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NEW RAPID METHOD OF CACO-2 CELL DIFFERENTIATION

Anna Olejnik, Monika Lewandowska, Włodzimierz Grajek, Katarzyna Czaczyk*

Department of Biotechnology and Food Microbiology, Agricultural University of Poznań, Poznań, Poland

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The Caco-2 cell line is used by many investigators as a cellular intestinal model. The aim of this work was to improve the conventional long--term 21-day Caco-2 cell culture to obtain a simple, less expensive and more rapid culture system. In the new culture system, the cells were grown on polycarbonate filters in serum-reduced medium containing 2% iron-saturated calf serum supplemented with combination of growth factors and hormones. The modified culture conditions were designed to induce rapid differentiation of Caco-2 cells. Using this system, the differentiated enterocyte-like monolayer was established within 4-6 days and characterized for cell morphology, growth rate, glucose consumption rate, lactate production rate, the monolayer integrity and activity of alkaline phosphatase.

INTRODUCTION

Caco-2 cells which are derived from a human colon adenocarcinoma have focused the great attention in recent years for their use as an *in vitro* model of the intestinal epithelium. In culture they form tight junctions, spontaneously differentiate, express relatively high levels of digestive brush border enzymes and display other morphological, structural and functional properties similar to intestinal enterocytes [Pinto *et al.*, 1983]. For this characteristics, the Caco-2 cell culture is widely used as a tissue model for studying bioavailability and transport processes [Tavelin *et al.*, 2001], adhesion of probiotic bacteria or pathogens [Moal *et al.*, 2002] and immune responses following allergen invasion [Kerneis *et al.*, 2000].

Successful use of cell culture models *in vitro* is closely related to culture method standardisation, therefore the first aim of the presented work was to characterize the growth kinetics of Caco-2 cell line in conventional culture. Caco-2 cells are generally grown in serum-containing cell culture medium, although serum-free media have been developed. Traditional Caco-2 cell culture requires a 21-day period to attain a differentiated monolayer. As a result of the long culture period and daily medium replacement, the Caco-2 cell culture is very expensive. Thus, the second and the main objective of this study was to develop a more rapid, serum-reduced and less expensive culture system for Caco-2 cell differentiation.

MATERIALS AND METHODS

Traditional cell culture. The established Caco-2 cell line was obtained from American Tissue Culture Collection (USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma), supplemented with 20% heat inactivated (56°C, 30 min) fetal bovine serum (FBS, Gibco BRL), 1% non-essential amino acids 100X (NEAA, Sigma) and 50 mg·L^{-1} gentamycin (Gibco BRL). The culture medium was changed daily in all experiments. Caco-2 cells were seeded at 0.2; 0.4; 1.0; 2.1; 4.2; 6.2 x 10^4 cells per cm^2 and grown as monolayers for 21 days in stationary T-flasks and six-well plates (Nunc) at 37°C, in a 5% CO_2/95% air atmosphere. The passages of Caco-2 cell line ranged from 20th to 60th.

Modified cell culture

Serum reduced cell culture system. Caco-2 cells after adaptation to serum-reduced environment were grown in medium, containing DMEM supplemented with 10, 4 or 2% FBS, 1% serum replacement 250X (Sigma), 1% NEAA and 50 mg·L^{-1} gentamycin at a seeding density of 1.0 x 10^4 cells per cm^2 of six-well plate surface. The cells were cultured at 37°C, in a 5% CO_2/95% air atmosphere for 21 days.

Short-term cell culture system. For the short-term cultures, Caco-2 cells were grown in a 1:1 mixture of DMEM/F-12, with 2% iron-supplemented calf serum and additional factors: bovine insulin (10 μg/cm^3), human transferrin (10 μg/cm^3), dexamethasone (10^{-8} M), EGF (ng/cm^3), ascorbic acid (50 μg/cm^3), cholera toxin (25 μg/cm^3), and bovine pituitary extract (50 μg/cm^3). The cells were seeded onto Millicell PCF (0.4 μm pore size) membranes (Millipore) and twenty four well cell cluster dishes at a seeding density of 2.12 x 10^5 cells per cm^2. The cells were cultured using 1 cm^3 of the supplement--containing medium, 0.6 cm^3 on the basolateral side and 0.4 cm^3 on the apical side. Medium was changed after 72 h, then daily for the remainder of the culture period. Caco-2 cells were cultured for 7-8 days, at 37°C, and in a 5% CO_2/95% air atmosphere.

*Author's address for correspondence: Włodzimierz Grajek, Department of Biotechnology and Food Microbiology, Agricultural University of Poznań, ul. Wojska Polskiego 48, 60-627 Poznań, Poland; tel.: (48 61) 846 60 02: fax: (48 61) 846 60 03; e-mail: grajek@au.poznan.pl

TABLE 1. Growth parameters of Caco-2 cell cultures at various inoculum densities and different serum concentrations.

Initial cell density [xE+04/cm^2]	Max. cell concentration [xE+04/cm^2] (±SEM)	Viability in maximal cell concentration [%]	Doubling time [h]
20 % serum concentration			
0.02	17.8 (±1.3)	91	50.8
0.42	21.7 (±0.7)	91	49.5
1.04	30.5 (±2.0)	92	42.5
2.08	31.8 (±4.0)	89	56.3
4.17	26.9 (±0.3)	91	52.0
6.25	28.9 (±2.6)	91	72.2
10 % serum concentration			
1.04	26.0 (±1.3)	89	70.0
4 % serum concentration			
1.04	8.6 (±0.2)	78	83.5

Analytical methods. Cell concentration was determined using Neunbauer hemocytometer and cell viability was evaluated by 0.4% trypan blue exclusion dye.

Glucose and lactic acid concentrations were analysed with HPLC method. Proteins contained in cell culture supernatant were precipitated by 5% trichloroacetic acid addition and incubated at 4°C for 1 h. Then samples were centrifuged (15 min, 8 000g) and filtered through a 0.22 μm filter (Millex GP, Millipore). Determination of glucose and lactic acid was carried out on MERCK-HITACHI system consisting of an autosampler (model L-7250), a pump (model L-7100), and a refractive index detector (model L-7490). Analyses were performed isocritically at a flow rate of 0.8 mL·min^{-1} at 30°C, on an Aminex HPX-87H, 300x7.8 mm column (Bio-Rad). As a mobile phase, 0.005 M sulfuric acid was used. Standard was used to identify peaks in the chromatograms, and peak area was used to determine the glucose and lactic acid concentration.

The specific rates of glucose consumption and lactic acid accumulation were calculated from the following equation:

$$q = [(dY/dt) / (dX/dt)]\,\mu,$$

where: Y - glucose/lactic acid concentration, X - viable cell density, t - time, μ - apparent specific growth rate.

Monolayer integrity was determined by using the Millicell Electrical Resistance System (Millipore). To monitor the evolution of confluency, the transepithelial electrical resistance (TEER) of Caco-2 cell monolayer was daily measured.

As a measure of cell differentiation, the activity of alkaline phosphatase (ALP) was determined in cell lysates using an Alkaline Phosphatase Kit (POCH, Cat. 178152149). Adherent cells were harvested in cold phosphate buffer saline solution (PBS, pH 7.4) and used for ALP assay. The enzyme's activity was expressed as units per μg of cellular protein. Cellular protein was determined spectrophotometrically by the Bradford's method with bovine albumin as a standard.

Statistical analysis. The results were analyzed by a one--way analysis of variance (ANOVA) to determine significant differences and by the Tukey and Kruskal-Wallis tests to determine the statistical differences between groups using STATISTICA PL. 5.1. software. The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Growth characteristics of Caco-2 cell culture

One of the critical parameters at proliferation of anchorage-dependent cells, like Caco-2 cell culture, is the initial number of cells per cm^2 of culture surface area. The number of cells used to inoculate the culture influences the plating efficiency of the cells. The cell plating efficiency depends on the stage of the culture cycle from which the inoculum is taken [Butler, 1991]. The results of our experiments with culturing of Caco-2 cells demonstrated that the cells in exponential growth phase had the highest plating efficiency (data not shown). The exponential stage of cell culture corresponded with approximately 80% confluence of growth surface and high cell viability ranging between 97-99%. Cells taken from an exponentially growing culture exhibited a short lag phase on subculture, whereas cells taken from a stationary culture exhibited an extended lag phase (data not shown).

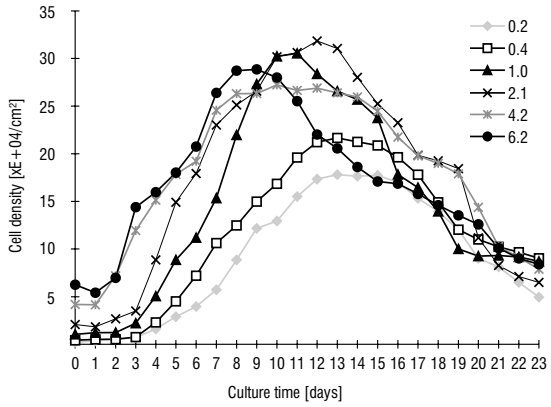

FIGURE 1. Growth kinetics of Caco-2 cells at different inoculum density.

The time courses shown in Figure 1 illustrate the growth cycle of Caco-2 cells at various inoculum densities ranging from 0.21 to 6.25 x 10^4 cells per cm^2. The shape of the Caco-2 growth curves is typical of animal cell cultures [Butler, 1991]. As shown in Figure 1 and Table 1, the initial cell density influenced the length of the lag period, the population doubling time in log phase, and the saturation density at plateau of culture. The cell proliferation began

after a lag period of 48 h at 1.04, 2.08, 4.17 and 6.25 x 10^4 cells/cm^2 inoculum density. Lower initial cell concentrations (0.21 and 0.42 x 10^4 cells/cm^2) caused elongation of adaptation stage from 48 to 72 h. At low cell densities of inoculation, the cell concentration was reduced and the total confluency of growth area was not reached. It involved considerable shortage of undefined factors released from growing cells into the medium. These factors are required for early initial cell growth and stimulation of cell proliferation [Butler, 1991]. The results of our studies on growth kinetics indicate that the culture of Caco-2 cells should be inoculated at cell concentrations higher than 0.4 x 10^4 cells/cm^2.

Seeding of cells at densities ranging from 1.04 to 6.25 x 10^4 cells/cm^2 had no significant influence on growth cycle (Figure 1), maximum cell concentration (Figure 2) and cell viability (Table 1). However, the average doubling times calculated following specific cell growth rates during exponential phase were different (Table 1). The log stage of culture inoculated at 1.04 x 10^4 cells/cm^2 exhibited the shortest doubling time of 42.5 h, thus this cell density was applied in further studies on improving the method of Caco-2 cell culture.

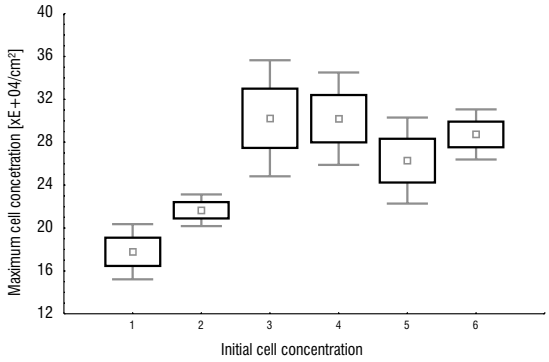

FIGURE 2. The effect of inoculum density on maximum cell concentration.

When the culture was inoculated with 1.04 x 10^4 cells/cm^2, the cell proliferation began after 48 h of a lag period. Confluency of growth surface was achieved after 7-8 days (15-22 x 10^4 cells/cm^2) and the stationary phase after 10 days (30 x 10^4 cells/cm^2). The monolayer of confluent Caco-2 cells was characterized by a burst of domes (blisters), which are typical of transporting epithelial cultures [Hughson & Hirt, 1996; Pinto *et al.*, 1983]. The domes were randomly formed and distributed all over the monolayer and increased in size with the time of culture. Pinto *et al.* [1983] noted that their diameter increased from 100 to 800 μm and their depth from 80 to 140 μm. The authors observed that their topography was varied, some domes appeared while others disappeared during the culture. The reason for blister developing is that the Na/K-ATPase in the cells is basolaterally located, and pumps ions downwards, drawing water with them. Because the thight junction between cells, a barrier across intercellular spaces is formed and that fluid accumulates under monolayer and, in places, forced it upwards [Hughson & Hirt, 1996].

To determine Caco-2 cell metabolic activity in culture started at 1.04 x 10^4 cells/cm^2 cell density, the glucose and lactate concentrations were measured and expressed as specific rates of glucose consumption or lactate production. The depletion of substrate or the accumulation of inhibitory metabolite may be responsible for the cessation of cell growth, thus an analysis of media changes during culture is necessary. The high specific rates of glucose consumption (9.6 x 10^{-7} mmol cell^{-1}·h^{-1}) and lactate production (2.5 x 10^{-7} mmol cell^{-1}·h^{-1}) during exponential growth phase indicated that daily medium replacement is required. The measurement of glucose and lactate concentration at the stationary phase of culture showed that extra energy is needed not only for cell proliferation but also for cell differentiation.

To determine the level of Caco-2 cells differentiation, the activity of alkaline phosphatase (ALP) as a specific brush border enzyme marker was estimated. The enzyme activity was low during the phase of active growth. Confluent but undifferentiated 7-day-old culture presented the low level of ALP activity. ALP activity markedly increased in 10-day-old monolayer and reached plateau in 18-day-old culture (1 300 mU/mg protein). These results are in agreement with previous reports which show an increase in ALP activity with time in culture [Dzierzewicz *et al.*, 2002; Pinto *et al.*, 1983].

Scanning electron microscopy of Caco-2 monolayer confirmed the culture maturation and differentiation. Early brush border development was apparent in 7-day-old culture. In the interval of days 7-21, the cells showed increasing signs of differentiation, the brush border became more apparent and more uniform. The brush border microvilli exhibited two different patterns: microvilli forming a thick carpet of brush border and microvilli forming flower-like clusters joint at their apical ends (Figure 3).

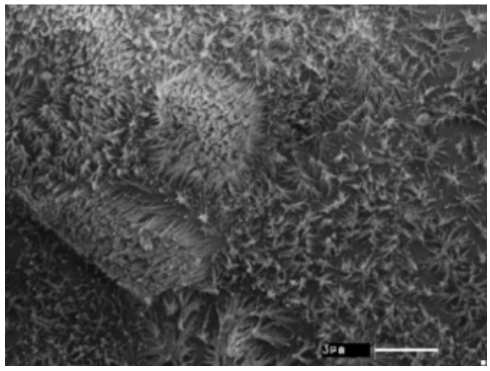

FIGURE 3. Scanning electron micrograph of 21-day-old Caco-2 cells grown in conventional culture supplemented with 20% serum concentration. Magnification: 11 800 x.

The results obtained indicate that traditional Caco-2 cell culture grown in the medium with 20% fetal calf serum concentration and seeded at 1.04 x 10^4 cells/cm^2 requires a 18-21-day period to form differentiated monolayer.

Modification of traditional cell culture

In the presented studies, two steps of culture modification were carried out: (i) adaptation of Caco-2 cell culture to growth in serum-reduced environment, (ii) induction of cell maturation and differentiation. The aim of the traditional culture modification was to reduce serum concentration in culture with no significance to cell proliferation and to induce the cell differentiation earlier than in 21-day-old culture.

Growth of Caco-2 cells in a serum reduced medium

Caco-2 cells were passaged and cultured with gradation of serum concentrations for 3-4 days. During serial passages the original serum supplement was gradually reduced from 20 to 10 and 4% and replaced with synthetic serum substitution. After adaptation process, the growth of the Caco-2 cells cultured in the presence of the different serum concentrations was analyzed (Figure 4). The results illustrated in Figure 4 indicate that proliferation of Caco-2 cells was modulated by serum addition. There was a significant difference in growth cycle and maximum cell concentrations obtained at various serum supplementation. The serum content markedly influenced the length of the lag period. The reduced serum concentration from 20 to 4% caused elongation of the adaptation stage from 48 to 144 h (Figure 4), which was reflected in 4-fold lower cell density and 2-fold longer doubling time of culture (Table 1).

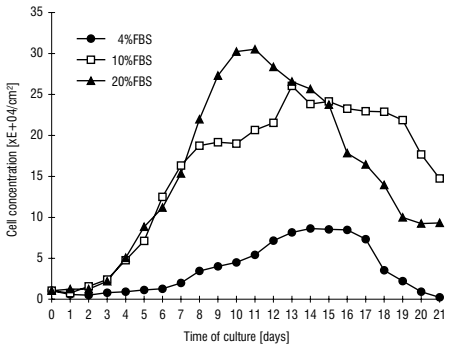

FIGURE 4. Effect of serum concentration on growth characteristic of Caco-2 cells.

The reduced serum concentration caused a decrease in efficiency of cell adhesion to the culture surface, probably by the lack of exogenous glycoproteins supplied with serum involved in the attachment process. Secondly, the shortage of growth factors and hormones of serum origin led to the inhibition of cell proliferation. The serum had also protective effect on cells and enhanced their viability in culture, especially FBS provided protease inhibitors which inactivate the trypsin used in routine subculturing procedures. The results obtained indicate that Caco-2 cells have not been successfully grown in medium containing less than 10% of serum. Fetal calf serum is a key factor effected on Caco-2 cell growth. In the absence of serum, adhesion and proliferation of Caco-2 cells were inhibited although serum replacer was added. Several studies indicate that a serum supplement is usually an essential component of culture media for animal cell culture and without serum many cell lines failed to proliferate [Artursson *et al.*, 1996; Butler & Jenkins, 1989; Maurer, 1992; Wiren *et al.*, 1998]. In Caco-2 cell culture it is usual to use a fetal calf serum in 20% concentration [Artursson *et al.*, 1996; Pinto *et al.*, 1983; Pontier *et al.*, 2001; Salvini *et al.*, 2002]. Fetal calf serum is a common supplement because of a high fetuin and biotin concentration and a low gamma-globulin and fat content. FBS is a source of growth promoting components which are not present in basal media formulations. On the other hand, FBS is very expensive, its composition is differentiated and undefined. For these reasons, great attention is focused on adaptation of cells from serum-based to defined serum-free medium [Butler & Jenkins, 1989; Maurer, 1992].

Cellular response to growth factor and differentiation inductors

On the basis of previous studies on the maturation of epithelial cell culture [Jenkins, 1991; Lentz *et al.*, 2000], we selected the key growth factors and inductors promoting cell differentiation which could help to reduce the time of Caco-2 cells culture. Caco-2 cells were grown in a 1:1 mixture of DMEM/F-12, with 2% iron-supplemented calf serum. The induction of cell differentiation was carried out by the addition of the following components: bovine insulin, human transferrin, dexamethasone, cholera toxin, epidermal growth factor, bovine pituitary extract, and ascorbic acid. Caco-2 cells were cultured on polycarbonate (PC) filters and plastic six-well plates using both traditional and modified 2% sCS medium. The cells were seeded at non typical, high concentration of 2.12 x 10^5 cells per cm^2. As shown in Figure 5, the time courses of Caco-2 cells grown in different media at the same culture dishes are nearly identical. It indicates that the modification of medium formulation had no influence on cells proliferation. However, a significant difference in the cell concentration between cultures grown on polycarbonate membranes and plastic dishes was observed. The cells originated from inoculum completely covered plastic growth surface and the cell number during the plate cultures was approximately constant. Whereas the concentration of cells grown on PC filters increased about 3-fold within 48 h of seeding. The lower cell density determined on plastic dishes was related with the blisters formed on the cell monolayer. It is interesting to note that the cell monolayer grown on permeable filters was uniform and blisters were not developed. The cells grew better on membrane than on plastic because they were nourished from both the apical and basolateral side. The growth, structure and function of cells cultured on permeable membranes seem to resemble more the tissue culture occurring *in vivo*.

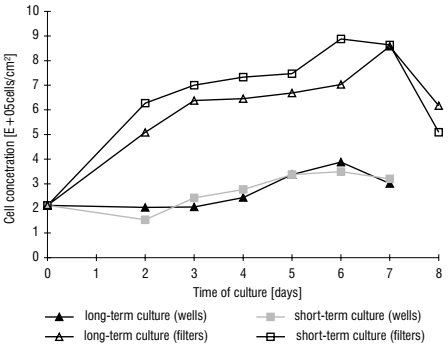

FIGURE 5. Time course of Caco-2 cells grown in different culture systems; **1**- 0.2; **2**- 0.4; **3**- 1.0; **4**- 2.1; **5**- 4.2; **6**- 6.2.

The cell monolayer integrity was confirmed by transepithelial electrical resistance (TEER) measurement. The TEER of Caco-2 monolayer increased with the time of culture, reaching the maximum after 48 h. In our study, TEER value of 150 (±7) Ω x cm^2 was obtained for Caco-2 cell monolayer at passage 40-60. In general, the TEER values ranging from 62 to 1 290 Ω x cm^2 have been reported for Caco-2 cells [Delie & Rubas, 1997]. The differences in TEER values are related to the use of different clones or passage numbers of Caco-2 cells [Delie & Rubas, 1997; Tavelin *et al.*, 2001]. Scanning electron microscopic

examination revealed the progressive formation of brush border with microvilli from 3- to 6-day-old Caco-2 cell culture grown in modified medium. As shown in Figure 6, microvilli were developed in 2% sCS culture system in 4 days. It was found that the Caco-2 differentiation characteristic of 4-day-old 2% sCS system can be comparable with 21-day-old 20% FCS system. It should be noted that a commercially available 3-day system for rapid differentiation of Caco-2 cells (BD BioCoat™ HTS Caco-2 Assay System, Becton, Dickinson and Company) can be purchased. However this system is significantly more expensive than that elaborated in our study.

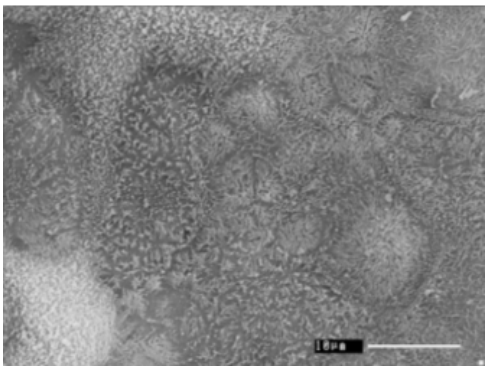

FIGURE 6. Scanning electron micrograph of 4-day-old Caco-2 cells grown in modified 2% sCS culture. Magnification: 3 740 x.

CONCLUSION

In conclusion, the proposed modified 2% sCS system of Caco-2 cell culture can be a considerable alternative to conventional 21-day system using 20% FBS. The new Caco-2 cell culture system enabled reduction of the serum concentration from 20 to 2% and the time of culture from 21 to 4-6 days. As a result, an expense of modified short--term culture is lower than that of traditional long-term culture. In further works, a new system will be applied in adhesion and transepithelial transport studies.

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