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Effects of lactate dehydrogenase suppression and glycerol-3-phosphate dehydrogenase overexpression on cellular metabolism

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Abstract

In order to conduct a physiological functional study of lactate dehydrogenase (LDH) and glycerol-3-phosphate dehydrogenase (GPDH), we engineered a CHO *dhfr*$^-$ cell, by overexpressing either the anti-sense LDH-A RNA (anti-LDH cells) or GPDH (GP3 cells), or both (GP3/anti-LDH cells). LDH activity in the cell cytosol, and lactate content and pH*e* change in the growth media were found to decrease according to the order: cell lines GP3/anti-LDH > anti-LDH > GP3 > CHO. Intracellular ATP contents, representing the extent of respiration rate, also decreased, according to a rank order as follows: GP3 > CHO > GP3/anti-LDH > anti-LDH. We also attempted to identify and characterize any physiological changes occurring in the cells which harbored diverse metabolic pathways. First, anti-LDH cells with heightened respiration rates were found to display a higher degree of sensitivity to the prooxidant tert-butyl hydroperoxide (tBOOH), and the mitochondrial complex III inhibitor, antimycin A, than the GPDH-expressing cells (GP3 and GP3/anti-LDH), which have a lower respiration rate. Second, the anti-sense LDH-A RNA-expressing cells (anti-LDH and GP3/anti-LDH) evidenced a higher degree of resistance to apoptosis by cell-cell contact inhibition, and a faster doubling time (∼19 h compared with ∼26 h) than the CHO and GP3 cells. Additionally, cell growth in an extended culture under HCO_3^--free conditions to induce a steep acidification could be maintained with the anti-sense LDH-A RNA-expressing cells, but could not be maintained with the CHO and GP3 cells. Third, we observed that the most appropriate cell line for the optical production of a certain therapeutic protein (Tissue-Plasminogen Activator) was the GP3/anti-LDH cells. Collectively, our data indicate a variety of physiological roles for LDH and GPDH, including cellular acidosis, oxidoresistance, apoptosis by both acidosis and cell-cell contact inhibition, cell growth, and the generation of recombinant proteins. (Mol Cell Biochem **284:** 1–8, 2006)

Key words: glycolysis, respiration rate, oxidoresistance, acidosis, cell growth, apoptosis, recombinant protein production

Introduction

Metabolic complex networks require rigorous regulation, but must also remain somewhat flexible, as the external environment to which cells are exposed is not constant. These pathways are designed for the maintenance of energy, pH, and cell growth [1–3]. Metabolic abnormalities, then, tend to contribute to several ageing-related diseases, including diabetes, tumors, and certain neurodegenerative diseases (e.g. Parkinson's disease). Diabetes is generally characterized by a loss of insulin activity, culminating in profound changes in glucose metabolism and gene expression [4]. As compared

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to normal cells, tumor cells exhibit enhanced glucose uptake and glycolysis rates [5, 6]. Glycolysis is rendered more efficient in hypoxic tumors, inducing the formation of internal clusters of solid tumors. Parkinson's disease is a neurodegenerative disorder which is characterized by aberrant motor activity. Parkinson's disease results in profound impairments in glucose uptake, as well as serious mitochondrial dysfunction and oxidative stress, ultimately inducing neuronal cell death [7].

It has been reported that several ageing-associated diseases are related to changes in NAD^+ level and NAD^+/NADH ratios in the metabolism [8–10]. NAD^+/NADH ratios tend to fluctuate in response to changes in metabolism. Both cytosolic and mitochondrial NADH are oxidized by glycerol-3-phosphate dehydrogenase (GPDH) and lactate dehydrogenase (LDH) in the glycolytic pathway [10], as well as by NADH dehydrogenase in the mitochondrial chain reaction [11]. Oxidized NAD^+ performs important functions as a metabolic regulator in the regulation of transcription [8] and glycolytic enzymatic activities [12, 13], such as glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase, and also in energy metabolism, which is related to the extension of lifespan due to calorie restriction [14, 15].

Glycolysis is ubiquitous in all living organisms, and has been well-characterized in the previous several decades. As there is only a limited amount of NAD^+ in the cell, NADH requires reoxidation back into NAD^+ in order that glycolysis may continue. The rate of conversion of the NADH/NAD^+ ratio is regulated by the activities of LDH and GPDH during glycolysis, as mentioned above. LDH is a tetrametric enzyme which catalyzes the interconversion of pyruvate and lactate, and which occurs in five isoforms (LDH-A_4, A_3B_1, A_2B_2, A_1B_3, and B_4). LDH-A favors the conversion of pyruvate to lactate with NADH as a cofactor, whereas LDH-B favors the conversion of lactate to pyruvate [16]. The lactic acid which is generated by the NADH-induced reduction of pyruvate has a pKa of 3.86, which ensures that it dissociates entirely to the lactate anion and a proton (H^+) at physiological pH, and induces both extra- and intracellular acidification, which is commonly known as lactic acidosis [17]. We recently reported that modification of the glycolytic pathway via the regulation of GPDH and LDH activity affects oxidant-induced apoptosis, resulting from mitochondrial dysfunction [18]. However, the physiological significance of GPDH and LDH remains poorly understood.

In order to characterize the cellular roles of GPDH and LDH, we attempted to regulate LDH and GPDH activity, using ectopic expressions of the anti-sense LDH mRNA and/or GPDH in the cell. We assessed physiological changes occurring in the cells, including oxidoresistance, apoptosis by acidosis and cell-cell contact inhibition, cell growth, and the generation of recombinant proteins.

Materials and methods

Cell culture

A dihydroforate reductase (*dhfr*)-deficient Chinese Hamster Ovary (CHO *dhfr*$^-$) cells were grown in sodium bicarbonate-buffered F-12 medium (Gibco-BRL) supplemented with 10% FBS at 37 °C, in a humidified HCO_3^--supplied incubator. In order to determine the effects of pH alteration, the cells were exposed to 1 mM Hepes-buffered F-12 medium, and cultured in a humidified HCO_3^--free incubator, at a temperature of 37 °C.

Transfection and selection

Vectors harboring GPDH or anti-sense LDH-A cDNA were prepared, transfected into CHO *dhfr*$^-$ cells using lipofectAMINE (Gibco-BRL), and then selected as previously described [19, 20]. In this study, the CHO *dhfr*$^-$ cells which harbored GPDH and anti-sense LDH-A cDNA were designated as GP3 and anti-LDH cells, respectively. For the GP3/anti-LDH cell line, GPDH and anti-sense LDH-A cDNA vectors were cotransfected into CHO *dhfr*$^-$ cells, then selected with G418 (Gibco-BRL) and methotrexate (MTX, Sigma) in order to amplify the anti-sense LDH-A gene.

Analysis of RNA-RNA duplexes

The formation of stable RNA-RNA duplexes between the sense and anti-sense LDH-A RNA was assessed via reverse transcription-polymerase chain reaction (RT-PCR), as previously described [20].

Lactate dehydrogenase A (LDH-A) activity

LDH activity was measured as previously described [20], but with some modifications. In brief, exponential growing cells were resuspended in a buffer (200 mM Tris-HCl, pH 7.4), harvested by scraping, then lyzed by several passages of cells through a 26-gauge syringe. The cell lysates were then separated into soluble and insoluble fractions via centrifugation. The reaction mixture included 30 μg of proteins of soluble fractions, 200 mM Tris-HCl, pH 7.4, 6.6 mM NADH, and 30 mM of sodium pyruvate. LDH-A activity in the soluble fractions was assessed by measuring the decrease in absorbance at 340 nm, reflecting NADH oxidation, and this was expressed as nmol/min/mg of protein.

Measurement of pHe and lactate in growth media

Cells (2×10^5 cells/4 ml/60-mm dish) were adapted in sodium bicarbonate-buffered medium in a humidified

HCO_3^--supplied incubator for 12 h, and then allowed to grow in 1 mM Hepes-buffered F-12 medium, in a humidified HCO_3^--free incubator for the indicated times. The pH*e* in the growth media was directly measured with a pH meter (CORNING pH meter 445). For the lactate assay, the medium was extracted from the culture dish, boiled at 80 °C for 15 min, then centrifuged at 12,000*g* for 10 min. The final supernatant was kept at −70 °C until use. Lactate concentration was then determined by measuring absorbance at 540 nm with a colorimetric assay kit (Sigma).

Growth curves

2×10^5 cells were seeded onto 60-mm dishes and adapted for the appropriate times. At each time point, the cells were washed once in PBS, and trypsinized. Viable cells were assessed via the trypan blue exclusion method.

ATP contents

Exponential growing cells were washed with ice-cold PBS, resuspended in a buffer (50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 4 mM EDTA, 1 mM DTT), and lysed by the passage of cells through a 26-gauge syringe. After the cell lysates had been centrifuged at 10,000*g* for 10 min, the resulting supernatant was removed from the proteins via the addition of trichloroacetic acid to a final concentration of 5%. The supernatant was then neutralized to pH 7.75, and assayed for ATP with a bioluminescence-based kit (Promega), using a luminometer (Turner Designs). Data were normalized according to protein concentrations.

DNA fragmentation test

Cells were grown on 60-mm culture dishes to confluence, transferred to fresh medium, then incubated for an additional 3 days. For DNA fragmentation, the cell populations (both adherent and floating cells) were spun down at 2,000*g* for 5 min and lysed in 700 μl of a buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 0.5% deoxycholate, 12 mM β-glycerophosphate, 10 mM sodium fluoride, 5 mM EGTA, 1 mM Na_3VO_3, 3 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin) for 30 min at room temperature. This solution was then extracted with phenol/chloroform, and precipitated with sodium acetate (pH 5.2) and ethanol. The final pellets were air-dried and dissolved in 200 μl Tris-HCl (pH 8.0), containing 50 μg/ml RNase A. 20 microliters of the fragmented DNA was separated on 1.2% agarose gel, stained with ethidium bromide, and visualized under a UV illuminator.

Tissue plasminogen activator (t-PA) assay

The t-PA cDNA was amplified by PCR, using template, pETPFR (ATCC 40403) containing tPA cDNA, and specific primers (sense primer; 5′-GAC GCT GTG AAG CAA TCA TG-3′, anti-sense primer; 5′-GAG GAG TCG GGT GTT CCT GGT CA-3′) and cloned into the pCR™2.1 vector (Invitrogen). The DNA fragment released by digestion of the pCR™2.1 vector containing tPA cDNA with *Spe*I and *Xho*I was subcloned into the mammalian expression vector pZeoSV(+) (Invitrogen) that had been digested with the same restriction enzymes. The resulting plasmid was transfected into the appropriate cells. The cells were selected with zeocin (Invitrogen).

To produce tPA, cells were cultured in MEM-α (Gibco-BRL) without ribonucleosides. In order to obtain the t-PA secreted from the cell, the medium was harvested at the indicated times, and maintained at −70 °C until use. tPA activity was measured as described [21, 22], with some modifications. Medium diluted in 100 mM Tris-HCl, at a pH of 7.5, containing 0.02% Triton X-100, was then incubated at 37 °C for 24 h in 200 μl of 100 mM Tris-HCl, at a pH of 7.5, with 0.02% Triton X-100, 10 μl of 0.2 U/ml plasminogen, 1 μl of 1 mg/ml fibrinogen, and 150 μM of synthetic substrate (D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride, Fluka) for plasmin. During this reaction, the conversion of plasminogen to plasmin by tPA in the presence of the synthetic substrate induces the release of the chromogenic product, *p*-nitroanilide. The amount of *p*-nitroanilide was measured at 405 nm with a microplate reader (BIORAD).

Results and discussion

Construction of cell lines with different glycolytic metabolism

It is difficult to amplify artificial gene copy number in mammals. One of defensible mechanisms to acquire the resistance to drug in mammalian cells is to increase copy number for specific genes, including *dhfr*. To amplify the targeted gene by treating MTX, which is able to block DHFR activity, we here designed a chimeric gene, *dhfr* gene linked to antisense *ldh* gene.

In order to develop a CHO *dhfr*$^-$ cell which was capable of modulating the glycolytic pathway, we engineered a CHO *dhfr*$^-$ cell by the overexpression of either anti-sense LDH-A RNA, GPDH, or both. Thus, we obtained four cell lines with different glycolytic pathway efflux: the wild type cells (CHO *dhfr*$^-$), anti-sense LDH-A RNA expressing cells (anti-LDH) [20], GPDH expressing cells (GP3) [19], and anti-sense LDH-A RNA and GPDH expressing cells (GP3/anti-LDH).

In order to access different metabolic pathways in the four cell lines, we initially measured the expression and enzymatic activities with regard to the transfected genes. The formation of stable duplexes of sense and anti-sense LDH-A RNA was detected by RT-PCR using the primer located within the duplex, as previously reported [20]. As expected, only the anti-sense LDH-A RNA-expressing cells (anti-LDH and GP3/anti-LDH) achieved complementarity between sense and anti-sense RNA (Fig. 1A, upper panel). These stable duplexes demonstrate that anti-sense LDH-A RNA can be reduced by LDH gene expression. Consistent with this finding, the LDH activities of the anti-LDH, GP3, and GP3/anti-LDH cells relative to that of the CHO cells indicated reductions of 29%, 10%, and 34%, respectively (Fig. 1A, lower panel). Also, we previously demonstrated that GPDH activity in GP3 cells increased to 12 times that of normal CHO cells [19]. As changes in cellular LDH activity tend to modulate lactate production and extra- and intracellular pH (pH*e* and pH*i*), we assessed the lactate concentrations and pH*e* changes in the medium, and pH*i* in the cell cytosol, under HCO_3^--free conditions. The lactate concentration and pH*e* levels were determined to have decreased according to the order: GP3/anti-LDH > anti-LDH > GP3 > CHO (Fig. 1B and C). Moreover, the pH*i* levels decreased from 7.305 ± 0.051 to 6.582 ± 0.002 in the CHO cells, 7.303 ± 0.068 to 7.21 ± 0.057 in the anti-LDH cells, and 7.42 ± 0.060 to 7.175 ± 0.007 in the GP3/anti-LDH cells. These phenomena were tightly dependent on LDH activity.

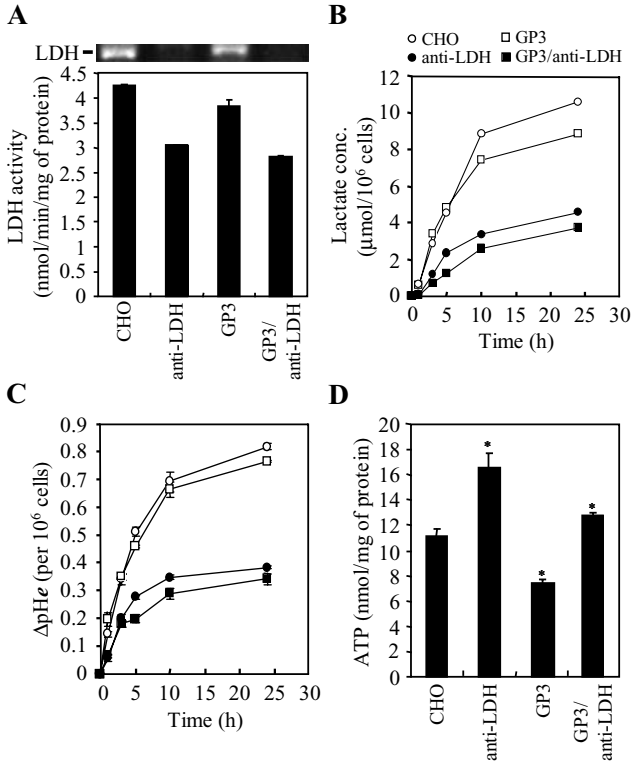

Fig. 1. Characterization of cells harboring altered metabolic pathways due to the overexpression of either anti-sense LDH-A RNA, GPDH, or both. A. Stable RNA-RNA duplexes between sense and anti-sense LDH-A RNA (upper panel) and LDH activity (lower panel). Anti-LDH-A and/or GPDH gene were/was transfected into CHO cells, selected with G418, and/or amplified by MTX. The disruption of wild type LDH gene expression by the formation of stable duplexes between sense and anti-sense LDH-A RNA was detected via RT-PCR. LDH activity was determined spectrometrically. B. Lactate contents in growth media. After the cells were transferred to a HCO_3^--free incubator, lactate concentration was assayed in medium at the indicated times. C. pH*e* changes in growth media under HCO_3^--free conditions. The pH*e* in culture medium at the indicated times was measured directly with a pH meter. D. Intracellular ATP contents. ATP concentrations were determined via the luciferin-luciferase method. Data are mean ± SD of triplicates from a representative experiment. $^*P < 0.01$ vs CHO cell (Student's *t* test).

Respiration rate also varied with modulation of the metabolic pathway. We evaluated ATP contents in order to characterize this phenomenon. The intracellular ATP contents of the CHO, anti-LDH, GP3, and GP3/ anti-LDH cells were: 11.101 ± 0.677, 16.525 ± 1.164, 7.42 ± 0.296, and 12.745 ± 0.223 nmol/mg of protein (Fig. 1D). ATP contents increased as the result of anti-sense LDH-A RNA expression, but decreased due to GPDH expression. From these results, we concluded that anti-sense LDH-A RNA and/or GPDH expression are/is capable of modulating the metabolic pathway. Since metabolic pathway has various patterns according to differences in species or cell types originated from various tissues, such as liver, brain, and muscle, it remains the question, at the present study, whether metabolic alteration by LDH and GPDH is able to apply to other cell types.

Effects of the alteration of the metabolic pathway on oxidoresistance

In a previous study, we had reported that ectopic GPDH expression increased oxidoresistance against hydrogen peroxide (H_2O_2) [19]. Therefore, we attempted to evaluate the effects of metabolic pathway bypass on oxidoresistance to an exogenous oxidative stimulus. The cGPDH-transfected cells were determined to be more resistant to tert-butyl hydroperoxide (tBOOH) than were the anti-sense LDH-A-transfected cells (Fig. 2A). Moreover, it was clear that electrons which leaked from complex III during the mitochondrial respiratory chain reacted with oxygen, forming the superoxide anion ($O_2^{\bullet -}$) and H_2O_2 via Mn-superoxide dismutase, and that these ROS thus induced cell death. This means that increased respiratory rates confers higher sensitivity to ROS [23–25]. As shown in Figure 1D, ATP contents were determined to decrease, according to the rank order: GP3 > CHO > GP3/anti-LDH > anti-LDH, thereby suggesting that the four cell lines in this study exhibit different cel-

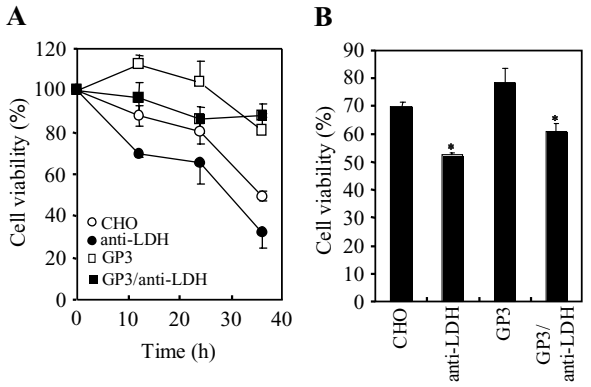

Fig. 2. The extent of cell oxidoresistance to oxidative stress. A. Sensitivity to *t*-BOOH. B. Sensitivity to antimycin A. Cells (2×10^5 cells/60-mm dish) were adapted for 12 h, then treated with 150 μM tBOOH for the indicated times, or 5 μM antimycin A for 24 h, after which cell viability was determined via the trypan blue exclusion method. $^*P < 0.05$ vs CHO cell.

lular respiration rates. We therefore attempted to determine whether different cellular respiration rates can influence the survival of these cells when they are exposed to antimycin A, a mitochondrial complex III inhibitor. Sensitivity to antimycin A was found to decrease, according to the rank order: GP3 > CHO > GP3/anti-LDH > anti-LDH (Fig. 2B). This supports the notion that enhanced respiration rates result in increases in sensitivity to oxidative stress.

Effects of anti-sense LDH-A RNA and/or GPDH expression under HCO_3^--supplied or HCO_3^--free conditions on cell growth and cell-cell contact apoptosis

Cell growth is normally inhibited by acidosis, oxidative stress, contact inhibition, nutrient deprivation, and waste products, including lactate and ammonia [25–26]. Also, the intracellular ATP contents are known to affect cell growth. As the alteration of the metabolic pathway effects diverse physiologic patterns, we attempted to determine whether this phenomenon could also affect cell growth under HCO_3^--supplied or HCO_3^--free conditions. Under HCO_3^--supplied conditions, the growth of both CHO and GP3 cells with low ATP levels was found to be inferior to that of both anti-LDH and GP3/anti-LDH cells with enhanced ATP, via anti-sense LDH-A RNA expression (Fig. 3A). Consistent with this finding, anti-LDH and GP3/anti-LDH cells exhibited a faster

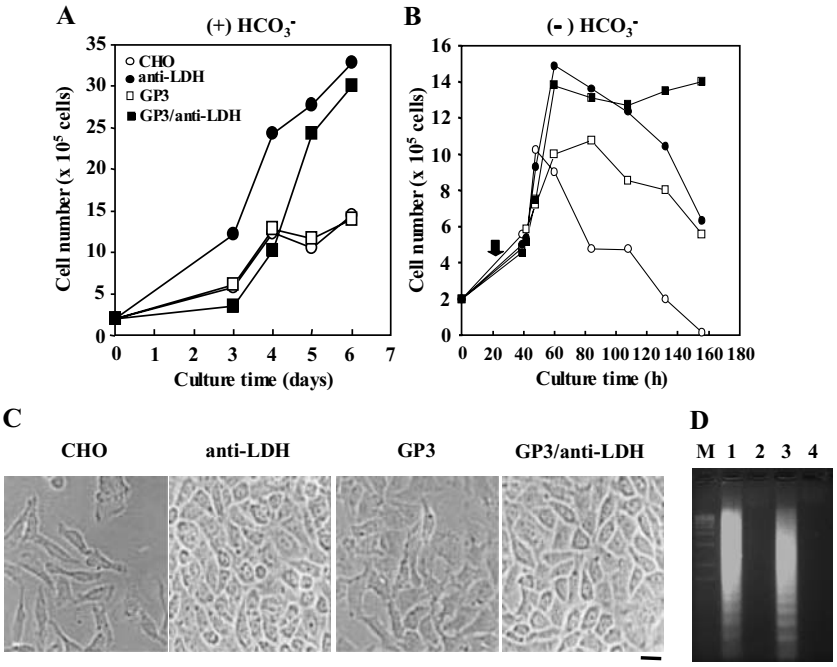

Fig. 3. Cell growth in HCO_3^--supplied or -free conditions, and apoptotic events due to cell-cell contact inhibition. A. Cell growth under HCO_3^--supplied conditions. B. Cell growth under HCO_3^--free conditions. After the cells were transferred to an HCO_3^--free incubator (arrow), the number of viable cells was counted. C. Morphological changes due to cell-cell contact inhibition. Cells were grown to confluence, incubated for an additional 3 days, and photographed under a light microscope. Bar; 25 μm. D. DNA fragmentation test. Cells were incubated as in C, and harvested by typsinization and centrifugation. DNA prepared from the total cell populations (floating and adherent cells) was fractionated on 1.2% agarose gel, stained with ethidium bromide, then visualized under a UV illuminator. Lane 1, CHO; Lane 2, anti-LDH; Lane 3, GP3; Lane 4, GP3/anti-LDH. Data are a representative result from three independent experiments.

doubling time, ∼19 h compared with ∼26 h, as compared to CHO and GP3 cells. Additionally, in order to assess the pattern of cell growth by acidosis, we subjected the cells to an HCO_3^--free incubator. Cell growth of the anti-sense LDH-A RNA expressing cells was maintained at higher levels than was the growth of the CHO and GP3 cells under HCO_3^--free conditions (Fig. 3B). We also found that the CHO and GP3 cells which were exposed to HCO_3^--free conditions exhibited increased rates of apoptotic death, by measuring the subdiploid DNA content (sub-G0/G1) of cells after propidium iodide staining and DNA fragmentation, relative to the anti-sense LDH-A RNA expressing cells (data not shown).

We then explored the effects of cell-cell contact inhibition on cells with diverse metabolic pathways. The apoptotic nature of cell death was morphologically confirmed via phase-contrast microscopy, showing that the CHO and GP3 cells grown to confluence exhibited membrane blebbing and the loss of their attachment to the dish, as compared with the anti-sense LDH-A RNA expressing cells (Fig. 3C). Figure 3D indicates that DNA fragmentation could also be detected in the CHO and GP3 cells 3 days after exposure to 100% confluence, but this was not true of the anti-sense LDH-A RNA-expressing cells. This indicates that cell growth and apoptosis as the result of unfavorable environments including acidosis and cell-cell contact inhibition depend on distinct cellular metabolisms.

Tissue-plasminogen activator (t-PA) production in metabolic-altered cells

The maintenance of intracellular protein, which is one of physiological characteristics in the cell, could be regulated by the extents of transcriptional and translational activity and the ratio between protein synthesis and breakdown. We obtained diverse cell lines which exhibited different patterns of cellular acidosis, ATP levels, oxidoresistance, and growth, which we determined to be the result of differing efficiency with regard to the generation of therapeutic recombinant proteins. Continuous mammalian cell lines constitute important hosts for the production of biological pharmaceuticals, because many of these therapeutic proteins require posttranslational modification for the performance of all of their biological functions, including glycosylation, phosphorylation, disulfide bond formation, folding, and endoproteolysis [31–34]. CHO cells have been widely used as a prominent mammalian expression host for the therapeutic protein production. Mammalian cell growth has proven to be sensitive to environmental changes, including alterations in nutrients, pH, temperature, oxygen, osmolality, and the waste compounds produced by the cell itself during normal growth [35]. In this

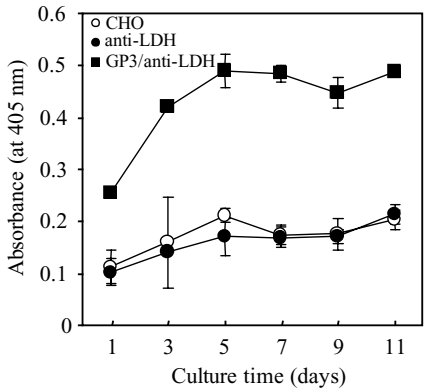

Fig. 4. tPA production in CHO, anti-LDH, and GP3/anti-LDH cells. After cells were cultured in growth media, the medium was harvested at 2-day intervals for 11 days, and kept at −70 °C until use. *In vitro* activity was assayed by monitoring the absorbance at 405 nm, as described in the Materials and Methods section. Data are expressed as the means ± SD of triplicate experiments.

study, the GP3/anti-LDH cells, which proved able to maintain the relative high energy and oxidoresistance to culture for long periods under steep acidification conditions, appear to be the most appropriate model system for the production of recombinant proteins (Fig. 4).

In summary (Fig. 5), the findings presented in this study indicate that (1) anti-sense LDH-A RNA-expressing cells represent decreases in lactic acidosis and cell death by cell-cell contact inhibition, and increases in ATP production, growth rates, and sensitivity to oxidative stress due to heightened respiration rates. (2) GPDH-expressing cells exhibit increases in oxidoresistance, and decreases in ATP production. (3) Cells with an adequate combination of two genes as described in our (1) and (2) results appear to be the most appropriate for the high-level production of recombinant proteins. Glycolytic enzymatic activity is implicated in various diseases, including diabetes and multiple sclerosis. It has been reported that the mitochondrial GPDH plays an important role in the regulation of insulin secretion and has been postulated as a candidate responsible for the pathogenesis of non-insulin-dependent diabetes mellitus [36]. GPDH seems to compensate for the loss of LDH-A subunit, resulted in recurrent rhabdomyolysis caused by decreased glycolysis [37]. We here have put forth understanding of metabolism by regulating glycolytic enzymatic activity, as a potential therapeutic target for metabolic diseases.

In this study, we determined that changes in the glycolytic pathway induced by alterations in the expression of GPDH and LDH-A RNA can served to regulate the extent of cellular acidosis, respiration, cell growth, oxidoresistance, and the generation of recombinant proteins.

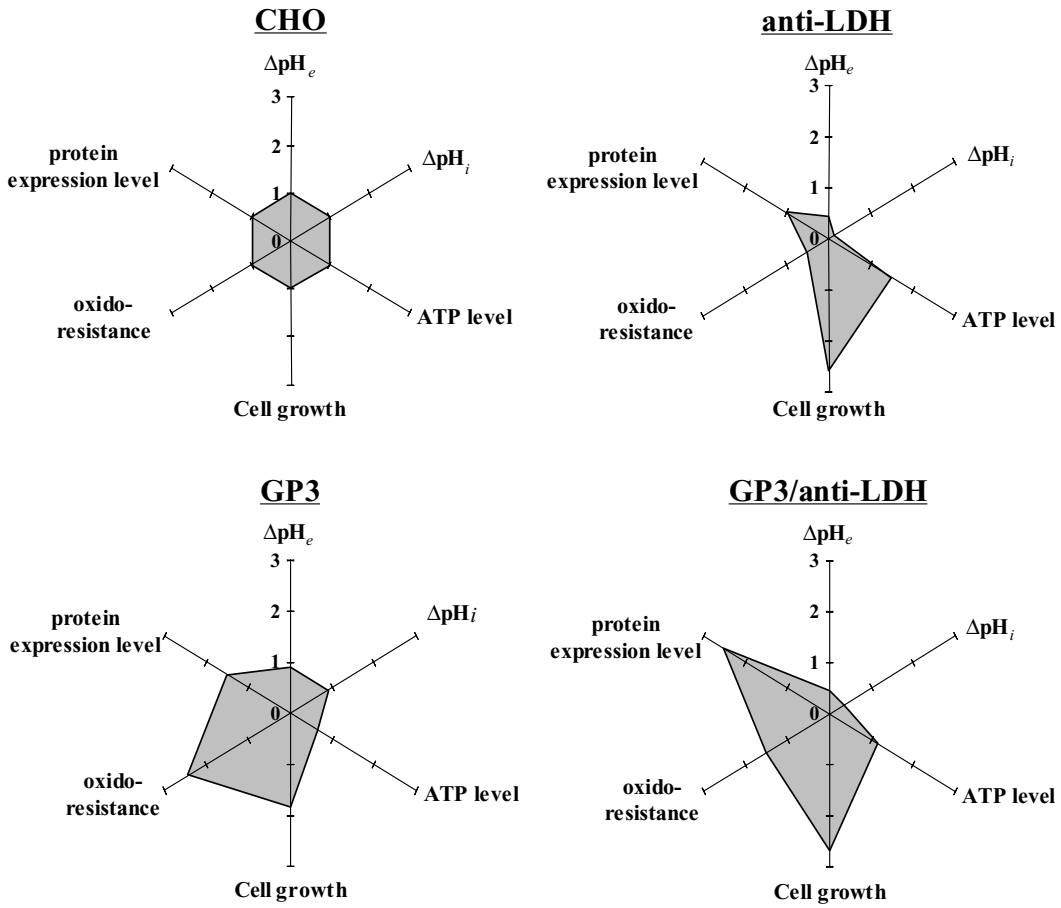

Fig. 5. Summarized diagrams of physiological changes due to the regulation of LDH and GPDH gene expression.

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