Characterization of Vero cell growth and death in bioreactor with serum-containing and serum-free media

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Received 9 July 2000; accepted 23 November 2000

Key words: apoptosis, cell cycle, lactate dehydrogenase, microcarriers, necrosis, serum-free medium, TUNEL, Vero cells

Abstract

The density of viable cells in a culture results from a balance between cell proliferation and cell death. The aim of this study was to characterize and compare these two phenomena in Vero cell cultures in one serum containing medium (ScA) and one serum free medium (SfB) in bioreactors. Cell growth was evaluated by cell counting (after crystal violet staining) and cell cycle analysis. Necrosis and apoptosis were characterized and quantified by measuring the release of LDH, trypan blue exclusion, annexin V-FITC/PI staining and TUNEL assay. ScA supported a higher maximal viable-cell density (2.3×10^6 vs. 1.8×10^6 cells ml^{-1}). However, cell cycle analysis showed that cell division was more active in SfB than in ScA. LDH release in the supernatant increased much earlier in SfB than in ScA (one vs. five days), but trypan blue counts showed no apparent difference in the viability of the cultures. Apoptosis, evidenced by annexin V-FITC/PI staining, could be detected in the population of suspension cells detached from microcarriers, but not among adherent cells; positivity of the TUNEL assay occurred later than that of the annexin V-FITC/PI staining. Our data indicate that the lower cell yield in SfB, compared with that in ScA, results from a higher cell death rate. Apparently, cells die from apoptosis followed by secondary necrosis.

Abbreviations: AV, annexin V; DCS, donor calf serum; FITC, fluorescein isothiocyanate; FL, fluorescence; FSC, forward scatter; LDH, lactate dehydrogenase; LDH_(I), intracellular LDH; LDH_(S), supernatant LDH; PBS, phosphate-buffered saline; PI, propidium iodide; SSC, side scatter; TUNEL, TdT-mediated dUTP nick end labeling; μ_{max} , maximum specific growth rate (h^{-1}).

Introduction

Cell cultures have been used extensively in the manufacture of biologicals for diagnostics, therapeutics, and vaccines (Werner and Noe, 1993a, b, c; Reiter and Blüml, 1994; Hu and Aunins, 1997).

The Vero cell line, derived from African green monkey kidney (Yasumura and Kawakita, 1963), can be grown on microcarriers in large bioreactors in a serum-containing medium for vaccine manufacturing

(Montagnon, 1984a, b; Kistner et al., 1998; Mendonça et al., 1993). Several researchers have identified parameters conducive to higher cell density: stirring (Fenge et al., 1993), oxygenation, concentration of microcarriers (Mendonça and Pereira, 1995), density of the cell inoculum (Forestell et al., 1991; Nahapetian et al., 1986), and type of growth surface (Cinatl et al., 1992; Nikolai et Hu, 1992) as well as chemical parameters, such as pH and medium composition (Mendonça and Pereira, 1997; Hassell et Engelhardt,

1977; Polastri et al., 1984). It has long been a desire of the industry not to use serum in cell cultures, as it is inconsistent in composition and can be the source of adventitious agents.

Indeed, Vero cells can grow in a serum-free medium as adherent cells (Merten et al., 1994), but at a lower rate, making scale up difficult. Conventional monitoring of cell cultures involves cell count, cell viability, and nutrient consumption. In addition, cell death has recently become a topic of interest. It has been demonstrated *in vitro* that cells die not only from necrosis, but also from apoptosis (Cotter and Al-Rubeai, 1995; Mastrangelo and Betenbaugh, 1995; Singh and Al Rubeai, 1998; Al Rubeai, 1998).

Our objective was to describe growth and death of Vero cells in bioreactors with serum-containing and serum-free media. First, we tried to define the most relevant parameters for the analysis of cell viability and mortality. We then compared the growth and death patterns of Vero cells in these two different media.

Materials and methods

Cell lines and culture media

ScA was Iscove's Modified Dulbecco's Medium (Hyclone) supplemented with 4% DCS (Hyclone); SfB 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, were seeded in bioreactors at passage 143 after a history of four passages in ScA and two passages in either ScA or SfB in Multitrays (Nunc, 6000 cm²).

Cultures in bioreactor

Four liter home-bioreactors were equipped with a marine propeller. Working volume was adjusted at 75% of the total volume and microcarrier concentration was 3 g l⁻¹. Vero cells were seeded at 4.05 × 10⁵ cells ml⁻¹ and cultures were kept for 15 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.

Cell count

Cells were counted daily using a Fuchs-Rosenthal hemacytometer, attached cells with the crystal violet technique (Sandford et al., 1951; Van Wezel, 1973)

and detached cells with the trypan blue exclusion technique (Phillips, 1973).

Calculation of specific rate of cell growth

The specific growth rate was calculated using the formula

$$\mu = (dx_t/dt)/x_a$$

where x_t is the sum of the concentrations of attached and of detached cells and x_a the concentration of attached cells.

LDH assay

Lactate dehydrogenase activity was determined by a colorimetric coupled enzymatic reaction, where the tetrazolium salt (INT) is reduced to formazan (Roche Diagnostics Cat. No. 1644793). The intracellular LDH content in attached cells (LDH_(I)) was quantified after trypsinization, count and lysis (1% Triton X 100 (Sigma) in PBS) of 1 × 10⁵ cells. The LDH activity in culture supernatants (LDH_(S)) was analysed in specimens collected after gravity sedimentation of microcarriers and detached cells, then subjected to centrifugation. A standard curve was plotted using an internal standard (Roche Diagnostics Cat. No. 759350). The variation coefficient of the method was about 5% and the lower limit of detection was 0.02 IU ml⁻¹.

Flow cytometry analysis

The data were generated with a FACScan (Becton and Dickinson) and processed using Cell Quest Software Program. For each experiment a minimum of 10 000 cells were analysed using an appropriate gating to exclude debris or doublets. For annexin V-FITC/PI staining and TUNEL assay FSC and SSC were measured in linear (lin) mode, annexin V-FITC, fluorescein-dUTP (FL-1) and PI (FL-2) in logarithmic (log) mode. For annexin V-FITC/PI staining electronic compensation was 17.9% of FL-1 subtracted from FL-2 and 0.5% of FL-2 subtracted from FL-1.

Cell cycle analysis

Attached cells (5 × 10⁵) were trypsinized, pelleted, then resuspended in 0.2 ml PBS and fixed in 1 ml of ice cold 70% ethanol in PBS. Fixed cells were pelleted, then gently resuspended in PBS, incubated for 30 min at 37 °C with 100 µg ml⁻¹ RNase (Sigma) and 20 µg ml⁻¹ PI (Sigma) and analyzed.

Figure 1. Density of attached Vero cells (crystal violet staining) cultured on microcarriers Cytodex 1, in a 3-L bioreactor, in ScA (●) and in SfB (▲).

Annexin V-FITC/PI staining

Annexin V-FITC/PI flow cytometry was performed according to Vermes et al. (1995). Detached cells were collected by centrifugation and attached cells by trypsinization. They were washed twice in PBS, resuspended in binding buffer (10 mM Hepes + 140 mM NaCl₂ + 2.5 mM CaCl₂, pH = 7.4) and adjusted to $2 \times$ to 5×10^5 cells ml⁻¹. 5 μ l of 250 μ g ml⁻¹ annexin V-FITC were added to 195 μ l of cell suspension and the mixture was incubated for 10 min at room temperature. Cells were washed once and resuspended in 190 μ l of diluted binding buffer, added with 10 μ l PI (Boehringer Ingelheim, Bioproducts France Cat. No. BMS306FI; 20 μ g ml⁻¹) and analyzed.

TUNEL assay

TUNEL assay was performed according to Gavrieli et al. (1992), using the *in situ* cell death detection fluorescein kit (Roche Diagnostics Cat. No. 1684795). One million cells were washed twice with 1 ml PBS, fixed in 4% (w/v) paraformaldehyde for 30 min on ice washed twice again, resuspended in 100 μ l of permeabilization solution (0.1% triton X-100 (Sigma) in 0.1% trisodium citrate dihydrate (Sigma), incubated on ice for 2 min, then washed twice with PBS. After addition of the TUNEL mixture (Terminal Deoxy Nucleotidyl Transferase (TdT): fluorescein-deoxy Uridine Triphosphate (fluorescein-dUTP) = 1:9), the attached cells and detached cells in suspension were incubated for 60 min at 37 °C, washed and analyzed.

Results and discussion

The performance of mammalian cell cultures in bioreactors depends on the growth rate and physiological status of the cells. Cell counting and trypan blue staining are routinely used to monitor cell density and viability, but these techniques do not allow to determine the relative contribution of cell proliferation and of cell death to the final cell concentration.

A combination of exploration protocols, to better monitor the behaviour of Vero cells, was applied to bioreactor cultures on microcarriers in a serum-containing (ScA) and a serum-free (SfB) medium. Cell growth was monitored by cell count and cell-cycle analysis. Necrosis was evaluated by LDH testing and trypan blue staining, whereas apoptosis was characterized using annexin V-FITC/PI staining and TUNEL assay.

Cell growth kinetics

Vero cells were cultured in batch mode in bioreactor. Figure 1 shows the kinetics of the density of attached cells in the two media. A similar 1-day lag was seen for both media. On day 2, the cells entered the exponential growth phase, with a higher specific growth rate in ScA ($\mu_{\max} = 0.035$ h⁻¹ at 15 h of culture) than in SfB ($\mu_{\max} = 0.026$ h⁻¹ at 24 h of culture), corresponding to doubling times of 18 and 26 h, respectively. After five days of culture, the attached cells reached a concentration of 2.3×10^6 cells ml⁻¹ in ScA and 1.8×10^6 cells ml⁻¹ in SfB. Then cell concentrations

Figure 2. Percentage of attached Vero cells in each phase of the cell cycle, in ScA □ and SfB ■, through a 15-day culture.

progressively decreased and levelled out in both media after 15 days of culture.

The lower maximal cell density in SfB, compared to ScA, was not explained by a different ratio of colonized to non-colonized microcarriers, as both cultures were homogeneous with virtually all microcarriers colonized.

The cell cycle was analyzed daily by flow cytometry. Figure 2 presents the evolution of the percentage of cells in each phase of the cell cycle along

the culture. The majority of cells in the inoculum was arrested at the G0-G1 phases as they came from confluent cell layers. After the first day of culture, 41% of cells (2.36×10^5 cells ml^{-1}) in ScA and 49% (2.61×10^5 cells ml^{-1}) in SfB were in the S and G2-M phases. Then contact inhibition progressively developed and caused a steady increase in the G0-G1 cell fractions. At confluence, after about five days of culture, fractions of cells in S and G2-M remained constant, though higher in SfB (20%/3.84 $\times 10^5$ cells

Figure 3. Evolution of Vero cells $LDH_{(I)}$ in ScA $\square$ and SfB $\blacksquare$. $LDH_{(I)}$ average $\pm$ 95% confidence interval was $2.11 \times 10^{-6} \pm 0.26 \times 10^{-6}$ U per cell in ScA and $1.3 \times 10^{-6} \pm 0.32 \times 10^{-6}$ U per cell in SfB.

ml^{-1}) than in ScA ($10\%/3.33 \times 10^5$ cells ml^{-1}). This residual percentage of cells in S and G2-M may be indicative of the persistence of cell growth until the end of the culture, because of the turnover of surface availability on microcarriers due to cell detachment.

These observations show that cell division was similar in both media. The lower final cell density in SfB might therefore be associated with a higher death rate rather than with a lower cell division. A more precise analysis of the cell death throughout the culture could provide some useful hints for process optimization.

Cell death quantification

Cell death is a significant parameter that can limit the optimal cell density in cultures (Akaton et al., 1985). Counts after trypan blue staining underestimate cell death, as they miss lysed cells. A more accurate way to assess cell death is to measure in the culture supernatant the lactate dehydrogenase $LDH_{(S)}$ released by dying cells (Racher et al., 1990; Wagner et al., 1991; Legrand et al., 1992; Falkenhain et al., 1998).

Having verified that LDH is stable in media at $37^\circ C$ for at least one week (data not shown), we assume that the $LDH_{(S)}$ content in supernatant is cumulative. We also determined the LDH content per attached cell at the different time points ($LDH_{(I)}$) to

calculate the cumulative number of dead cells in the supernatant from the measured $LDH_{(S)}$. As seen in Figure 3, $LDH_{(I)}$ fluctuated between 1 and 2 LDH units per 10^6 cells, was not influenced by the time, and was higher in ScA than in SfB. Goergen et al., 1992 showed that $LDH_{(I)}$ can vary depending on the cell origin and culture conditions; in our conditions, the average values $\pm$ 95% confidence interval were $2.11 \times 10^{-6} \pm 0.26 \times 10^{-6}$ U per cell in ScA and $1.3 \times 10^{-6} \pm 0.32 \times 10^{-6}$ U per cell in SfB. The number of dead cells on each day of culture was then calculated as the ratio between the $LDH_{(S)}$ value obtained on that day and the average $LDH_{(I)}$ content per cell.

Figure 4 shows the evolution of the number of attached cells (determined by crystal violet staining), of the number of detached dead cells in suspension (determined by trypan blue staining), of the amount of $LDH_{(S)}$, and of the total number of dead cells (quantified by LDH method) in ScA medium (A) and SfB medium (B).

In the ScA medium, the dead cell count was low throughout the culture; the $LDH_{(S)}$ level remained low for the first five days, then increased constantly. In the SfB medium, the $LDH_{(S)}$ activity kept increasing from the first day of culture while trypan blue counts were low and stable. There was no correlation, between the number of trypan blue positive cells and the $LDH_{(S)}$

Figure 4. Evolution of attached Vero cells (crystal violet staining) (▲), LDH (●), dead detached Vero cells in suspension (trypan blue staining) (■) and total number of dead Vero cells ($LDH_{(S)}/\text{average of } LDH_{(I)} \text{ per cell}$) (+). A: ScA; B: SfB.

increase, suggesting that dead cells lysed rapidly and were not recorded by trypan blue staining.

The higher levels of $LDH_{(S)}$ for the first five days of culture and the lower $LDH_{(I)}$ (see Figure 3) are indicative of a higher number of dead cells in SfB than in ScA.

This observation is in agreement with reports on the protective effect of serum on cells in culture (Elias et al., 1995; Kunas and Papoutsakis, 1989). In ScA, the $LDH_{(S)}$ activity remained low and relatively constant from cell inoculation to the stationary phase, indicating that this medium supported a good cell viability. Beyond day 5, there was a marked correlation between the increase of cell death, evidenced by an increase of $LDH_{(S)}$ and the decrease of viable attached cells.

Vero cell apoptosis characterization

The trypan blue and LDH methods are indicative of the alterations of cell membrane integrity that occur during necrosis.

Several laboratories have suggested that cells may die in bioreactors from apoptosis (Cotter and Al-Rubeai, 1995; Mastrangelo and Betenbaugh, 1995; Singh and Al Rubeai, 1998; Al Rubeai, 1998); others extended this observation to Vero cell cultures (Inward et al., 1995; Sadzot-Delvaux et al., 1995; Pugachev and Frey, 1998). Cells undergoing apoptosis maintain membrane integrity until the end of the apoptotic program. So, the release of LDH comes too late to be a suitable indicator of apoptotic cell death (Loo et al., 1993). Various methods can be used to specifically monitor cell apoptosis. Annexin V-FITC/PI followed

Figure 5. Bivariate frequency distributions of annexin V-FITC/PI 2-parameter flow cytometry of Vero cell culture in ScA and SfB. The lower left quadrant shows viable cells that exclude PI and are negative for annexin V-FITC adherence. The lower right quadrant shows apoptotic cells that exclude PI and bind annexin V-FITC. The upper right quadrant shows cells that have acquired necrotic characteristics. These cells do not exclude PI. A and B: annexin V-FITC/PI staining of attached Vero cells removed by trypsinization in ScA and in SfB respectively at five days of culture. C and D: annexin V-FITC/PI staining of detached Vero cells in suspension in ScA and SfB, respectively, at five days of culture.

by flow cytometry allows to detect the translocation of phosphatidylserine groups to the outer layer of plasma membrane which occurs early in the apoptotic programme and to assess membrane integrity. Cells positive only for annexin V-FITC staining are considered apoptotic; cells positive for both annexin V-FITC and PI staining are considered secondary necrotic cells; viable cells are double negative (Figure 5). We used the flow cytometry TUNEL assay to confirm apoptosis as it detects internucleosomal DNA fragmentation occurring at the end of the apoptotic programme (Figure 6).

We first applied these methods to cells anchored to microcarriers.

Annexin V-FITC stained approximately 20% of attached cells throughout the culture whatever the medium (Figure 5; see upper right quadrant dot blot A and B). This level is unexpectedly high and it was

surprising that the proportion of positive cells was constant throughout the culture. This was likely caused by the injury associated with trypsinization step necessary to process the cells for the analyses. This may reflect the unsatisfactory results reported by Pugatchev and Frey (1998) obtained with the annexin V-FITC/PI test on attached cells. We conclude that annexin V-FITC/PI is not suitable for the determination of apoptosis on attached cells. The TUNEL assay showed a constant low level (1%) of stained cells. This is incoherent with the increase of mortality after five days of culture and indicates that cells entering apoptosis detach from the microcarrier before to be positive to TUNEL assay.

We then tested the two protocols on detached cells in suspension.

As shown in Table 1, annexin V-FITC stained 12 and 17% of detached cells in suspension in ScA (vs

Figure 6. Analysis of chromatin fragmentation by TUNEL assay followed by flow cytometry. At five days of culture, in ScA (histogram A) and in SfB (histogram B), we controlled the autofluorescence of attached cells (a) incubation of attached cells without fluorescein-dUTP or terminal transferase), we then performed a negative control (b) fixed and permeabilized attached cells were resuspended in fluorescein-dUTP solution without terminal transferase, instead of TUNEL reaction mixture) and finally we evaluated DNA fragmentation on attached Vero cells (c) and detached Vero cells in suspension (d) incubated with the TUNEL reaction mixture.

20 and 31% in SfB), on days 2 and 3, respectively; in the same specimens, annexin V-FITC/PI stained 34 and 59% of cells (vs 61 and 63% in SfB). On day 8, annexin V-FITC/PI stained 65% of cells in ScA and 77% in SfB.

As shown in Table 2, TUNEL stained 62 and 40%

of detached cells in suspension in ScA, on days 5 and 8 respectively (vs 71 and 86% in SfB). Figure 6 illustrates the difference in the staining of attached cells (c) and of detached cells in suspension (d). Fragmentation of DNA was detected in an average of 50% of detached cells in suspension in ScA and about 70%

Table 1. Annexin V-FITC/PI staining profile of detached Vero cells in suspension in ScA and SfB. Results are expressed in percentage

Time (d)	ScA				SfB			
	Not stained	PI	AV	AV/PI	Not stained	PI	AV	AV/PI
2	53	1	12	34	19	0	20	61
3	23	1	17	59	6	0	31	63
4	8	0	32	60	4	1	13	82
5	8	0	32	60	1	0	33	66
6	3	2	26	69	1	1	16	82
8	7	1	27	65	2	1	20	77
11	5	0	14	81	1	1	10	88
15	8	1	18	73	13	4	12	71

Table 2. TUNEL staining profile of detached Vero cells in suspension in ScA and SfB. Results are expressed in percentage

Time (d)	ScA		SfB	
	Negative cells	Positive cells	Negative cells	Positive cells
5	38	62	29	71
6	36	64	27	73
8	60	40	14	86
11	74	26	45	55
15	43	57	33	67

in SfB (Table 2). So the number of detached cells in suspension tested positive in SfB was higher than in ScA.

We have a good correlation between detached cells stained by annexin V-FITC/PI on day five (60% in ScA vs 66% in SfB) and the percentage of cells positive to TUNEL reaction mixture (62% in ScA vs 71% in SfB).

However the total percentage of positive detached cells detected by annexin V-FITC and annexin V-FITC/PI on day five was significantly higher (92% in ScA vs 99% in SfB). Similar observations were made for cells in suspension (Vermes et al., 1995; Overbeeke et al., 1998) and suggest that annexin V-FITC reveals an earlier signal of cell apoptosis than does the TUNEL assay. In principle, apoptosis can be detected by staining with annexin V-FITC/PI and confirmed by the TUNEL assay. Applied to detached cells in suspension, the annexin V-FITC/PI assay was easy to perform and gave information not only on the extent of apoptosis, but also on the percentages of necrotic and viable cells. By comparison, the TUNEL assay appeared tedious and expensive and should therefore

be used for confirmation studies. These tools did not detect apoptosis in adherent cells, likely because cell detachment closely follows the apoptotic signal. Vero cell cultures in media with and without serum showed different profiles in terms of apoptosis and necrosis.

Conclusion

The purpose of this work was to evaluate and compare the behavior of Vero cells on microcarrier in serum-containing and serum-free media in bioreactors.

Cell proliferation was monitored by cell count and cell cycle analysis. Cell death was assessed by measuring membrane injury by the trypan blue exclusion procedure and LDH release. Apoptosis were followed by annexin V-FITC staining and by TUNEL analysis.

LDH monitoring in cell culture supernatant proved a more relevant parameter for the quantification of cell necrosis than trypan blue staining. Apoptosis was detected and characterised only in the population of detached cells in suspension and not among cells adherent to microcarriers. As of the first day of culture

and during the exponential growth phase, we observed a significantly higher cell death in SfB than in ScA, although the number of dividing cells was equal in both media. No evidence of cell injury could be detected on attached cells, may be due to the lack of proper markers. The coexistence in the supernatant of viable cells (negative to annexin V-FITC/PI test and TUNEL analysis), apoptotic cells (positive to annexin V-FITC and TUNEL analysis, negative to PI) and necrotic cells (positive to annexin V-FITC/PI test) suggest a sequence of events from the detachment as viable cells (very early after entry in apoptosis) to apoptosis and finally to necrosis. It does not exclude death by only necrosis where cells are subjected to damaging (as shearing). However, most of Vero cells seem to have died in bioreactor from apoptosis followed by secondary necrosis. It would be useful to investigate the early steps of apoptosis (in attached and in viable detached cells), in this way caspase activity quantification and/or mitochondrial potential changes could be an interesting approach.

A higher death rate in a serum-free medium (vs. serum-containing medium) can be explained by insufficient protection of cells, by the depletion of essential nutrients, and/or by the accumulation of cytotoxic catabolites. Evaluation of the protective effect of macromolecules and/or antiapoptotic agents, like caspase inhibitors, could be explored to reduce cell death in serum-free-medium cultures. At the same time, media formulation must be adapted to the cell culture requirements to eliminate large depletions of essential nutrients known to induce the cell apoptosis signal.

Acknowledgements

The authors would like to thank Dr Yves Richard (Veterinary School of Lyon) for helpful discussions, Nicole Pascal for her help with FACScan, Hugues Graf and his collaborators for supplying the Vero cell suspensions and the Serum-free Medium Unit of Aventis Pasteur, especially Christophe Chevalard and Bernard Pinson for technical support. This work was funded by Aventis Pasteur.

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