Alteration of Tumor Glucose Metabolism after Radiotherapy in MCF-7 Breast Cancer Cell Lines

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ABSTRACT

Cancer cells utilize anaerobic glycolytic way to compensate their faster metabolism when compared to normal cells. The purpose of this study is to investigate the effect of radiation on tumor metabolism.

MCF-7 breast cancer cell lines were divided into 4 groups, including 2 control groups and aerobic and anaerobic study groups (were irradiated 600 cGy by Co-60 teletherapy unit), incubated with radiolabelled glucose for 4 hours. One control group was for aerobic, and the other was for anaerobic group after KCN addition. Radiolabelled CO_2 produced by the cells was isolated and collected in specially designed sintillation vials. In supernatant the measurements of other end-products of carbohydrate catabolism including lactate, pyruvate, acetate were performed on a liquid scintillation analyzer after they were collected via anion-exchange chromatography. Finally glucose in supernatant was measured enzymatically by glucose oxidase method. Glycogen consumption and lactate production were significantly higher in anaerobic and radiation groups ($p<0.01$). Whereas CO_2 production was significantly higher in control group ($p<0.01$). Taken all results together radiation lead tumor cells more anaerobic glycolysis with high glycogen consumption, high lactate production and low CO_2 production. Radiation itself has led tumor cells to produce energy by anaerobic glycolysis, meaning radiation exposed cells become more hypoxic.

Key Words: Radiation, Tumor metabolism, Hypoxia, MCF-7 cell line

ÖZET

Radyoterapi Sonrası MCF-7 Meme Kanseri Hücre Kültürlerinde Glukoz Metabolizması Değişiklikleri

Kanser hücreleri hızlı metabolizmalarını kompanze etmek için normal hücrelere göre daha fazla anaerobik glikoliz yaparlar. Bu çalışmanın amacı radyasyonun tümör metabolizması üzerine etkisinin belirlenmesidir..

MCF-7 meme kanseri hücre kültürleri 4 gruba ayrıldı. İki kontrol grubu (aerobic ve anaerobik), iki çalışma grubu aeorbik ve anaerobik (hücre kültürleri 600 cGy tek doz, Co-60 teleterapi cihazıyla ışınlandı) ve radyoişaretli glukozla 4 saat inkübe edildiler. Anaerobik gruplar ise KCN eklenerek hazırlandı. Radyoişaretli CO_2 izole edildi ve özel tüplerde toplandı. Ayrıca süpernatandan anion-exchange kromotografi kullanılarak elde edilen glukoz metabolizlması son ürünleri olan laktat, piruvat ve asetat sıvı sintillasyon yöntemiyle ölçüldü. Süpernatanda bulunan glukoz, glukoz oksidaz enzimatik yöntemiyle ölçüldü. Glikojen yıkımı ve laktat üretimi anaerobik ve radyasyon gruplarında anlamlı olarak daha fazla bulundu ($p<0.01$). CO_2 üretimi kontrol grubunda anlamlı ölçüde daha yüksekti ($p<0.01$). Bütün bulgular bir arada incelendiğinde radyasyonun tümör hücrelerini, yüksek glikojen yıkımı, yüksek laktat aretimi ve düşük CO_2 üretimiyle, daha anaerobik glikolize ittiğini söyleyebiliriz. Radyasyon tek başına tümör hücrelerindeki anaerobik glikolizi artırmış ve hücrelerin daha hipoksik olmasını sağlamıştır.

Anahtar Kelimeler: Radyasyon, Tümör matebolizması, Hipoksi, MCF-7 hücre kültürü.

INTRODUCTION

Glycolysis plays a central role in cell oxygen consumption, Adenosine Tri-phosphate (ATP) production, carbohydrate consumption and metabolic pathways. Mammalian cells whether normal or malignant use the same metabolic pathways to produce ATP. There are two pathways of glycolysis that environmental oxygen and glucose concentrations determine the metabolic pathway as anaerobic or aerobic glycolysis. In high oxygen concentration, glucose degradation continues with Krebs cycle in mitochondria in which electron transport chain located on its inner membrane (1). In the absence of oxygen, mitochondrial part of glycolysis does not take place and less energy and lactate are produced. Oxygen dependence of energy metabolism is known as "Pasteur Effect" (2).

Energy metabolism of the cell does not only depend on oxygen, but also glucose concentration of the environment determines pathway. Tumor cells, microorganisms, parasites prefer anaerobic glycolysis in presence of large amount of glucose even in the presence of oxygen that is known as "Crabtree Effect" (3,4). Neoplastic cells shift to aerobic metabolism in low glucose concentration. Cancer cells produce energy in a very short way with few enzymatic reactions instead of long and large pathway. This is somehow beneficial to cancer cells, however harmful to host organism because of large expenditure of body resources. Moreover anaerobic pathway cause increase in lactate concentration which make cell to shift lower pH. Malignant cells adapt themselves to glucose concentration of the environment and survive in every glucose concentration (5).

The importance of glycolysis for tumor progression and for treatment is not clear. However, 85% of the intracellular oxygen has been utilized for energy metabolism (6). Dominancy of anaerobic glycolysis means hypoxic cell. Holthusen reported radioresistance of anaerobiosis in ascaris eggs (7). Later, Crabtree and Cramer reported reduced radiation effects in hypoxic cells (8). Attempts to exploit the acquired knowledge for an improvement of radiotherapy, however, have met with disappointingly little success: Neither hyperbaric oxygen breathing nor the use of hypoxic cell sensitizers like misonidazole were able to remarkably improve treatment outcome (9,10).

Hypoxia in solid tumors has often been anticipated to cause excessive lactate production and lactacidemia, and lactate has been frequently considered a surrogate of hypoxia (11). On the other hand, recent molecular research has provided strong evidence for aerobic lactate production to be direct consequence of malignant conversion of glycolysis by activation of oncogenes and/or inactivation of tumor suppressor genes (12-14). These studies have confirmed Warburg's hypothesis of malignancy to be associated with metabolic shift from oxidative to the glycolytic energy production (15).

There is no data how radiation itself affect the tumor metabolism which in part, means oxygen consumption of the cell. In our study, we aimed to determine the effect of radiation on tumor metabolism.

MATERIAL AND METHODS

Groups

MCF-7 breast cell lines were used for this in vitro study and 4 groups were prepared: aerobic control group (Ac), anaerobic control group (KCN), radiation group (RT) and anaerobic+radiation group (KCN+RT). Surviving fractions in KCN+RT group is measured and compared with the radiation group. Due to technical limitations we can run biochemical analysis in first three groups.

Chemicals and Biomaterials

D-[6-C14] glucose was purchased from Amersham (Bucks, UK) Company and hexokinase and glucose-6-phosphate dehydrogenase enzymes were from Boehringer (Manheim, Gemany). MCF-7 breast cancer cell lines maintained at 37 °C in the medium of 5% CO_2 and medium of RPMI 1640 supported by fetal calf serum 10%, 2 μM L-Glutamine, 100 μg/mL streptomycine and 100 U/ml penicilline.

Radioactive Incubations and Analysis of Excreted End Products

A total of 16 T25 flasks, 4 cultures in each group were prepared. Radioactive incubations were performed by glucose in which sixth carbon was labeled with radioactive Carbon 14 (D-[6-C14] glucose). Amount of labeled glucose added to each culture was 25μCi D-[6-C14] glucose. Before the in-

cubation procedure, the MCF-7 cell cultures were made up to adequate concentrations and separated into three groups. The first one was for the aerobic culture the second one was for the anaerobic culture after KCN addition defined by Tielens (16) and the last one was for the determination of the radiation effects on the cells. Both aerobic and anaerobic cell cultures were immediately incubated with radiolabelled glucose for 4 hours in a specially designed chamber at 37°C. The cultures which were separated for the determination of zero time glycogen and protein levels were not incubated with radiolabelled glucose. Incubated cell cultures which catabolize externally given radioactive glucose through glycolysis convert it into products including lactate, acetate and propionic acid. Following incubation, the generated radioactive CO_2 was collected in scintillation vials via nitrogen gas. Then, the content was separated as supernatant and pellets. By using supernatant layer, end-products of glycolysis (lactate, acetate and piruvate) were collected in scintillation vials by anion-exchange chromatography, and were calculated on standard graphics in Microsoft Excel program.

In pellets, protein was determined by modified Lowry method and glycogen by Hassid and Abraham's enzymatic method (17). Glucose in supernatant was measured enzymatically by glucose oxidase method. Glycogen consumption and CO_2 production for each gram of protein were calculated by using data obtained by the measurement of glycogen and protein found in the pellets.

Radiation

All cell cultures were brought to radiotherapy department to eliminate effect of transfer. Radiation group and anaerobic (KCN+RT) radiation group received a total of 600 cGy radiation via Co-60 teletherapy unit (Theratron 780 C) in a single fraction with an output of 152.78 cGy/min. To increase cell culture dose to the maximum level a 5 mm wax bolus was placed on the radiation portal.

Statistical Analyses

Statistical analysis of the data was done with 9.0 SPSS Package programme for computer. Kruskal-Wallis and Mann-Witney U tests were used for the difference between groups. $P<0.05$ was assumed to be significant.

RESULTS

Table 1 and 2 show the amounts of glycogen consumption, labeled and total end products of glycogen metabolism, CO_2 and lactate, and external glucose degradation as percentage of internal glycogen degradation in MC-7 breast cancer cell line in aerobic control, anaerobic (KCN) media and radiation treated aerobic cell culture. Glycogen consumption was significantly higher in anaerobic culture than aerobic one ($p < 0.01$). Labeled and total amounts of the end product lactate were higher ($p < 0.01$ and $p < 0.01$, respectively), and labeled and total amounts of the end product CO_2 were lower ($p < 0.01$

Table 1. Cell counts and glycogen consumption

Cell Culture	Addition	Cell count incubation	Glycogen at the begining	Glycogen consumtion
		$x10^6$+SE	nmolglucose/h/1000 cell+SE	nmolglucose/h/1000 cell+SE
Aerobic		41,3+0.92	83,71+1.8	2,63+0.05
Anaerobic	KCN	46,3+0.83	79,43+1.3	5,71+0.33
Aerobic	Radiotherapy	12,3+0.78	72,6+0.9	6,39+0.15
		p<0.05	p<0.01	p<0.01

*SE: Standart Error of Mean

Table 2. Glucose metabolism end products

Cell Culture Groups	CO_2 production	Lactate production	Total CO_2	Percentage of External Glucose Consumption/
	pmol/h/1000 cell ±SE	pmol/h/1000 cell ±SE	pmol/h/1000 cell ±SE	internal glycogen consumption
Aerobic control	11,38+0.01	0,09+0.005	490,62+2.1	0,433
Anaerobic KCN	2,25+0.08	0,42+0.01	305,50+2.3	0,039
Aerobic radiation	2,04+0.02	0,57+0.03	236,97+2.8	0,032
	p<0.01	p<0.01	p<0.01	p<0.01

*SE: Standart Error of Mean

and $p < 0.01$, respectively) in anaerobic culture when compared to aerobic one.

When radiation treated aerobic MC-7 breast cancer cell culture was compared with aerobic normal cancer cell culture, glycogen consumption was found to be significantly higher ($p < 0.01$), labeled and total end product lactate was found to be higher ($p < 0.01$) and labeled and total end product CO_2 was found to be lower in cancer cell cell culture ($p < 0.01$). When radiation treated aerobic MC-7 breast cancer cell culture was compared with anaerobic control group, glycogen consumption, both labeled end products lactate and CO_2 were similar.

Cell count in the aerobic radiation treated group was 44.3×10^6 prior to radiation and 12.3×10^6 after radiation. In aerobic cell line, addition of the radiation led to dead of 73% of the total cell count. However, in the anaerobic group (KCN) addition of the radiation caused dead of 53% of the total cell count. Initial cell count was 48.6×10^6 and, after radiation it was 22.4×10^6. Surviving fraction in anaerobic group after radiation is 43%. There is significant difference between aerobic and anaerobic radiation treated groups in regard to surviving fraction ($p < 0.05$).

DISCUSSION

Our experimental study showed that the radition switched tumor metabolism to anaerobic side and caused significantly more cell death in aerobic tumor cells. Anaerobic metabolism in tumor is triggered by high glucose concentration (crabtree effect) (3) or poor oxygenation (pasteur effect) (2). Our study, on the other hand, gives a clue that irradiation also produces a shift of tumor metabolism from aerobic to anaerobic state. Though clinical significance of this shift is not known, it can give us an insight for clinical radiation response and progress in radiotherapy.

Anaerobic shift phenomenon is particularly important for the fractionated radiation therapy, since the hypoxic tumors are more radioresistant and more aggressive. Because the 85% of the intracelluler oxygen is utilized for aerobic metabolism, next fractions of the radiation might be delivered to a hypoxic tumor which is more radioresistant. So next fractions of radiation will not be as effective as the first fractions. It is believed that reoxygenation takes place during fractionated radiotherapy which leads to sensitization of hypoxic cells to later fractions of radiation. The mechanism that is suggested is the death of aerobic cells and beter oxygenation of the remaining cells due to closer migration to

blood vessels (18). However our results do not confirm this. Aerob cells exposed to 6 Gy of irradiation shifted cell metabolism to the anaerobic state, even in the presence of large amount of oxygen. It may cause a vicious cycle that radiation limits its efficiousy by itself.

Our conclusion is based on glycolytic use of oxygen in the tumor cells. However there is an important question that how about remaning 15% of the oxygen? If tumor perfusion is running well, and tumor cell is undergoing anaerobic glycolysis, can we accept cell as hypoxic? Although most of the literature accept as hypoxic, for radiation response, remaining 15% of the oxygen might be more important than 85% oxygen used for glycolysis. Neverthless, lactate is shown to be an important metabolite for tumor progression and prognosis (15).

Malignant transformation is associated with an increase in glycolytic flux and in anaerobic and aerobic lactate excretion. In all investigated tumor entities, high concentrations of lactate were correlated with a high incidence of distant metastasis (19-21). Low lactate tumors were associated with a longer overall and disease-free survival when compared with high lactate lesions (22,23). Numerous biological activities of lactate that can enhance the malignant behavior of cancer cells. Hence lactate accumulation not only mirrors but also actively enhances the degree of tumor malignancy.

Our findings of this metabolic shift might be due to couple of reasons. Because, in addition to the recognized effects of radiation on the integrity of DNA and cell survival, radiation also impairs mitochondrial function. Previous studies have demonstrated altered mitochondrial function in cells exposed to radiation, such as loss of enzymatic activity, oxidative phosphorylation, and onset of lipid peroxidation (24-26). Zolzer et al. examined the effect of radiation on the integrity of DNA in the mitochondria and confirmed the greater susceptibility of mtDNA to radiation-induced damage (27). Mitochondrial DNA is responsible for synthesis of couple cytochrome enyzmes that are required for krebs cycle to run. Inability to synthesize cytochrome enzymes may activate anaerobic metabolism and more lactate production.

Nuclear DNA damage, although it is repaired, might cause cessation of synthesis of glycolytic enzymes. Instead of running a huge enzyme system, it might be a good way to stop synthesizing too many glycolytic enzymes and to work on repair enzymes and proteins.

Another explanation for this event is that there might be no metabolic shift at all. In a cell culture radiation might selectively kill aerobic cells and keep anaerobic cells alive. Erronously, one can just measure metabolic activities and reach such a conclusion. Neverthless, in our experiment we also irradiate a completely anaerobic cell culture and look the cell count. Even in complete anaerobic population 53% of cells die, in aerobic cell culture 73% of cells die. If we consider surviving fraction in anaerobic group (47%), and assume that all aerobic cells killed by radiation, more than 55% of tumor cells in aerobic culture should be in anaerobic status at the begining. Such condition of anaerobic glycolysis for more than half of the cells in MCF-7 culture under optimal conditions is not expected. In spite, no firm conclusion could be driven, we do think that although this mechanism might partly be true, there is stil metabolic shift to anaerobic site. We are planning to take this experimental trial as a reference for our future trial.

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