論文の内容の要旨

論文題目　Metabolic remodeling of mitochondrial electron transport chain under hypoxia and hyponutrition in a human pancreatic cancer cell line
（低酸素・低栄養下におけるヒト膵臓癌由来細胞株の
ミトコンドリア呼吸鎖再構築による適応代謝）

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An essential role of mitochondria is energy transduction. Mitochondrial dysfunction is involved in various diseases ranging from neuronal disorders such as Parkinson's disease, hereditary encephalopathy and glioma to ischaemia-reperfusion injury in myocardial infarction or stroke. Embedded in mitochondrial inner membrane are electron transport chain (ETC) complexes, namely complex I, II, III and IV. In a normoxic condition, electrons are transferred to complex I, III, IV or II, III, IV in these orders.

A role of mitochondria in tumour formation is implied by the fact that tumour cells harbor mutations in the genes that encode enzymes of the TCA cycle. Dominant mutations in the genes encoding complex II predispose carriers to carotid body paragangliomas, adrenal gland pheochromocytomas, and gastrointestinal stromal tumours. As to the mechanism of tumourigenesis caused by mutated complex II, growing evidence suggests the pro-tumorigenic effects of succinate. Tumour often forms a mass, the centre of which is hypovascularized, causing hypoxic and nutrient-deprived condition known as tumour microenvironment. It has been reported that succinate accumulates in cancer cells under such microenvironment, although we do not have direct biochemical evidence. Neither do we have an accurate, easy nor fast assay method for succinate concentration determination since mass spectrometry is a time-consuming and expensive method for this purpose.

In order to reveal the mechanism of succinate accumulation, this study has established a novel cyclic assay system for succinate concentration and examined the mechanism as to how cancer cells accumulate succinate.

Using this newly established method, the hypothesis that succinate accumulates in cancer cells and promotes cellular proliferation was tested through three following experiments. In the first experiment, the effect of succinate on cellular proliferation was examined and its promotive effect on proliferation in Panc-1 cells in hypoxia was confirmed. In the second experiment, we evaluated the intra- and extracellular succinate concentration of DLD-1, Panc-1 and HDF cells under normoxic/nutrient-rich and hypoxic/nutrient-deprived conditions. Under hypoxic/nutrient-deprived conditions, the cells secreted more succinate than they did under normoxic/nutrient-rich conditions. In the third

experiment, succinate level treated with ETC inhibitors was measured in order to identify the source of electrons flow to ETC, under normoxic/nutrient-rich and hypoxic/nutrient-deprived conditions. From this experiment, the accumulation of succinate during hypoxia was due to increased quinol–fumarate reductase (QFR) activity of complex II. In hypoxia, electrons from complex I by oxidizing NADH are transferred to complex II, where its QFR activity reduces fumarate to succinate. This may be an adaptive cellular mechanism to produce ATP in the absence or in the presence of low concentration of oxygen.

This study also demonstrated that in hypoxic/nutrient-deprived conditions, electrons produced at dihydroorotate dehydrogenase (DHODH) also flow to complex II, making possible the biosynthesis of pyrimidine even in the absence of oxygen. In hypoxic/nutrient-deprived conditions, the contribution of complex I and DHODH as electron donors to QFR activity of complex II is comparable to each other, and they can be two main electron donors. It was also confirmed that in HDF, both hypoxia and acidic condition (pH=6.9) are the essential elements for complex II to work as QFR to produce succinate, although in Panc-1 cells hypoxia alone can induce succinate production.

DHODH is an enzyme involved in the *de novo* biosynthesis of pyrimidines. The fact that DHODH functions during hypoxia as an electron donor to QFR activity of complex II suggests that in hypoxic cancer cells, “metabolic remodeling” occurs, in which electron flow alters, and that this remodeled metabolism links ETC with nucleotide metabolism. The two main electron donors to complex II, namely complex I and DHODH were revealed to be the drug target for cancer therapy, because inhibiting these complexes leads to decreased succinate level and thus less proliferation rate of cancer cells.

Next this study focused on succinate-driven reactive oxygen species (ROS) production. Ischemia-reperfusion (IR) injury is a universal phenomenon observed in various pathological events such as liver transplantation, hepatectomy, heart attack and stroke. Although reperfusion of ischemic tissue is essential for survival, it also triggers excess oxidative damage. IR injury attributes the tissue damage to the radical oxidative stress responses induced by a short period of ischemia and subsequent reperfusion. Mitochondria-derived ROS are the important mediators of oxidative cellular injury. It has been reported that during IR, superoxide is generated at complex I, although the production site of other ROS, especially hydroxyradical (OH•), which is the most radical ROS, remains unclear.

The aim of the second half of the study is to gain deeper insights into hydroxyradical production site in IR which will be a potential therapeutic target for IR injury. To attain this goal, this study focused on highly reactive oxygen species (hROS) which include hydroxyradical and peroxynitrite (ONOO-), but not superoxide. To examine hROS

production level *in vitro*, its specific marker, Aminophenyl Fluorescein (APF) was used. At the same time, this study evaluated extracellular succinate concentration during IR using our novel ASCT-SCS succinate cycling assay. This study also investigated the effects of various mitochondrial complex inhibitors on hROS production to elucidate the contribution of each ETC complex to hROS production and mitochondrial electron flow during IR.

To investigate when hROS is most generated after IR, the hROS level was analysed by observing the cells at various time-points (20min, 30min, 1h, 2h) after transient exposure to hypoxia. In Panc-1 cells, the hROS level continued to rise until 30min, when it reached its peak, and continued to decline after 30 min irrespective of the pH. Large increase of ROS production was observed at pH 6.9 rather than at pH 7.4. Similar pattern was observed in HDF cells at pH 6.9 while at pH 7.4 the hROS level showed only a modest increase 30 min after IR-mimicking treatment. It was also found that accumulated succinate was consumed during normoxia, indicating that hROS production is driven by accumulated succinate during hypoxia.

Panc-1 cells showed significantly lower hROS level when treated with dimethyl malonate (5 mM), indicating hROS is produced by the flow of electron generated at complex II by oxidizing succinate which had been accumulated in hypoxic period. Atpenin A5 (5 μM) treatment did not reduced hROS level to the dimethyl malonate level, meaning that significant amount of hROS are produced at either the FAD or [Fe-S] subunit of complex II, since malonate competes with succinate at the FAD site and atpenin A5 inhibits complex II at its ubiquinone binding site (IIQ). In Panc-1 cells, rotenone (5 μM) treatment did not diminish hROS production compared to the control group, leaving little possibility of hROS production at complex I at least at 30 min after IR. Antimycin A (5 μM), a post-ubisemiquinone (Q_i) site inhibitor of complex III, caused more decrease in hROS production than atpenin A5 did. This gap of the decreasing between the two in hROS amount may be attributed to hROS produced at complex III Q_i site but not pre-ubisemiquinone (Q_o) site when electron is transferred from complex II.

DHODH inhibitor diminished the hROS level in Panc-1 cells but not in HDF, indicating that the contribution of DHODH to hROS production was specific to Panc-1 cells. This result suggests that in Panc-1 cells during IR-mimicking condition, in addition to complex II, DHODH directly fuels hROS production.

From the study on hROS, it was elucidated that in Panc-1 cells IR induces succinate accumulation which results in the electron flow in the following orders; complex II - III - IV, and DHODH - complex III - IV. The former flow may be attributed to succinate accumulation during hypoxic period, and the latter flow may be accounted as follows: in cancer cells, under hypoxic/nutrient-deprived conditions, pyrimidine biosynthesis is totally dependent upon the

de novo pathway, because pyrimidine intermediates are depleted and the salvage pathway does not work. This may be the reason why considerable electrons flow from DHODH which results in hROS production.

In conclusion, complex II inhibitor dimethyl malonate and DHODH inhibitor were suggested as potential therapeutic drugs for IR injury. Also, acidic condition at pH 6.9 promoted succinate production at complex II under hypoxia, followed by larger amount of hROS generation, suggesting that keeping the local tissue pH at 7.4 may prevent hROS production after IR.

Succinate-mediated hROS production can be a leading candidate for intervention and the hROS production mechanism revealed in this study has a significant impact on the intervention to a range of ischemic disorders.