ALA-Porphyrin Science

The Effect of Aerobic Respiration Activity on Porphyrin Metabolism in Tumor Cells

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Summary

Our previous research have demonstrated that 5-aminolevulinic acid (ALA), the precursor in the porphyrin synthesis pathway leads to heme, promotes aerobic energy metabolism via up-regulation of protein levels of mitochondrial respiratory chain complex IV (cytochrome *c* oxidase, COX) in mouse. On the other hand, tumor cells exhibit the characterized metabolic phenotype, the Warburg effect, which is a shift of ATP generation from oxidative phosphorylation to glycolysis. Moreover, tumor cells have a limited capacity of heme synthesis. Therefore ALA induces the accumulation of porphyrin in tumor cells. Hence, in the present study, we focus on energy and porphyrin metabolism in tumor cells. We examined aerobic respiration activities and porphyrin accumulation levels of human gastric cancer cell lines. COX enzyme activity and oxygen consumption rate in the cell culture medium were measured. The porphyrin accumulation level in tumor cells incubated with ALA was quantitatively determined by fluorescence measurement. COX activities in tumor cells were lower than that in normal cells. These results indicates that a reduction of COX activity is critical factor in the Warburg effect. Furthermore, differences in COX activities, oxygen consumption rates and porphyrin accumulation levels were observed among the cell lines. The cell line which showed high oxygen consumption rate exhibited the low porphyrin accumulation. On the other hand, the cell line which showd low oxygen consumption rate exhibited the high porphyrin accumulation. In the result, these data suggest that there is an inverse correlation between aerobic respiration ability and porphyrin accumulation level.

Keyword

5-aminolevulinic acid, tumor, cytochrome *c* oxidase, porphyrin metabolism, electron transport chain

Introduction

Tumor cells exhibit the characterized metabolic phenotype, the Warburg effect, which is a shift of ATP generation from oxidative phosphorylation to glycolysis even in the presence of oxygen [1]. The use of glycolysis provides the tumor cell with ATP in a less efficient manner than that produced by oxidative phosphorylation. It might also confer a growth advantage in hypoxic conditions, allowing cancer cells to survive in the absence of oxygen.

In normal tissues, the main ATP synthesis pathway is oxidative phosphorylation in mitochondria. During oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors such as oxygen. These redox reactions generate energy, which is used to convert ADP to ATP in mitochondrial inner membrane with a series of protein complexes called electron transport chain. Complex IV, cytochrome *c* oxidase (COX), plays a key role at the end of the respiratory electron transport chain [2]. COX is a hemoprotein containing hemes as active site and performs an oxidation reaction of cytochrome *c* and a deoxidation reaction at the same time. In most tumor cells COX activities are suppressed. Moreover, tumor cells have a limited capacity of heme synthesis and COX is a typical heme protein. Therefore, limited capacity of heme synthesis and COX activity were unique characters for tumor.

It is well known that tumor cells accumulate protoporphyrin (PpIX) by the administration of 5-amonolevulinic acid (ALA) because of limited capacity of heme synthesis. The resulting PpIX fluorescence can be visualized using a modified neurosurgical microscope and is used for photodynamic diagnosis or therapy [3]. Hence, in the present study, we studied the relationship of the COX activity and PpIX accumulation after ALA administration.

Experimentals

Cells

The human normal cell line Human Embryonic Kidney 293 (HEK293) cells, the human T lymphoma cancer cell line Jurkat cells, the human gastric cancer cell lines NKPS, TMK-1, MKN45 cells and the human cervical epithelioid carcinoma cell line HeLa cells were used in this study. These cells were grown in Dulbecco's modified Eagle's medium (HEK293, Jurkat and HeLa) or RPMI-1640 medium (NKPS, TMK-1 and MKN45), supplemented with 10% fetal bovine serum (FBS) and ABAM, at 37°C in an incubator with a controlled humidified atmosphere containing 5% CO_2.

Measurement of COX activity

Measurement of COX activity was performed as previously described with some modifications [4]. Mitochondrial fractions were obtained using a Mitochondria Isolation Kit (MITOISO2, Sigma-Aldrich). In brief, cells (5×10^7 cells) were washed and homogenized in extraction reagent. The homogenate was centrifuged at $600 \times g$ for 5 min, and then the supernatant was further centrifuged at $11,000 \times g$ for 10 min. The pellet was suspended in storage buffer and used as a mitochondrial fraction. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, CA). COX activity was measured using a Cytochrome *c* Oxidase Assay Kit (Sigma-Aldrich). In brief, 100 μg of the mitochondrial fraction were diluted with enzyme dilution buffer containing 1 mM n-dodecyl β-d-maltoside. Ferrocytochrome *c* (reduced cytochrome *c* with dithiothreitol) was then added to the sample, and COX activity was measured by the decrease in absorption at 550 nm. The difference in extinction coefficients between reduced and oxidized

cytochrome *c* is 21.84 at 550 nm [5]. One unit of COX activity was defined as the oxidization of 1.0 μmole of ferrocytochrome *c* per min at pH 7.0 at 25°C.

Measurement of dissolved oxygen levels in liquid medium

Dissolved oxygen levels in liquid medium were determined by using a 24-channel SDR SensorDish® Reader (PreSens). Cells were seeded in a 24-well OxoDish® (5×10^4 cells / well) and cultured under 5% CO_2 gas at 37°C for 16 h. Subsequently liquid paraffin was added as gas barrier followed by the measurement of dissolved oxygen levels for 24 h in 2 h intervals.

Measurement of intracellular PpIX accumulation levels

Cells (5×10^5 cells) were incubated with 1 mM ALA in RPMI-1640 medium under 5% CO_2 gas at 37°C in the dark for 4 h. Cells were washed and lysed by 0.1 M NaOH. The lysates were extracted by adding the same volume of perchloric acid : methanol (1 : 1, v / v) solution. The obtained mixture was centrifuged to remove protein. The supernatant samples were quantitatively determined by measuring their fluorescence in a Hitachi Fluorescence Spectrophotometer Type F7000 (Hitachi High-Technologies, Tokyo, Japan). To measure fluorescence intensity, excitation and emission wavelengths of 405 nm and 605 nm, respectively, were used.

Incubation of human gastric cancer cells with ALA and exposure to LED irradiation

Cells were seeded in a 96-well plate (1×10^4 cells/well) and cultured under 5% CO_2 gas at 37°C for 24 h. ALA was added to the culture medium at increased concentrations, and incubation was continued in the dark for 4 h. Thereafter, cells were exposed to LED irradiation for 5 min (630 nm, 3.6 mW / cm^2) by placing the cell culture plate below an LED irradiation unit that had been provided by SBI Pharmaceuticals CO., LTD. (Tokyo, Japan). The light spot of LED was equally illuminated 128 mm × 86 mm in size which covers the whole area of 96-well culture plate. Cells were further incubated in the dark under 5% CO_2 gas at 37°C for 24 h, and cell viability was then measured by the MTT assay as described previously [6].

Results and Discussion

Aerobic respiration capacity in tumor cells

We have first tested whether there are differences of COX activity in normal or tumor cells *in vitro*. The human normal cell line HEK293 cells, the human T lymphoma cancer cell line Jurkat cells, the human gastric cancer cell lines NKPS, TMK-1, MKN45 cells and the human cervical epithelioid carcinoma cell line HeLa cells were used. Each cell line was incubated in normal condition, and mitochondrial fractions were harvested from 5×10^7 cells. Using mitochondrial fractions of each cell line, COX enzyme activity was measured by the decrease in absorption at 550 nm of ferrocytochrome *c*.

COX activities in tumor cells were significantly lower than that in normal cells (Figure 1). The activity of COX, the key enzyme in the electron transport chain, is fundamentally important in aerobic energy metabolism. Many diseases are characterized by defects in the oxidative phosphorylation system. Therefore, this result indicates that a reduction of COX activity is critical factor in the Warburg effect.

In addition, the COX activities were not same levels among the cancer cell lines. Hence, we challenged evaluation of aerobic respiration capacity in tumor cell lines. Decreasing of dissolved oxygen levels in medium during cell culture were measured as oxygen consumption. The time dependent response of the liquid-phase oxygen levels in cell culture medium were measured (Figure 2). There were differences in oxygen consumption rate among the gastric cancer cell lines. Moreover, NKPS cells had highest rate, followed by TMK-1 and MKN45 cells.

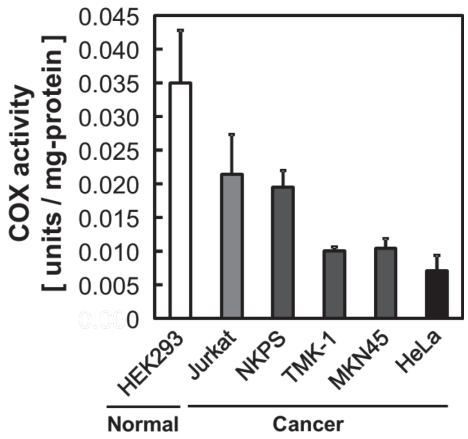

Figure 1. Cytochrome *c* oxidase activity of human normal or cancer cell lines. Determination of COX activity is based on the observation of a decrease in absorbance at 550 nm of ferrocytochrome c oxidized by COX. COX activity of normal HEK293 cells is significant higher than the other cancer cells. This data indicates that COX is critical factor of Warburg effect. Furthermore, different activity levels were observed among cancer cell lines.

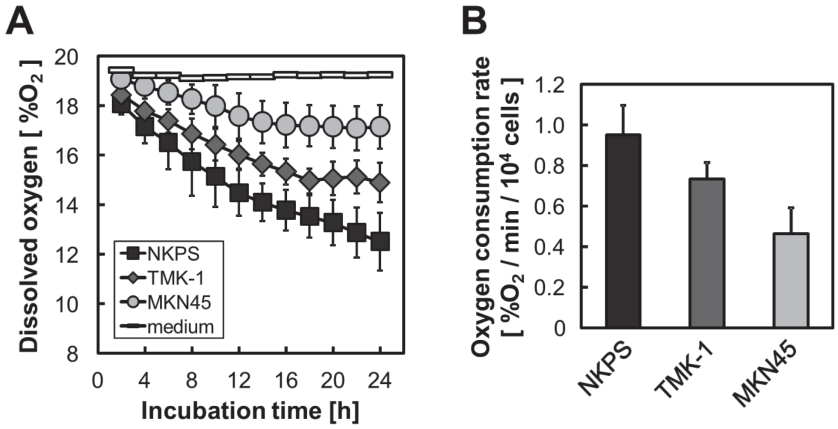

Figure 2. Oxygen consumption rate of human gastric cancer cell lines. Dissolved oxygen levels in cell culture medium were determined for 24 h by using a 24-channel SDR SensorDish® Reader (PreSens). Cells were seeded in a 24-well OxoDish® (5×10^4 cells / well) and liquid paraffin was added as gas barrier.

Porphyrin accumulation levels in tumor cells

In order to explore the correlation between aerobic capacity and porphyrin accumulation levels, we evaluated the intracellular biosynthesis and accumulation of PpIX in those three gastric cancer cell lines. The cells were harvested after incubation with 1 mM of ALA for 4 hours. The amount of PpIX produced in the cells was determined by measuring its fluorescence. There were differences in intracellular PpIX accumulation levels between three cell lines (Figure 3A). In addition, MKN45 cells showed highest accumulation level, followed by TMK-1, NKPS cells.

The efficacy of ALA-based photocytotoxicity is considered to depend on the intracellular concentration of PpIX that can produce cytotoxic singlet oxygen and other ROS via photoactivation reactions. In this context, we tested the cells viability after incubation in the presence of ALA at

increased condition for 4 hours followed by exposure to LED irradiation for 5 minutes (1080 mJ / cm^2, Figure 3B). As a result, MKN45, which had the highest PpIX accumulation levels, showed the highest level of sensitivity to ALA-based photo-cytotoxicity PpIX among the three cell lines. In contrast, NKPS which had lowest level of PpIX showed the lowest level of sensitivity. In other words, the ALA-based photo -cytotoxicity was found to be correlated with intracellular PpIX levels.

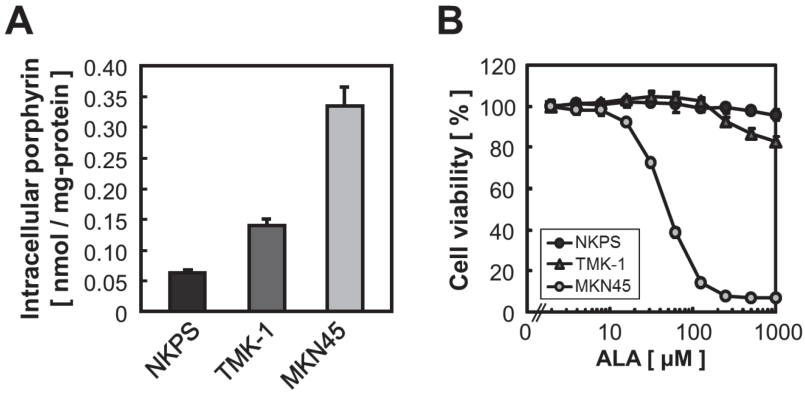

Figure 3. Porphyrin accumulation and ALA-based photocytotoxicity of human gastric cancer cell lines. (A) The porphyrin accumulation level in tumor cells incubated with 1 mM ALA for 4 h was quantitatively determined by fluorescence measurement. There were differences in porphyrin accumulation levels between three cell lines. (B) The cells viability after incubation in the presence of ALA at increased condition for 4 h followed by exposure to LED irradiation for 5 min (1080 mJ / cm^2).

These data indicate that the cell line which had high oxygen consumption rate exhibited the low porphyrin accumulation (Figure 2 and 3). On the other hand, the cell line which had low oxygen consumption rate exhibited the high porphyrin accumulation. In other word, low aerobic cells might show high sensitivity to ALA-based photocytotoxicity. Our study indicates that tumor cells, which have low capacity of oxygen consumption, could be sensitive target to ALA-based photodynamic diagnosis or therapy. As a rule, in most solid malignancies, the O_2 status in tissue is lower than in normal tissue. Hypoxic area in tumors has been shown to contribute to resistance to standard radiotherapy, chemotherapy. Therefore, our study suggests a potential of ALA-induced photodynamic therapy and photodynamic diagnosis.

Conclusion

Our experiments *in vitro* revealed the differences in COX activities, oxygen consumption rates and porphyrin accumulation levels among the cell lines. Furthermore, it is observed in three gastric cancer cells that reverse order of the cell line which had high oxygen consumption rate and high porphyrin accumulation. In the result, these data indicate that there is an inverse correlation between aerobic respiration ability and porphyrin accumulation level.

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