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Research paper

Evaluation of mitochondrial function and metabolic reprogramming during tumor progression in a cell model of skin carcinogenesis

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ABSTRACT

Metabolic reprogramming from mitochondrial aerobic respiration to aerobic glycolysis is a hallmark of cancer. However, whether it is caused by a dysfunction in the oxidative phosphorylation pathway is still under debate. In this work, we have analyzed the bioenergetic cellular (BEC) index and the relative cell ability to grow in the presence of either galactose or glucose as sources of sugar (Gal/Glu index) of a system formed by four epidermal cell lines with increasing tumorigenic potentials, ranging from nontumorigenic to highly malignant. We find that the BEC index gradually decreases whereas the Gal/Glu index increases with tumorigenicity, indicating that a progressive metabolic adaptation to aerobic glycolysis occurs in tumor cells associated with malignancy. Interestingly, this metabolic adaptation does not appear to be caused by damaged respiration, since the expression and activity of components of the respiratory chain complexes were unchanged in the cell lines. Moreover, the corresponding mitochondrial ATP synthetic abilities of the cell lines were found similar. The production of reactive oxygen species was also measured. A shift in ROS generation was found when compared nontumorigenic with tumorigenic cell lines, the latter exhibiting about threefold higher ROS levels than nontumorigenic cells. This result indicates that oxidative stress is an early event during tumor progression.

1. Introduction

In 1927, Otto Warburg put forward the hypothesis that cancer cells undergo a shift in their metabolism from mitochondrial aerobic respiration to glycolysis followed by lactic acid fermentation that would occur even under normoxic conditions. Warburg also proposed that this altered metabolism is caused by defects in the oxidative phosphorylation (OXPHOS) pathway [1]. A bioenergetic signature of cancer cells was proposed by Cuezva and collaborators who showed that the expression of mitochondrial and glycolytic enzymatic markers varies significantly when comparing human carcinomas with paired normal tissues [2]. These authors analyzed a wide variety of tumors, including liver, kidney, colon, breast, lung, gastric and esophageal carcinomas, and concluded that OXPHOS function is reduced during neoplastic transformation. They defined a bioenergetic cellular index, the BEC index, which could be used to estimate the mitochondrial status in human tumors. However, the concept that the respiration of cancer cells is damaged is highly debated, in particular, since the discovery that proto-oncogenes and tumor suppressor genes besides regulating signal transduction pathways involved in cell cycle control also affect cellular metabolism [3,4]. Nowadays, the idea that metabolic reprogramming is a hallmark of cancer is well established, regardless of whether the cause is or is not a defective respiration [3,5].

In this work, we have analyzed the OXPHOS status relative to glycolysis in a model of mouse skin carcinogenesis formed by four cell lines with increasing tumorigenic abilities. Mouse skin chemical carcinogenesis is a well-characterized model system to study the genetic and biochemical changes associated with tumor initiation, promotion and progression. In this model, tumors are induced on the skin of mice by treatment with chemicals, either by the "two-stage" or "complete" carcinogenesis protocols [6]. The

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two-stage protocol involves treatment with a single dose of a carcinogen; i.e., 7,12-dimethylbenz(*a*)anthracene (DMBA) followed by repeated applications of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which gives rise to the appearance of benign papillomas. A small fraction of papillomas spontaneously progress to malignant squamous cell carcinomas (SCCs), which lately can undergo an epithelial–mesenchymal conversion and form highly aggressive spindle cell carcinomas (SpCC) [7]. Complete carcinogenesis, on the other hand, involves repeated applications of the carcinogen DMBA. Here, DMBA-induced cell lines corresponding to different stages of skin carcinogenesis were used to evaluate whether OXPHOS dysfunctions that lead to increased glycolytic activity occur associated with tumor progression.

2. Materials and methods

2.1. Cell lines, culture conditions and Gal/Glu index determination

MCA3D cells were derived by treatment of a primary mouse epidermal keratinocyte culture with DMBA, followed by selection in high calcium medium that induces terminal differentiation of normal keratinocytes [8]. PB cells were obtained from a papilloma induced by two-stage carcinogenesis [9]. The A5 cell line was derived from the spindle component of a skin carcinoma induced by complete carcinogenesis [10], and CarC cells were explanted from a carcinoma induced by two-stage carcinogenesis [11]. A summary of the origin and characteristics of the cell lines is presented in Supplementary material (Table S1). MCA3D and PB cells have normal *H-Ras* genes, while both CarC and A5 cell lines exhibit an identical mutation at codon 61 of *H-Ras* [8,11,12]. Moreover, CarC cells show complete loss of the normal *H-Ras* allele [11] and have the *Ink4* locus encoding the cell cycle regulators $p15^{Ink4b}$, $p16^{Ink4b}$ and $p19^{Arf}$ deleted [13]. A5 cells, on the other hand, have inactivated the tumor suppressor *Trp53* gene encoding p53 [10], whereas CarC has normal *Trp53* [14]. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)–Ham's F12 medium (1:1) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics: 100 μg/ml ampicillin, 60 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Cells were grown at 37 °C in a 5% CO_2-humidified atmosphere.

To compare the cell abilities to grow in the presence of either glucose or galactose as a sugar source, cells were transferred to sugar-free DMEM medium (Gibco) containing glucose (4.5 g/l) or galactose (0.9 g/l) plus the supplements cited above. Cells were incubated up to 8 days, refreshing the medium every 2 days. Doubling times were calculated by applying the suitable formula $DT = \log 2 \times (t - t_0)/\log N - \log N_0$, where $(t - t_0)$ is the total days of incubation and N_0 and N, the number of cells at the beginning and end of incubation, respectively. The Gal/Glu index is the ratio of the corresponding doubling times.

2.2. Tumorigenicity assay

Cells were collected, washed with PBS (without calcium) and intradermally/subcutaneously injected at the two flanks of 8 week-old Balb/c female athymic nude mice (Harlan). Each animal received approximately 2×10^6 cells/site. Tumor development was measured about twice per week and the size of tumors calculated from caliper measurements of two orthogonal diameters.

2.3. Western-blot analysis and BEC index determination

Collected cells were centrifuged and pellets frozen at −20 °C until use. Pellets were lysed in cold RIPA buffer supplemented with complete protease inhibitors cocktail (Roche). The homogenates were kept in ice for at least 15 min and, if necessary, passed through a needle (0.4 × 13 mm) to clarify the extracts before centrifuging at 13,000 rpm and 4 °C for 10 min. The supernatants were saved and the protein content determined with the BCA protein assay kit (Pierce). Extracts were stored frozen until use.

Protein extracts (20 μg) were separated in 10% SDS-PAGE gels and electrotransferred to either Immobilon-P or nitrocellulose membranes (Millipore). In order to determine the BEC index, membranes were probed with a polyclonal antibody against β-ATPase [15] and, subsequently, with monoclonal antibodies recognizing Hsp60 and GAPDH (Stressgen). Appropriate secondary antibodies coupled to horseradish peroxidase were used, and peroxidase activity was developed using either an enhanced chemiluminescence (ECL) kit (Amersham) or the Odyssey detection system (nitrocellulose filters). The signals obtained for each enzyme were quantified by densitometric analysis with Image J program. The BEC index was calculated dividing the ratio β-ATPase/Hsp60 by the value obtained for GAPDH [2].

Determination of protein levels of the respiratory chain subunits was achieved as indicated above using 50 μg protein extracts and monoclonal antibodies specific for COXI and NDUFA9 (Mitosciences). A monoclonal antibody against β-actin (Sigma) was used as loading control.

2.4. Determination of the activity and blue native electrophoresis of the respiratory chain complexes

Enzymatic activity measurements were performed with homogenates obtained from 2×10^6 cells grown as described in Section 2.1, washed in PBS and resuspended and sonicated in SETH buffer (250 mM sucrose, 2 mM EDTA, 100 U/L Heparin, 10 mM Tris–HCl, pH 7.4). The amount of protein was determined using the DC Protein Assay kit (Bio-Rad). The activities of the respiratory chain complexes I, II, III and IV and the mitochondrial mass marker citrate synthase were measured by spectrophotometric methods as previously described [16].

Blue native electrophoresis was performed as previously reported [17]. Mitochondrial protein extracts were obtained by treatment of the cells with 2 mg/ml digitonin followed by solubilization of the native complexes with 2% lauryl maltoside. Mitochondrial extracts (40 μg) were loaded on native 4–15% polyacrylamide gradient gels. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore) and probed with the specific complex I monoclonal antibody NDUFA9 (Mitosciences).

In-gel activity assays were performed as described [17]. For complex I activity assay, gels were incubated 1–2 h at 37 °C in 2 mM Tris–HCl, pH 7.4, 0.1 mg/ml NADH, and 2.5 mg/ml nitrotetrazolium blue (NTB). Stained gels were washed with distilled water and scanned.

2.5. ATP steady state levels

ATP measurements were carried out as previously described [18], with some modifications. Briefly, 2×10^5 cells were grown for 24 h in glu/gal medium in 35 mm dishes to approximately 80% confluence and then incubated for 2.5 h in a solution (156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM $KH2PO_4$, 2 mM $CaCl_2$, 20 mM HEPES, pH 7.35) containing a combination of substrates and inhibitors: 5 mM 2-deoxy-D-glucose/1 mM pyruvate (for oxidative ATP) and 2-deoxy-D-glucose/pyruvate/oligomycin (for residual ATP). After incubation, cells were washed with PBS and lysed with 1 ml of boiling 100 mM Tris, 4 mM EDTA, pH 7.75. Lysates were collected and incubated for 2 min at 100 °C, centrifuged 10 min at 13,000× *g* and 4 °C and supernatants frozen. ATP measurement was carried out with an Optocomp I luminometer (MGM instruments