oxygen (a process known as Warburg effect) to yield lactic acid as the end-product instead of pyruvate. The accumulation of lactic acid creates a condition termed *lactic acidosis,* which has been described as an oncologic emergency due to its association with poor prognosis and high mortality rates in patients. The effects of lactic acid on immune cells have been reported, however there are no available reports regarding endothelial cells, except studies describing the individual effects of lactate or acidity. Acidity can protect endothelial cells from apoptosis and cause them to escape the effects of antiangiogenic drugs by down-regulating the VEGF receptor 2 (VEGFR2). This render drugs which target VEGF and VEGFR2 ineffective. Exogenous lactate acts on endothelial cells to stimulate their migration and tube formation. In the tumor microenvironment, TECs may be exposed to high amounts of tumor-derived lactic acid, yet tumor angiogenesis is activated and not impeded. Based on this understanding, in this study, it was hypothesized that TECs possess unique properties that promote their survival in a lactic acid-rich and acidic environment.

Cancer cells have devised various ways to avoid the harmful effects of lactic acid that they produce. They show upregulated expression of proton-coupled monocarboxylate transporters (MCTs) such as MCT1 and MCT4 to help regulate intracellular lactate and proton levels. Moreover, to maintain a stable intracellular acid-base balance, they have upregulated the expression of pH regulators, including sodium/hydrogen exchanger 1 (NHE1), sodium/bicarbonate (Na^+/HCO_3^-) co-transporters, proton-sensing G-protein-coupled receptors (GPCRs), vacuolar ATPases (V-ATPase) and carbonic anhydrases (CAs) including CAIX, CAXII and CAII. CAIX and CAXII are well studied tumor-associated CAs, while CAII is reported to be uniquely expressed in the endothelium of many cancers. This distinct expression of CAII in the tumor endothelium may suggest a significant role of CAII in either the establishment of the tumor endothelium or the biological activity of TECs. However, the unique function of CAII in the tumor endothelium and pH regulators in TECs has not been elucidated. The aim of this study was to investigate the role of pH regulators in the survival of TECs under lactic acidosis. The objectives set to achieve this aim included an investigation of the effects of lactic acidosis on the proliferation and angiogenic activity of TECs, analysis of the expression of pH regulators in TECs and their contribution to TEC survival and proliferation in the presence/absence of lactic acidosis, and finally the identification of potential therapeutic targets of tumor angiogenesis.

In this study, TECs isolated from A375-SM tumor xenografts and NECs from the dermis of nude mice were used. The metabolomes of TECs and NECs were analyzed by the capillary electrophoresis-mass spectroscopy (CE– MS). Tissue and culture medium lactate contents were determined enzymatically with a lactate measurement kit. The mRNA expressions of pH regulators and transporters were determined by real time-quantitative polymerase chain reaction (RT-qPCR). Small interfering RNAs (siRNAs) of selected genes were used to target mRNA and the effect on TEC proliferation in complete medium (medium containing glucose,

glutamine and endothelial cell supporting growth factors), lactic acidosis (20mM lactate, pH 6.9), and lactosis (20 mM lactate, pH 7.3) was determined. Cell proliferation was measured either by cell counting or the MTS assay. The expression of CAII in mouse kidney, xenograft tumors and human renal cell carcinoma (RCC) tissues was determined by immunohistochemically or by immunofluorescence staining with anti-CAII antibody and anti-CD31 antibody to identify the blood vessels. To identify the factors that may be responsible for CAII upregulation in TECs, immortalized HMVECs (iHMVECs) were recruited as normal endothelial cells. The iHMVECs were treated with A375-SM tumor-conditioned media (tumor-CM) and the effect on CAII mRNA expression was analyzed. In some cases the tumor conditioned medium was heat-inactivated or pre-treated with Ki8751 to inhibit VEGFR2 signaling. Akiyama and others reported in 2012 that the conditioned medium obtained after culturing A375-SM tumor cells contained significantly high amounts of VEGF. Therefore the iHMVECs were also stimulated with VEGF to verify its contribution to CAII upregulation in TECs. For pharmacological inhibition assays, monocarboxylate transport was targeted with α-cyano-4-hydroxycinnamate (CHC) and carbonic anhydrases (CAs) with acetazolamide. A combination of acetazolamide and Ki8751 (VEGFR2 inhibitor) was also used to target TEC proliferation *in vitro*. The effect of CA inhibition on tumor angiogenesis was investigated by implanting A375-SM tumor-cells into nude mice. After the tumors were visible, treatment with acetazolamide (CA inhibitor) was commenced. A daily dose of 80mg/kg acetazolamide was administered for 10 days by intraperitoneal injection.

It was observed that during culture of the endothelial cells TEC culture medium became pale with a more acidic pH, whereas NEC culture medium maintained a color similar to medium without endothelial cells. The acidity of the TEC medium however did not affect TEC proliferation. This observation suggested that TECs can maintain their proliferation regardless of the pH of the extracellular environment. The metabolome analysis revealed a higher glycolytic flux in TECs, with higher levels of glycolytic metabolites than in the NECs. TECs

also produced more lactate than NECs. Measurement of tissue lactate content showed a significantly higher lactate level in tumor tissue as compared to skin and kidney normal tissues. Furthermore, TECs proliferated in lactic acidosis, whereas this condition did not support NEC proliferation. The analysis of pH regulators showed that MCT1 and MCT4 which are respectively involved in lactate import and export were both expressed in TECs. In complete medium TECs were more sensitive to CHC inhibition of MCT function in the cells. MCT1 and not MCT4 knockdown caused a decrease in TEC proliferation in complete medium. In lactic acidosis, both MCT1 and MCT4 knockdown decreased the proliferation of the TECs. TECs also showed an upregulated expression of CAII, CAIII, and NHA2 as compared to NECs. CAIV, CAIX, CAXII, NHE1, Gpr4, Gpr65 expression levels were not unique to only TECs and in some cases the expression was comparable between the TECs and NECs. Further analysis showed that TEC proliferation was significantly decreased by NHA2 knockdown and more significantly by CAII knockdown. Only CAII knockdown and not NHA2 knockdown led to a decrease in TEC proliferation in lactic acidosis and lactosis. It was observed that CAII was expressed in the endothelium of A375-SM and stage III RCC tumor tissues. In the normal kidney tissues and a stage I RCC specimen, endothelial CAII expression was not observed. Furthermore, it was demonstrated that protein factors in the A375-SM tumor-CM including VEGF contribute to CAII upregulation in TECs. This upregulated CAII expression by tumor derived factors was cancelled when the cells were treated with heat-inactivated tumor CM or tumor-CM that contained Ki8751 (VEGFR2 inhibitor). *In vitro* analysis of the effects of carbonic anhydrase inhibition showed significant decrease in TEC proliferation. Furthermore a combination of acetazolamide and Ki8751 led to a more significant decrease in TEC proliferation. Unexpectedly *in vivo* treatment with acetazolamide neither affected tumor growth nor angiogenesis.

The findings of this study showed that TECs can proliferate in lactate rich environments, and their growth is not stunted by low extracellular pH. Consequently, TECs proliferated in lactic

acidosis. TECs expressed various classes of pH regulators. Among them MCT1, NHA2, and CAII were vital to TEC proliferation under conditions of nutrient availability. This may imply that the activity of pH regulators play an important role in the proliferation of TEC. In lactic acidosis MCT1 and MCT4 knockdown decreased TEC proliferation; however, CAII knockdown caused a more significant decrease in TEC growth. Carbonic anhydrases have been shown to interact with MCT1 and MCT4 to facilitate efficient proton transport by these lactate transporters. CAII upregulation in TECs and the significant effect of MCT knockdown on TEC proliferation may suggest that CAII could enhance MCT lactate coupled proton transport in these cells. CAII is reported to enhance the proton exporting activity of NHEI, similarly it may interact with NHA2 in TECs to facilitate proton extrusion. CAII is an endothelium associated CA, and in this study, its importance to TEC function, particularly proliferation has been shown. These findings may suggest a significant role of TEC-specific CAII to the *in vivo* angiogenesis process in tumors. However this needs to be further investigated. In this study it was shown for the first time that tumor derived-factors such as VEGF can upregulate CAII expression in endothelial cells. This observation suggests that CAII expression in endothelium is under the influence of the VEGF and VEGFR2 signaling. This shows that VEGF/VEGFR2 signaling may affect pH regulation in endothelial cells. Successful inhibition of *in vitro* TEC proliferation by targeting CA and VEGFR2 suggests that combination of carbonic anhydrase inhibitors and anti-VEGFR2 drugs are therapeutic strategies which need to be explored. It was observed that of the two RCC specimen used, CAII expression was evident only in the tumor endothelium of a stage III tumor and not in stage I tumor. CAII expression has been linked to malignant progression of meningioma in studies by other authors and this supports the expression of CAII in the tumor endothelium of the advanced stage RCC patient in the current study. Furthermore, the two RCC specimen were from different cell types, so the differential CAII staining pattern may suggest that the tumor cellular origin may influence CAII expression in the associated endothelium. This also needs further investigation. Although only one specimen was analyzed for each cancer stage, it can be proposed that CAII may contribute to tumor malignancy in some

types of cancers including RCC.

The present study has shown that the function of pH regulators is necessary for the growth of TECs in nutrient available conditions and in lactic acidosis. Additionally, CAII may work together with other pH regulators like MCT4 and NHE1 to ensure TEC survival in lactic acidosis. The effect of CAII knockdown under all the conditions studied implies that CAII is very vital for the survival of TEC hence its unique expression in the tumor endothelium. CAII is required for TEC proliferation under lactic acidosis as well as in conditions of nutrient availability. These findings point to a potential role of pH regulation in facilitating angiogenesis *in vivo*, making CAII and intracellular pH control prospective targets of tumor anti-angiogenic therapy.