Kinetics and metabolic specificities of Vero cells in bioreactor cultures with serum-free medium

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Abstract

The aim of this study was to understand the metabolism kinetics of Vero cells grown on microcarriers in bioreactors in serum-free medium (SFM). We sought to determine what nutrients are essential for Vero cells and how they are consumed. Contrary to glucose and to most of the amino acids, glutamine and serine were very quickly depleted in this medium and can be supposed to be responsible for cell apoptosis. Lactate and ammonium ions did not reach toxic levels for Vero cells. We paid more attention to the lactate metabolism. Usually we observed that after about 2 days lactate was consumed in serum-containing media, but its concentration plateaued in SFM. Moreover, the addition of serum in SFM provoked lactate consumption and the rate of glucose and glutamine consumption was twice as high as in the SFM not supplemented with serum. The depletion of glutamine and serine and the metabolic deviations leading to a shortage of intermediate products required for other metabolic pathways probably contribute to the lower cell yield and higher cell death rate in SFM.

Abbreviations: Ala – Alanine, Arg – Arginine, Asn – Asparagine, Asp – Aspartic acid, DCS – Donor Calf Serum, Gln – Glutamine, Glu – Glutamate, Gly – Glycine, His – Histidine, Ile – Isoleucine, Leu – Leucine, Lys – Lysine, Met – Methionine, Pro – Proline, SFM – Serum-Free Medium, Ser – Serine, Tyr – Tyrosine, Thr – Threonine, Val – Valine, Phe – Phenylalanine

Introduction

The Vero cell line, derived from African green monkey (*Cercopithecus aethiops*) kidney (Yasumura and Kawakita 1963) has been used extensively in the manufacture of human vaccines, in particular against poliomyelitis and rabies (Mendonça et al. 1993; Montagnon et al. 1984a, 1984b).

The optimization of culture media that are free from raw materials of animal origin requires a better understanding of the growth (Clark et al. 1982; Mer-

ten et al. 1994), of the death (Al-Rubeai 1998), and of the metabolism of animal cells (Doverskog et al. 1992) cultivated under various conditions. Metabolic behaviours of hybridoma cells (Bonarius et al. 1995; Europa et al. 1999), CHO cells (Nyberg et al. 1999) and BHK cells (Cruz et al. 1999; Linz et al. 1997) have been studied extensively, but little is known about Vero cells. (Lee and Engelhardt 1977; Mendonça and Pereira 1997; Nahapetian et al. 1986; Polastri et al. 1982). These studies led to the adjustment of the concentration of nutrients to prevent their

depletion and to limit the production of cytotoxic metabolites – two phenomena conducive to cell death in culture (Duval et al. 1992; Papaconstantinou et al. 1998; Sakagami et al. 1998).

In a previous article we showed that the lower Vero cell yield in serum-free medium, compared to serum-containing medium, was not due to a decreased rate of cell division but to a higher rate of cell death (Quesney et al. 2001). In order to identify the parameters responsible for this phenomenon, we have now quantified their metabolism in bioreactor cultures on microcarriers performed in serum-free medium (SFM), as indicated by the specific rates of essential nutrients consumption as well as by the production of cytotoxic metabolites.

Materials and methods

Cell lines and culture media

SFM was a 1:1 (v:v) mixture of VPSFM (Life Technologies) and William's E medium (Life Technologies). Vero cells, derived from a serum-containing cell bank at passage 137, were seeded in bioreactors at passage 144 after a history of four passages in serum-containing medium and two passages in SFM in Multitrays (Nunc, 6000 cm²).

Cultures in bioreactor

In-house designed bioreactors were equipped with a marine propeller. Culture volume was 3 l, i.e. 75% of the vessel volume. Microcarrier concentration was 3 g/l (Cytodex™ 1, Amersham-Pharmacia). Vero cells were seeded at 4.1×10^5 cells/ml and cultures were kept for 9 days in a batch mode. The pH was set at 7.2, the temperature at 37 °C, the dissolved oxygen at 25% of air saturation (by sparging) and stirring at 30 rpm. Attached cells were counted daily using a Fuchs-Rosenthal hemacytometer and the crystal violet technique (Sandford et al. 1951; Van Wezel 1973).

Cultures in static flask

1.25×10^6 cells were seeded into 10 ml of SFM in 25-cm² culture flasks (Corning). Cells detached after trypsinization were counted by the trypan blue exclusion technique in a Fuchs-Rosenthal hemacytometer (Philips 1973).

Biochemical analyses

Samples were collected daily. They were then centrifuged and the supernatants were aliquoted and frozen at -20 °C. Metabolites (glucose, lactate, glutamine, and NH₄⁺) were assayed using a Nova BioProfile 200 analyzer (Nova Biomedical, Waltham USA). BioProfile controls were systematically included in the analyses. Amino acids were assayed by ion exchange liquid chromatography with a Beckman 6300 analyzer (Biochemistry Pediatrics Department headed by Monique Mathieu, Debrousse Hospital, Lyon, France).

Calculation of specific rates and yields

A polynomial curve was fitted to experimental data. The polynomial fitting allowed smoothing of experimental points and generation of intermediate points. The Smooth macro computed a moving average of the curve and of the mean values of the N-points surrounding the current data points. The variation of specific rates was calculated from the derivative with reference to the time of the filtered curves. More precisely, due to the batch mode, the specific growth rate could be calculated using the formula $\mu = (dx_t/dt)/x_a$ where x_t is the sum of the concentrations of attached and detached cells in bioreactor and x_a is the concentration of attached cells. The specific nutrient consumption rate was calculated using the formula $\nu = (dS/dt)/x_a$ and the specific metabolite production rate using the formula $\pi = (dP/dt)/x_a$. The lactate/glucose ratio was calculated using the formula $R = (d(\text{lactate})/d(\text{glucose}))$.

Results and discussion

Previous studies in literature have shown that the growth and survival of mammalian cells in culture depend on the availability of nutrients (Zielke et al. 1984), the characteristics of the cell-culture surface (for adherent cells) (Abercrombie and Ambrose 1962), the accumulation of cytotoxic metabolites (Duval et al. 1992), and the oxygen concentration (Fenge et al. 1993; Nahapetian et al. 1986; Oller et al. 1989). In this study, we focused on the nutrient consumption and metabolite production of Vero cells cultured on microcarriers by following the kinetic rates and yields in serum-free medium (SFM).

Figure 1A and B show the kinetics of the con-

centration and specific growth rate of Vero cells grown in batch mode in bioreactors on Cytodex™ 1 microcarriers in SFM. After five days of culture, the attached cells reached a concentration of 1.8×10^6 cells ml^{-1} , then cell concentrations progressively decreased. The specific growth rate peaked after 31 hr in SFM (0.026 h^{-1}), then decreased progressively and was no longer measurable after about 5 days of culture (Fig. 1B).

We previously showed that the lower cell density in SFM was the result of a higher cell mortality rather than a lower cell division (Quesney et al. 2001). In the present study, we tried to find out whether the high cell mortality and the fast onset of cell senescence in SFM could be explained by the cell metabolism.

Characterization of Vero cell metabolism in bioreactor

The energy necessary for growth and cell survival is provided by several metabolic pathways including the glycolysis, the Krebs cycle and the respiratory chain. The predominance of glycolysis over the other metabolic pathways depends on the culture conditions and particularly on the initial concentration of glucose and availability of oxygen (Glacken 1988; Guppy et al. 1993; Newsholme et al. 1985; Wagner et al. 1991). Glutamine is the most consumed amino acid because of its easy entry into the Krebs cycle (Zielke et al. 1980, 1984) and of its role in the biosynthesis of other amino acids and of purine and pyrimidine bases.

Glucose and glutamine metabolism

Figure 1C and E show the evolution of glucose and glutamine concentrations in SFM, while Fig. 1D and F present their specific consumption rates. The amount of glucose consumed after 5 days of culture was about 10 mM. Glucose concentration was not a limiting factor in the cell growth phase, as at five days of culture residual glucose concentration was about 5 mM. The specific rate of glucose consumption was highest at the beginning of the culture (at $0.75 \text{ mmol}/(10^8 \text{ cells} \times 24 \text{ hr})$) and then decreased to negligible values at the end of the exponential phase. This can explain why glucose concentration was maintained at about 5 mM after five days of culture.

Glutamine was almost completely depleted after 3 days in SFM. The specific rate of glutamine consumption was also highest at the beginning of the culture (at $0.27 \text{ mmol}/(10^8 \text{ cells} \times 24 \text{ hr})$). This high con-

sumption of glutamine observed in SFM may explain this early depletion after 3 days of culture.

Glutamine depletion is known to induce cell apoptosis (Pardridge and Casanello-Ertl 1979). Glutamine can prevent apoptosis either as an essential source of energy by maintaining a sufficient concentration of ATP or by maintaining a glutathione concentration that minimizes the formation of free radicals. Apoptosis caused by glutamine deficiency can be prevented by the addition of antioxidants (e.g. dimethyl sulfoxide) to the media (Papaconstantinou et al. 1998). Glutamine depletion could therefore be one of the causes of apoptotic cell death in SFM, where glutamine was depleted early. In a previous study we demonstrated that the percentage of apoptotic cells increased after 3 days of culture in SFM concurrently with glutamine depletion (Quesney et al. 2001).

Amino acids metabolism

Although glutamine is the most relevant amino acid in cell culture, it is important to consider the metabolism of other amino acids, especially when major nutrient concentrations, like glucose and glutamine concentrations, were low. The main cause of consumption of amino acids is their integration in peptides during protein synthesis. They also participate in cell metabolism by sustaining the Krebs cycle (Arg, Leu, Ile, Lys, Val, Thr, Phe, Tyr, His and Met). Most of them cannot be synthesized by the cell and must be provided in culture medium. Some amino acids appear in the medium during the culture. We have studied more precisely the amino acid metabolism of Vero cells cultivated in SFM.

Figure 2A presents the initial amino acid concentration in SFM and the amount consumed at 8 days of culture. Figure 2B and C give the evolution of the specific rates of amino acid consumption. The amount of the amino acids was sufficient except for glutamine and serine. Consumption rates show that the decrease in consumption of amino acids was slow. Consumption rates were negligible at the end of the exponential growth phase, although amino acids were still present in sufficient quantity in SFM. The specific consumption rates of amino acids do not seem to depend only on the initial amino acids concentrations, as the initial concentration of serine is more than 2 fold lower than valine or isoleucine for identical consumption rates profiles. The specific consumption rates of serine explain the rapid depletion of this amino acid in SFM. Serine residual content was negligible after 8 days of culture, in contradiction with the synthesis reported

Figure 1. Kinetics of a Vero cells culture grown on Cytodex™ 1 microcarriers in a 3-liter bioreactor in SFM. Concentration of attached cells (▲) and specific growth rate (—) are shown as a function of time in panel A and B respectively; glucose (■) and lactate (+) concentrations are shown in panel C; glutamine (●) and NH_4^+ (◇) concentrations in panel E. The specific rates of glucose consumption and of lactate production are shown in panel D and the specific rates of glutamine consumption and of NH_4^+ production in panel F.

by Polastri et al. (1982). The high serine consumption can be explained by the depletion of glutamine (Cruz et al. 1999) as the decrease in glucose or glutamine

concentrations cause an increased serine requirement for biosynthesis; serine can also be converted to pyruvate. Serine depletion is known to induce apop-

tosis in HL-60 cells (Sakagami et al. 1998), which consume serine and arginine very rapidly, leading to a slowdown of their growth and then apoptosis. Sakagami et al. (1998) showed that addition of serine prolongs the exponential growth phase of these cells.

Arginine, abundantly consumed, is known to be a precursor of nitric oxide that can also induce or inhibit cell apoptosis in culture. Arginine consumption and especially the depletion of glutamine and serine in SFM might have contributed to the high cell death rate in this medium.

Glutamate, alanine, proline, and ornithine were produced by Vero cells (Fig. 2D). Overall, 5.35 mM of amino acids, including glutamine, were consumed and 1.71 mM were produced. Glutamine was transformed into glutamate and glutamate into α -ketoglutarate, which integrates the Krebs Cycle. Alanine and proline are usually produced in the presence of an overflow metabolism, which is consistent with the observed results. Alanine derives from glutamic acid and pyruvate; at high glucose concentrations, the increase in pyruvate production led to an increase in alanine production; at low glucose concentration, the pool of pyruvate is lower and alanine production is consequently reduced. Glutamine depletion could be responsible for the high alanine production as it is known that low glutamine concentration enhances alanine production. During the urea cycle, arginine is transformed into urea and ornithine. This metabolic way is confirmed by the consumption of arginine and the production of ornithine (Fig. 2A and D) by Vero cell along the culture.

The specific rates of glutamate and alanine production (Fig. 2E) confirm that glutamine was consumed mostly at the beginning of culture. These results evidence a rapid glutaminolysis and a greater transformation of glutamine into glutamate and alanine. The high alanine production rate coupled to lactate accumulation in SFM certainly caused a loss of acetyl CoA at the beginning of the Krebs cycle and consequently a reduction of energy production.

Lactate and NH_4^+ ion metabolism

The consumption of glucose and glutamine often results in the accumulation of toxic metabolites accumulation in culture medium such as lactate and ammonium ions. The evolution of these two metabolites has been followed (Fig. 1C and E) and their specific production rates are reported in Figure 1D and F.

Lactate concentration reached a peak (9.1 mM), on

day 5, then plateaued. The specific rate of lactate production during the first two days of culture was higher than that of glucose consumption, probably due to the high glutamine consumption. When Vero cells were cultured in serum-containing medium (data not shown) we first observed an initial accumulation of lactate, followed after two or three days of culture by a decrease of its concentration, whereas its concentration plateaued in SFM.

Metabolization of glucose via glycolysis produces ATP and reduces intermediate products, such as NADH. Glycolysis also sustains the Krebs cycle through pyruvate. Metabolization of glucose via Krebs cycle may be low compared with lactate formation, as less energy is produced because of the incomplete degradation of glucose. The calculation of the lactate/glucose ratio allows the quantification of the amount of glucose transformed into lactate and the assessment of energy loss. In our experiments, the lactate/glucose ratio peaked at 1.14 after 1 day of culture. This ratio is not very high and does not indicate a shortage of oxygen.

The maximal lactate concentration measured under our experimental conditions (about 9 mM) was below the limits of tolerance described by Hassel et al. (1990) so that it did not reach toxic concentrations; nevertheless, the accumulation of lactic acid certainly brought about a shortage of intermediates such as pyruvate or acetyl CoA that are necessary for other metabolic pathways.

The consumption of glutamine and other amino acids brought about an accumulation of ammonium ions (1.5 mM). This accumulation, which extended after glutamine depletion, was probably due to the metabolism of other amino acids. The profiles of specific rates of NH_4^+ production, as well as the specific rates of glutamate production, confirm the rapid consumption of glutamine in SFM. Ammonia was produced at nontoxic concentrations. Hassel et al. (1990) observed that ammonium chloride at a concentration of 25 mM did not reduce Vero cell growth.

Particular lactate metabolism in Vero cells

As we observed some important differences of lactate metabolism in Vero cells cultivated in bioreactors with or without serum, we have decided to study two parameters that could potentially affect this behaviour. Thus we have performed different cultures in SFM, which were either initially supplemented with sodium lactate or with donor calf serum (DCS).

Figure 2. Amino-acids consumed and produced by Vero cells cultured on Cytodex™ 1 microcarriers in a bioreactor in SFM. Initial medium concentration (C_0) of amino-acids (■) and the concentration of amino acids consumed at 8 days of culture (C_0-C_8) ($\square$) are shown in panel A. The specific rates of amino acids consumption are shown in panels B and C. Amino acids produced by Vero cells are shown in panel D (initial medium concentration (C_0) (■) and amino acids produced at 8 days of culture (C_8-C_0) ($\square$)). The specific rates of amino acids production are shown in panel E.

Effect of sodium lactate addition in SFM

We first observed the effect of the addition of sodium lactate in SFM at five days of culture on the cell growth (Figure 3). The initial addition of sodium lactate from 0 mM up to 30 mM did not induce a significant decrease of Vero cell density after 5 days of culture (from 8 to 7×10^6 cells / 25 cm^2), whereas at 60 mM final Vero cell density was reduced by two fold. Hassel et al. (1990) have shown that the growth of Vero cells was little influenced by the addition of up to 20 mM of sodium lactate. It should be underlined, however, that these experiments have been conducted with sodium lactate, a neutralized form of lactate which induces very small variations of pH in contrast to the lactic acid released during culture.

Secondly, we verified the effect of initial addition of sodium lactate on lactate and glucose metabolism (Figure 3). Figure 3 shows the glucose concentration consumed at five days of culture and the concentration of lactate at 5 days of culture minus the concentration of lactate at days 0. The addition of sodium lactate from 0 mM to 30 mM reduced lactate accumulation after 5 days from about 17 mM to 2 mM. At 60 mM of sodium lactate, Vero cells consumed 21 mM of lactate, concurrently with a decrease of density from 8×10^6 to 4×10^6 cells per static flask. The quantity of glucose consumed was not affected by the addition of

sodium lactate (Figure 3). In SFM the toxicity of added sodium lactate may be due to its high consumption by Vero cells.

Effect of DCS addition in SFM in bioreactor

Figures 4A and 4B show the kinetics of the density of attached cells and their specific growth rate in SFM supplemented with 4% DCS. By comparison with the data presented in Figure 1, the maximum cell density (2.6×10^6 cells ml^{-1}) was higher than that obtained in non-supplemented SFM; accordingly, the maximum specific growth rate was also higher (0.047 h^{-1} after 15 hr of culture).

Figures 4C and 4E show the kinetics of the concentration of glucose, lactate, glutamine, and NH_4^+ in SFM supplemented with 4% DCS. The glucose and glutamine consumption was higher in supplemented medium during the proliferation phase. This was confirmed by the higher specific rates of glucose and glutamine consumption induced by DCS supplementation (Fig. 4D and F). The maximum specific rate of glucose consumption ($1.48 \text{ mmol}/(10^8 \text{ cells} \times 24 \text{ hr})$) was twice as high as that obtained in non-supplemented SFM. This high glucose consumption, which brought about an accumulation of lactate of approximately 16 mM after two days of culture (Fig. 4C) and a high lactate/glucose ratio of 1.9, seems

Figure 3. Effect of added lactate on the final Vero cell density and on glucose and lactate metabolism in SFM. 1.25×10^6 cells were seeded into 25-cm^2 culture flasks in 10 ml of SFM containing various concentrations of neutralized sodium lactate. The final cell density per 25 cm^2 flask at five days of culture (▲), the glucose consumed (C_0-C_5) at five days of culture (■) and the lactate concentration (C_5-C_0) (+) are shown.

Figure 4. Kinetics of a Vero cell culture grown on CytodexTM 1 microcarriers in a 3-liter bioreactor in SFM supplemented with 4% DCS. Concentration of attached cells ($\blacktriangle$) and specific growth rate (—) are shown as a function of time in panel A and B, respectively ; glucose ($\blacksquare$) and lactate ($+$) concentrations are shown in panel (C); glutamine ($\bullet$) and NH_4^+ ($\blacklozenge$) concentrations in panel E. The specific rates of glucose consumption and of lactate production are shown in panel D and the specific rates of glutamine consumption and of NH_4^+ production in panel F.

mainly to depend on the culture performance due to the presence of serum and not on the glucose concentration. The cells rapidly colonized the microcar-

riers and passed from an extensive transformation of glucose into lactate to a lactate consumption (a very high lactate/glucose ratio of about 2 suggests that all

glucose was transformed into lactate within 48 hr of culture). Owing to the addition of serum to SFM, the cells consumed 5.4 mM of lactate between the third and the seventh day of culture. Glutamine was almost totally depleted after 2 days of culture. Glutamine consumption induced an accumulation of NH_4^+ to about 1.4 mM (Fig. 4E). Similar to glucose consumption, the glutamine consumption rate of 0.56 mmol/ (10^8 cells $\times$ 24 hr) in SFM supplemented with 4% DCS was twice as high as in non-supplemented SFM (Fig. 4F).

From the qualitative viewpoint, the addition of serum to SFM induced similar characteristics of glucose metabolism (lactate accumulation followed by its consumption, maximum lactate/glucose ratio after 2 days) to those previously observed in other serum-containing media (data not shown). This particular glucose metabolism does not seem to be dependent on the residual concentration of glucose observed after serum addition. Indeed in other experiments lactate was consumed in a serum-containing medium when 18 mM of glucose still remained in the medium (data not shown). From the quantitative viewpoint the addition of serum to SFM increased all kinetic rates of metabolite consumption and production. Moreover, we observed a very different glucose-lactate metabolism in static flasks in comparison to cultures performed in a bioreactor using the same culture media and, in particular, we did not observe the lactate consumption in serum-containing media at later culture stages (data not shown). We suppose that a limited aeration or the absence of oxygen regulation in flasks was conducive to anaerobic glycolysis throughout the culture regardless of the presence of serum. This could result in an inhibition of lactate consumption and thus could have an effect opposite to that of serum addition. Consequently, the presence of serum is not the only factor affecting the lactate metabolism in Vero cells.

This lactate consumption has already been described in the literature without precise reference to the factors responsible (Mendonça et al. 1993). Evidence of lactate consumption, influenced by the volume of medium, has been found in other kidney cell lines, such as LLC-PK1 (Gstraunthaler et al. 1999). This indicates an effect of oxygenation on the oxidative metabolism of lactate. Other authors suggest that lactate accumulation may be a result of the shortage of aspartate/malate transporters that carry NADH and H^+ from cytosol to mitochondria. The hydrogen in excess would then be eliminated by reducing pyruvate

into lactate (Sanfeliu et al. 1997). Irani et al. (1999) introduced a cytoplasmic pyruvate carboxylase into cells to create a new cytoplasmic metabolic pathway and thereby promote a more extensive use of pyruvate for malate formation, as well as the transfer of NADH and H^+ and the reduction of the amount of lactate produced. It has also been proposed that the sugar type and its level may affect the lactate consumption. Wagner et al. (1991) showed that a slowdown of glycolysis, linked to the presence of galactose, induced lactate consumption by human kidney TCL 598 cells. Ozturk et al. (1997), Zielke et al. (1984) reported that lactate consumption started when glucose concentration fell below a critical value. Our results do not confirm this hypothesis because the reduction of the glucose concentration after 8 days of culture in SFM did not induce lactate consumption. Thus, lactate consumption by Vero cells is not unequivocally linked to the glucose concentration.

Conclusion

Our results pointed out a different metabolic behaviour of Vero cells cultivated in SFM by comparison with a medium supplemented with serum. This was probably due to the physiological state of the cells when they were seeded into a bioreactor, and in particular due to the protective effect of serum vis-à-vis trypsinization. Nevertheless, glucose and most of the added amino acids were not completely depleted and did not reach limiting levels that could induce cell death. Also, no toxic levels have been reached for cytotoxic metabolites such as lactate and ammonium ions.

The most important depletions of substrates were observed for glutamine and serine, two depletions which are known to induce cell apoptosis. Metabolic deviations, inducing shortages of intermediate products required for the Krebs cycle and the respiratory chain, as well as nutrient depletions, rather than the toxicity of lactate and ammonia, seem to be responsible for the lower Vero cell yield and greater cell death in SFM. A further investigation of the influence of glutamine and serine depletions could provide some important insights into the role of these amino acids in Vero cell death. The growth and metabolism of these cells can, no doubt, be optimized by the adjustment of the initial concentration of these nutrients and their addition throughout the culture to match the needs of the cells.

We also demonstrated that addition of serum to serum-free medium again promoted lactate consumption when cells were cultured in bioreactors. On the contrary, we observed that lactate was not consumed in static culture flasks, where there was no oxygen regulation, this also in the presence of serum. Serum and oxygen levels are therefore two elements affecting the lactate metabolism of Vero cells and, in addition, the specific rates of nutrient consumption and metabolite production. Moreover, we could show that the addition of high concentrations of sodium lactate could also induce lactate consumption in flasks, even in the absence of serum and oxygen regulation.

This study represents a further improvement for the optimization of Vero cell culture in serum-free media. The Vero cell physiology, which is different in serum-free medium, compared to serum-containing medium should be considered for metabolic optimization of industrially relevant culture processes. This approach may prove to enhance not only industrial cell culture performance but fundamental knowledge concerning cell metabolism.

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