Dependence of Energy Metabolism on the Density of Cells in Culture

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ABSTRACT. Metabolism in cultured cells strongly depends on cell density, thus cell density may be an important factor in biotechnological maintenance of cell cultures. Therefore the energy metabolism of three related cell types with different proliferation activity has been characterised: density controlled primary or secondary fibroblasts [pmf], immortalized, but still density controlled 3T3 cells, and SV40-transformed 3T3 lacking growth control. The investigations revealed the decrease of oxygen consumption, net lactate production, ATP-content, NAD-content and NAD-redox potential, and F-actin content with increasing culture density in pmfs, less distinct in 3T3 cells and in SV40-3T3 cells. The main decrease of these factors is related to cell-cell contacts rather than to proliferation which ceases at least two division cycles after most cells contacted a neighbouring cell. SV40-3T3 cells also at preconfluent densities exhibit relatively low metabolic activity as revealed by the above mentioned factors. Supply with metabolites for catalytic processes seems to be the rate limiting factor as deduced from a decreasing NADH/total NAD ratio.

SV40-3T3 cells lack a contact mediated reduction of energy metabolism which is in accord with the missing contact inhibition of motility and proliferation in SV40-3T3 cells. Because of a possible association of glycolytic enzymes with actin, F-actin content has been determined. No correlation, however, was revealed by the F-actin/lactate ratio. This may be due to the fact that about 50% of the lactate released into the culture medium originated from glutaminolysis rather than glycolysis. Only in SV40-3T3 cells were respiration and lactate production insensitive to glutamine deprivation and in these cells both these parameters did not change significantly, therefore they did not allow testing as to whether F-actin content and glycolytic activity are correlated.

Fractionated release of adeninnucleotides revealed that energy charge in the cytosolic fraction is the only factor which strictly correlates with the cessation of proliferation. Thus energy charge of the cytosol seems to be the only factor of these studied here, which can be used as an indicator of a culture’s commitment for further proliferation. To compare energy metabolic parameters among different cell types one always has to take into account the density dependence of these factors.

The problem is stated

When reaching confluency many cells in culture are subjected to inhibition of proliferation, i.e., density dependence of growth (13, 15), which seems to be closely related to adhesion mediated control of proliferation and to another phenomenon, the contact inhibition of motility (2). Although this behaviour is typical for primary and secondary cultures (just after explantation of the tissues, and after first passages of these cultures), it also is common in immortalised cell lines. The proliferation of most tumour cells, however, is not affected by increasing cell density, i.e., these cells continue dividing also when all the available space is covered by other cells.

Since the findings of Warburg (1926) that tumour cells have higher rates of “aerobic glycolysis” terminating in increased lactate production in the presence of oxygen than normal cells, the question of the relationship between aerobic glycolysis, deficient respiration and uncontrolled growth has been discussed. Decreased pyruvate transport from the cytoplasm into the mitochondria (20, 32) might be one of the reasons for this behaviour. In addition, cell cycle dependent glycolytic activity, respiration and ATP turnover has been described for Ehrlich ascites tumour cells (37). Whether these characteristics of the energy metabolism constitute a mechanism for growth control or whether they are epiphenomena of another, different control system, remains to be solved. In addition, the relatively high glucose content (in the range of vertebrate blood glucose levels) of culture media (about 5.5 mM) has to be considered which increases lactate production compared to

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Recent findings indicate that glycolysis is not a process taking place in the cytosol exclusively. Rather it is being influenced by the association of glycolytic enzymes with elements of the cytoskeleton thus forming large multienzyme complexes allowing channeling of metabolites from one enzyme to the other and also to ATP-consuming structures and processes (5, 14, 28, 36). The more diffuse actin fibrillar system in most tumour cells and the changes of F-actin organisation with cell density and during the cell cycle may function as modulators of glycolysis thus shifting the relative significance of glycolysis and respiration in ATP-production (8). Therefore the question of the involvement of the energy metabolism in proliferation control in various cell types can be analysed in a multiparameter system, including cytoskeletal organisation.

**The test system**

The main parameters for the characterisation of cellular energy metabolism are oxygen consumption, lactate production, NADH-redox potential and adeninnucleotide content. We investigated these parameters in fibroblastic cells in culture. Primary mouse fibroblasts (pmf), the immortalized mouse cell line 3T3 and a 3T3 line transformed with SV40 virus represent a series with increasing loss of proliferation control by cell density and adhesion, and thus seem to be an ideal model system to follow proliferation control related changes in metabolism.

Energy metabolism of cells has been investigated at three levels of integration. The first is represented by mitochondria isolated from tissues under various conditions, the second is permeabilization of cells and tissues, and the third is respirometry of living cell cultures.

Isolated mitochondria are easy to investigate, and all metabolites and inhibitors can easily be applied. However, the structure of isolated mitochondria in most cases does not resemble their structure in the living cell (4). Interaction of insoluble enzyme systems in their normal structural environment can be analysed in permeabilized cells. The levels of substrates and effectors can be varied deliberately as is the case with isolated mitochondria (19). The method is more difficult to perform; however, the structural performance of the system is superior to isolated mitochondria. Whole cell measurements with a perfusion system allows one to manipulate all the external parameters, the cells are alive and are allowed to maintain the metabolite concentrations typical for each situation. However, the plasma membrane acts as a barrier for most substrates and energy rich phosphates cannot be controlled directly. Therefore this method yields data which represent the whole energy metabolism rather than the behaviour of mitochondria. In addition, our perfusion system allows cells to remain attached to a solid substratum. This maintains their normal cytoskeleton. Because all light microscope methods can be applied, NAD-content by microfluorometry and morphological studies can be performed synchronously.

**MATERIALS AND METHODS**

**Cells**

Swiss 3T3 cells, an immortalized mouse fibroblast line and 3T3 cells transfected with SV40 virus (which have been kindly provided by Prof. Dr. Adam, Konstanz), were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% newborn calf serum (NCS). Primary/secondary mouse fibroblasts were derived from the skin of NMRI mouse embryos prepared according to Freshney (18) and kept in DMEM with 10% NCS. Only passages 1–10 after explantation have been used. All media were obtained from Gibco (Glasgow, UK) and were supplemented with antibiotics (penicillin, streptomycin, neomycin).

**Methods**

Determination of oxygen consumption and simultaneously of the aerobic lactic acid production provide information on all energy-delivering metabolic pathways. Oxygen consumption was measured with a microperfusion respirometer (35). Subsequently, lactic acid production was determined enzymatically from the perfusate using a test kit from Boehringer (Mannheim, F.R.G.).

ATP-content: Total ATP content was measured either after extraction of cell cultures with hot Triton X 100 (1%) using the luciferin-luciferase method or the adeninnucleotides were collected by fractionated extraction followed by HPLC-analysis. The elution buffer (60 mM PIPES, 25 mM HEPES, 2 mM EDTA, 1 mM MgCl2, 120 mM KCl, 0.5 mg/ml dispase; pH 7.2–7.3) was supplemented with the appropriate detergent. Dispase was used to destroy ATPases. Fraction I ("cytosolic", freely soluble): Cultures were washed twice with 0.5 ml ice cold elution buffer, then immediately 0.5 ml ice-cold NRS-permeabilisation medium (6 parts elution buffer plus 1 part NRS = nucleotide releasing reagent, LUMAC®, The Netherlands) was poured on the cultures and evenly distributed by gently moving the culture dishes on ice. After 2.5 min the elution medium was sucked off and stored at −20°C until further analysis. Fraction II ("loosely bound", associated): Following treatment with NRS, cultures were exposed to elution buffer with 0.1% Triton X 100 for a further 2.5 min at room temperature. Again the sample was stored deep frozen. Fraction III ("bound"): The same detergent buffer as for fraction II was used at 95°C for a further 2.5 min.

The samples of elution buffer were first centrifuged at 13,000 rpm for 15 min (Biofuge, Eppendorf, Hamburg), then the supernatant was filtered through Millipore filters 0.45 μm. Twenty μl of the filtrate were fractionated on a HPLC-system with reversed phase column (Ultropak ODS-TSK 120, 5 μm,
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Pharmacia LKB, Lund, Sweden). The flow rate of the buffer (0.1 M phosphate buffer, pH 6.0) was 1 ml/min. Nucleotides were quantified at 253 nm using UVicord S (Pharmacia LKB). For each set of experiments the system was calibrated with a nucleotide mixture of known composition.

F-Actin content of cells was quantified by microfluorometry of cultures stained with TRITC-phalloidin (16) according to a standardized schedule which gave reproducible results under constant culture conditions (11). NAD also was quantified by scanning microfluorometry after reduction in the presence of substrate and 4 mM KCN (21).

RESULTS

Morphology and F-actin pattern

In sparse cultures with single cells, pmfs exhibit a prominent stress fibre pattern (Fig. 1a), in 3T3 cells stress fibres are a bit less developed (Fig. 1d) and SV40 transformed 3T3 cells are almost devoid of stress fibres (Fig. 1g) and single cells occupy a smaller projection area than the other two types, thus a comparable degree of slide coverage is reached at relatively higher densities. With increasing culture density some overlap occurs among the cells in all types which is most prominent in very dense SV40-3T3 cells. Stress fibres become reduced, so that after reaching confluency they disappear in the immortalized lines (Fig. 1f, li). Also the F-actin content (Fig. 2) decreases considerably in pmfs and slightly in 3T3 cells with increasing cell densities. In preconfluent SV40 3T3 cells F-actin content is low and stays on a level corresponding that of the other two cell types after having reached confluency.

Cell density dependence of respiration and aerobic lactic production

Oxygen consumption as well as aerobic lactate production (measured simultaneously) are high in sparse cultures of pmfs (up to 6.5 nmol/h 1,000 cells O2 consumption and 90 nmol/h 1,000 cells lactate production) and decrease to very low levels on reaching confluency (mean oxygen consumption: 0.52±0.20 nmol/h 1,000 cells, lactate production: 3.5±1.84 nmol/h 1,000 cells) (Figs. 3 and 4).

This behaviour is much less expressed in 3T3 and SV40-3T3 cells. Sparse cultures of 3T3-cells consume only up to 4 times as much oxygen than confluent cultures (1.5 versus 0.41±0.13 nmol/h 1,000 cells), in SV40 transformed 3T3 cells at densities <195 cells/mm2 (the cultures reach confluency between 1,000 and 1,000 cells/mm2) no density dependence of respiration was found (mean oxygen consumption: 0.21±0.07 nmol/h 1,000 cells). The simultaneous aerobic lactic acid production resembles the course of the oxygen consumption. In confluent cultures net lactate production in 3T3 cells was 2.18±0.92 nmol/h 1,000 cells and 1.52±0.60 nmol/h 1,000 cells in SV40-3T3 cells.

Lactate can be produced along different metabolic pathways. The main pathways are glycolysis and glutaminolysis (26, 27). Replacing glucose in the medium with desoxyglucose (in the presence of glutamine) or perfusing with glutamine-free medium in the presence of glucose, shows the degree of lactate production originating from either pathway. In pmfs and 3T3-cells about 50% of the net lactate production depends on glutamine consumption, while SV40-3T3 cells do not respond to the lack of glutamine either by lactate production or by respiration (values not shown).

Regulatory principles

What is the regulatory principle for these density related changes in respiration and lactate production in pmfs and — to a minor degree — also in 3T3 cells? In general terms the limitation for the control of energy metabolism can be exerted by substrate supply or by the requirements for energy-rich phosphates. The redox state of NAD is a good measure to determine whether substrate supply might be rate limiting. All catabolic pathways use NAD as an electron acceptor. The NAD/ NADH redoxpotential is in equilibrium with other redox potentials such as that of glutathion and NADP. Therefore the percentage of reduced NAD (from total NAD) provides a measure whether a cell is well supplied with redox-equivalents from catabolic reactions or not. Because of almost identical fluorescence spectra NADH cannot be distinguished from NADPH by fluorometry.

ATP-level and energy charge (1), on the other hand, indicate whether energy requirements of endergonic processes (such as Na/K-ATPase, protein synthesis, actomyosin-ATPase and others) can sufficiently be supplied by ATP derived from oxidative phosphorylation or substrate-linked phosphorylation.

The total amount of NAD per cell also decreases with increasing density in the three cell types. However, the high values are reached only in cultures with almost all single cells (i.e., 32 cells/mm2) and a constant (density independent) level is reached before the cultures become confluent (data not shown). The percentage of reduced NAD seems to be the more significant parameter because it is a measure of turnover. This parameter again gives the same picture. Only in very scarce cultures 90% or more of the total NAD are in the reduced form, at higher densities in pmfs about 60%, in 3T3 cells and SV40-3T3 cells about equal amounts of oxidised and of reduced NAD are found (Fig. 5). This clearly indicates that once the maximum turnover rate for NAD-linked redox processes is reached, no more redox equivalents can be provided by catabolic processes in dense cultures. Thus catabolic reactions represent the rate limiting processes.

The physiological significance of overall adeninnucle-
F-actin pattern at different densities of mouse primary fibroblasts (pmf; a, b, c), 3T3 cells (d, e, f) and SV40-transformed 3T3-cells (g, h, i); a, d, g: almost single cells, b, e, h: almost confluent cultures, c, f, i: densely grown cultures. Stress fibers become reduced with increasing cell density and are almost missing in SV40-3T3 cells. Bar: 50 μm in a–c; 30 μm in d–i.

otide content of cells — which shows about the same density relationship as does lactate production — is not very great because energy metabolism is supposed to be controlled by either the ATP/ADP ratio, the phosphorylation potential or the energy charge. ATP/ADP ratio does not include the potential of ADP to act as a source for ATP via myokinase activity. The phosphorylation potential includes all three adeninnucleotides and the phosphate concentration. The latter is not exactly known for living cytoplasm. Therefore we use the "ener-
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Fig. 2. Dependence of F-actin content on cell density. F-actin has been determined by quantifying fluorometrically bound TRITC-phalloidin (11).

Fig. 3. Dependence of oxygen consumption on cell density in different cultures as measured in a perfusion system.

Fig. 4. Dependence of net lactate production on cell density in different cultures. In the case of negative values, the cells consume more lactate from the culture medium than is being produced by their metabolism. At extremely low cell densities high lactate production is obvious in pmfs.
Fig. 5. Changes of NADH/NAD redox state with increasing cell density. In confluent cultures the oxidized and the reduced stage are approximately the same size. In cultures containing preferentially single cells, pmf and 3T3 cells contain about 90% reduced NAD.

Fig. 6. Energy charge in the freely soluble cytosolic fraction ("free": open signs) and in the associated fraction ("associated": solid signs) and their dependence on cell density in 3T3 cells and in SV40-3T3 cells. For each culture dish three independent determinations have been made. They did not vary by more than 3% from the mean value. The mean values of each of these determinations have been plotted. Because of the variance of cell density in the different cultures no statistics are provided. However, those density values which are close together show very small variation in energy charge derived from different cultures. The extremely high density values are difficult to obtain because cells have the tendency to lose adhesion.

Energy charge (1) as an index (dimension-less) for the energy status of a cell. Energy charge includes all three adenine phosphates to characterize the situation for the energy supply in cells (Fig. 6). It is defined as:

$$\text{energy charge} = \frac{[\text{ATP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

In addition, nucleoside phosphates can be supposed to be compartmentalized. The following compartments have to be considered: A freely soluble cytosolic fraction, an inner mitochondrial fraction, a fraction associated with cytoplasmic structures, the cytoskeleton in particular and finally, a fraction of nucleotides which are intimately embedded in macromolecules. An example of the latter group is the ADP bound to actin or the GDP and GTP bound to tubulin.

Only those molecules which can freely interact with each other, can physiologically participate in the energy charge (or any other nucleotidphosphate ratio). With fractionated release of nucleotides (ANPs=adeninnucleoside phosphates) we tried to tackle this problem. The first fraction represents the easily soluble cytosolic fraction, the second one contains the mitochondrial ANP-content and all those ANPs which are loosely associated with the wealth of large and charged surfaces of
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macromolecules. This fraction may not be easily accessible to metabolic reactions rather than being involved in activities of macromolecular complexes to which they are attached. The existence of such a fraction of ATP and ADP may be related to the association of glycolytic enzymes with F-actin (38). Furthermore, experiments following the distribution of ADP injected into well spread cells provided some evidence for restricted mobility in the cytoplasm (9).

In cultures of varying cell density the energy charge showed considerable differences (Fig. 6): In 3T3 cells only at very thin densities the energy charge in the cytosolic fraction exceeded that of the associated fraction, and at maximum cell densities the cytosolic energy charge decreased significantly, whilst that in the associated fraction remained constant or slightly higher. In SV40-3T3 cells cytosolic energy charge slightly exceeded that of the associated fraction, but both were high. They decrease only at very high cell densities when cultures degenerate or finally cells are released into the culture medium. Although no statistics can be made because of variations in exact culture density, the coincidence of the values achieved for similar densities is a clear indication that in those cases where energy charge differences have been observed, these differences are real. Thus, energy charge in the cytosolic fraction is the only factor which seems to correlate with the cessation of proliferation.

DISCUSSION

Characterisation of the energy metabolism of three related cell types with different proliferation activity (density controlled primary or secondary fibroblasts [pmf], immortalized, but still density controlled 3T3 cells, SV40-transformed 3T3 lacking growth control) revealed the decrease of oxygen consumption, net lactate production, ATP-content, NAD-content and NAD-redox potential, and f-actin content with increasing culture density in pmfs, less distinct in 3T3 cells and in SV40-3T3 cells. Although the courses of the relationships follow the same exponential decay \( y = A \cdot x^b \) (\( x = \)culture density, \( A \) and \( b \) are reaction and cell type specific correlation factors) it is difficult to attribute to these factors a direct cause-effect relationship in growth control. Most of the decrease happened at densities long before cessation of proliferation. However, the decay of most of these parameters was parallel to the degree of confluency which is reached at densities of about 200 cells/mm\(^2\) in pmfs and around 500 cells/mm\(^2\) in 3T3 cells, SV40-transformed 3T3 cells become fully confluent around 1,000 cells/mm\(^2\). However, in this cell type the relation of the above mentioned parameters to cell density is almost missing. The experiments show that it is the cell contact which evokes the reduction of the parameters important in energy metabolism. The switches reducing proliferation may not act immediately because also in other cell types a delay of about two replication rounds was observed between reaching confluency and cessation of growth (6).

Both substrate availability and ATP requirements seem to be down regulated, and relative NADH concentration is high only in cultures consisting mostly of single cells. As soon as cells come into contact with each other, the total amount and the redox potential drop, indicating an increase in turnover of single molecules at diminished total NAD concentrations. No differences are found among the cytosolic and mitochondrial NAD-fractons, consistent with a synchronous decrease of oxygen consumption and net lactate production. Such parallel changes in glucose utilisation and lactate production may be typical for the condition of high glucose concentrations (>1 mM) in the culture medium. Substrate (i.e. glucose) uptake has not been determined. However, previous studies using XTH-2 cells revealed contact dependent decrease of Rb\(^+\) influx effected by the Na/K-ATPase (17), pointing to a decreased uptake of small organic molecules via symport with sodium ions. This reduction in substrate supply seems to be balanced by diminished ATP requirements as is shown by almost constant energy charge just before further proliferation is inhibited. The reduction of two important energy consuming processes may be involved in the reduction of ATP requirements, cell motility (2, 22, 23, 40) including the turnover of cytoskeletal elements and the Na/K-ATPase (17, for review see 7), both of which are reduced by cell-cell contacts. Kajstura and Korhoda (1982) postulate depletion of basic metabolites for macromolecular synthesis by the reduction of respiration (citric acid cycle). This hypothesis is consistent with the delay between reduction of energy metabolism and inhibition of proliferation.

An example for the relationship of energy metabolism and proliferation is the stimulation of Na/K-ATPase by factors activating quiescent cells to enter the cell cycle again, followed by an increase in aerobic glycolysis (8) which can be short-circuited by phorbol esters stimulating proliferation without enhancing Na or K fluxes (for review see 3).

An important result was the lack of glutaminolysis and the general low metabolic activity of the SV40-3T3 cells being equal to the activities found in the two other cell types after reaching confluency. This finding is in accord with the contact mediated reduction of motility (missing in SV40-3T3 cells (40)) and with the assumption of an only indirect role of energy metabolism in growth control. Other reports emphasize high metabolic activities of tumor cells (30). However only a few comparative investigations have been made which revealed diminished oxygen consumption by neoplastic cells.
Indeed, another way of interpretation seems to be more adequate: Single cells in general have high metabolic requirements. At the moment they contact each other, reduction of ATP requirements by reduction of cytoplasmic motility and/or by metabolic cooperativity may decrease to a level which seems to be about the same in the three cell types under investigation. Obviously SV40-3T3 cells exhibit the largest fraction of freely soluble ANPs, i.e., ADP and ATP are easily accessible for all metabolic processes. We suppose the reduced microfilament equipment of these cells is responsible for the higher mobility of ANPs in these cells, a question presently under investigation.

Role of the cytoskeleton
Cytoskeletal fibrils provide a huge surface for binding of metabolites and enzymes. Regarding the well established association of some glycolytic enzymes with F-actin (10, 14, 28, 31, 36) and considering the differences in actin organization between normal and many malignant cell types, the total amount of F-actin has been determined in parallel to the metabolic parameters. Contrary to our expectations in pmfs only weak correlation was found between F-actin content and net lactate production. However, in glutamine-free medium lactate production in preconfluent cultures was reduced by about 50% indicating that half the lactate has been produced via glutaminolysis (26) rather than by glycolysis. Only the latter pathway contains the enzymes associating with actin. Because in SV40-3T3 cells neither actin nor lactate production changed with cell density, no relationship could be tested.

Whether ANPs are associated with actin (in this case we discuss transient association, not the well known strong binding of ATP or ADP to actin) or to other cytoplasmic structures is not clear at the moment. However, the cytoskeleton is one of the most potent candidates for such an association and in preliminary experiments we found a retarded release of ATP by cytochalasin D (shifting F-actin bundles to random coil fibrillar pattern). Fractionated release of ANPs from the cultures showed considerable differences between the free cytosolic and the structure associated fraction which, in general, was the larger one. Significant reduction of energy charge occurred only in the cytosolic fraction of 3T3 cells. This decrease coincided with the cessation of proliferation. In SV40-3T3 cells energy charge in both fractions decreases only when the cultures reach densities where the cells start to degenerate (Fig. 6).

In conclusion, all the density dependent decreases of parameters of the energy metabolism observed in the three cell types are temporarily coincident with the formation of contacts. Contact inhibition of motility is an immediate reaction. Growth inhibition is caused by a time consuming signal transduction process including changes in adhesion, organisation of the cytoskeleton and the supply with metabolites for macromolecule syntheses. Non-associated ANPs and the energy charge of the cytosol are the only factors studied here which can be used as an indicator of a culture’s commitment for further proliferation. Oxygen consumption, net lactate production, NAD content and redox-potential decrease long before cessation of proliferation and thus may influence commitment of cells to pass through a further cell cycle by very indirect mechanisms only. For the expansion of cell cultures in bioreactors and for comparison of energy metabolic parameters among different cell types the density dependence of these factors has to be taken into account.

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