Study of Vero Cell Attachment and Growth in A Modified Serum-Free Medium (SFM01-M) Supplemented with Different Concentrations of Vitamin C

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Abstract

New and reused tissue culture flasks (T-flasks) without precoating with adhesion factors (e.g. fibronectin, laminin, collagen) were used to subculture Vero cells for 3 passages (P1, P2, P3) by using a modified serum-free medium (SFM01-M). A comparison of the cell yields obtained from those passages of new or reused T-flask cultures was not significantly different. The effect of vitamin C on Vero cell growth showed that the cell yields obtained from SFM01-M supplemented with 25-50 mg/L of vitamin C significantly decreased in contrast to those obtained from a lower concentration. The analysis of secreted proteins using SDS-PAGE in SFM01-M supplemented with different concentrations of vitamin C (0, 10, 25, 50 mg/L) during the first 24 h of cultures showed that all cultures gave the similar protein bands with a molecular weight (MW) of approximately 146 kDa. This protein band specifically reacted with monoclonal anti-collagen type I using a direct enzyme-linked immunosorbent assay (ELISA). In microcarrier culture, the attachment of Vero cells on Cytodex^{TM}1 microcarriers could be observed as spherical shape during the first 3 h of culture. The maximum cell concentration was 14.90 ± 0.56 x 10^4 cells/mL at day 6 of culture. From this study, the attachment and growth of Vero cells on new and reused T-flasks including on Cytodex^{TM}1 microcarriers can be achieved by using SFM01-M.

Key words: Modified serum-free medium, Vitamin C, Collagen, Microcarrier, Vero cells

Introduction

To develop serum-free media for established cell lines or stem cells, a number of serum substitutes have been widely used, especially non-animal derived hydrolysates obtained from plants or yeast (Jan et al., 1994; Keen and Rapson, 1995; Heidemann et al., 2000; Franěk et al., 2000; Sung et al., 2004; Chun et al., 2007; Rourou et al., 2009; Lobo-Alfonso et al., 2010; Michiels et al., 2011) as well as other basic components such as insulin, transferrin, sodium selenite, and ethanolamine (Taub et al., 1979; Murakami et al., 1982; Kovář and Franěk, 1989;

Eto et al., 1991; Chen et al., 1993; Okamoto et al., 1996). In addition to basic vitamins used, vitamin C is found to be essential for developing an animal-component free medium, though it was added at a low concentration (Rourou et al., 2009). Vitamin C or L-ascorbic acid is a micronutrient responsible for several biological functions not only as an important cofactor of certain enzymes involving in the collagen biosynthesis but also in the glycosaminoglycan biosynthesis (Kao et al., 1990; Arigony et al., 2013). Vero cell line, derived from African green monkey kidney, has been widely used for viral vaccine productions as recommended by The World Health Organization (WHO, 1998). In this study, the effect of vitamin C on Vero cell attachment and growth in new and reused T-flasks without precoating with adhesion factors were studied. Additionally, the effect of vitamin C on the secretion of extracellular matrix proteins – collagens was also studied. The extracellular matrix proteins – collagens secreted into culture medium were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting and a direct enzyme-linked immunosorbent assay (ELISA). In microcarrier culture in a spinner flask, the attachment of Vero cells on microcarriers was observed during the first 3 h of culture and the concentration of free amino acids during the course of microcarrier culture in SFM01-M was also analyzed.

Objectives

The objective of this study was to study the effect of vitamin C on Vero cell attachment and growth on both static and microcarrier cultures and to study the effect of vitamin C on the production of extracellular matrix proteins – collagens.

Research Methodology

Cell line

Vero cells (ATCC® CCL-81™) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in serum-containing medium (SCM) that consisted of Eagle's minimal essential medium (MEM, "*Nissui*" No.1, Nissui Pharmaceutical Co., Ltd., Japan), 10 % (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) MEM non-essential amino acids (100X), 1 mM sodium pyruvate and 2 mM L-glutamine, at 37 ^{o}C, 5 % CO_2 in a CO_2 incubator until confluent. Monolayer cells were washed once with phosphate buffered saline (PBS, Ca^{2+}, Mg^{2+}-free PBS, pH 7.4), and then detached with the trypsin-EDTA solution (0.25 % w/v trypsin and 0.02 % w/v EDTA) for 2-5 min at 37 ^{o}C. Cells were subcultured in SCM for at least 3 passages before subsequent use. For all of the SFM01-M cultures, Vero cells were adapted to SFM01-M for at least 3 passages and TrypLE™ Select (1X)(Gibco) was used instead of the trypsin-EDTA solution during the subculture of Vero cells under serum-free condition.

Cell cultures

In this study, the original SFM01-M was fundamentally comprised of Bacto™ yeast extract (0.1 g/L), Bacto™ soytone (0.1 g/L), vitamin C (9.719 mg/L), vitamin B12 (0.173 mg/L), SITE liquid media supplement (0.1 % v/v), recombinant human epidermal growth factor (rhEGF)(0.058 mg/L), MEM non-essential amino acids (100X)(1.0 % v/v), sodium pyruvate (1.0 mM), L-glutamine (4.0 mM), sodium hydrogen carbonate (2.2 g/L), and MEM medium (9.4 g/L). SFM01-M was prepared as a liquid form. Additionally, SFM01-M was not added with any adhesion factors such as fibronectin, laminin or collagen before used in the experiments. SFM01-M has been developed for Vero cell culture as described previously (Posung et al., 2016a, 2016b).

Subcultures of Vero cells in new and reused T-flasks

The effectiveness of SFM01-M in supporting Vero cells during the attachment and growth in new and reused T-flasks was firstly studied. An initial cell density of 4 x 10^4 cells/cm^2, derived from the last passage (P3) of SCM cultures, was inoculated in T-flasks (25 cm^2) (Thermo Fisher Scientific, Jiangsu, P.R. China) containing 5 mL of SCM medium. Cells were grown at 37 ^{o} C and 5 % CO_2 for 4 days. After 4 days, monolayer cells in T-flasks were washed once with PBS, T-flasks were added with 0.2 mL of TrypLE™ Select (1X) to detach the cells at 37 ^{o} C for 2-5 min. Dislodged cells in T-flasks were added with 1.0 mL of SFM01-M and then transferred to 1.5 mL centrifuge tube. Cells were separated by centrifugation at 1,000 rpm for 5-10 min. Cell pellets were added with 1.0 mL of SFM01-M to resuspend the cells. The cells derived from this first culture were named as P0 because cells were still contacted with serum. To perform the experiments, new T-flasks were inoculated with the initial cell density of 4 x 10^4 cells/cm^2 and 5 mL of pre-warmed SFM01-M, cells were grown at 37 ^{o} C and 5 % CO_2 for 4 days. For new T-flask cultures, subcultures were carried out repeatedly for 3 passages (P1, P2, P3). In reused T-flask cultures, the starter cells used at P1 were obtained directly from P0 (Table 1) as well as that used in P1 of new T-flask cultures. The initial cell density and a volume of medium used were the same as performed in new T-flask cultures. However, the reused T-flask was washed once with PBS before used in the subsequent culture at P2 and P3. During the subcultures of Vero Cells in both cultures, TrypLE™ Select (1X) was used in place of the trypsin-EDTA solution and the detachment method was carried out as the same as mentioned above for P0. Vero cell morphology was observed by a phase contrast microscope (Nikon, Japan) and recorded as photographs. Both of T-flask cultures were carried out in duplicate.

Effect of vitamin C on Vero cell growth and on the secretion of extracellular matrix proteins – collagens

A concentration of vitamin C in the original SFM01-M was varied as follows: 0, 10, 25, and 50 mg/L. The starter cells were obtained directly from those grown in the SFM01-M cultures at passage 4 (P4). Prior to use in each experiment, the starter cells were washed once in each medium formulation and then separated by centrifugation at 1,000 rpm for 5-10 min. Cell pellets were well resuspended in each medium formulation before inoculated in new T-flasks (25 cm^2) with the initial cell density of 4 x 10^4 cells/cm^2 and 5 mL of medium. Cell cultures were carried out in duplicate at 37 ^{o}C and 5% CO_2 for 4 days. The extracellular matrix proteins – collagens secreted by Vero cells was analyzed using SDS-PAGE according to the manufacturer's instructions (Bio-Rad). Two milliliters of each culture medium, derived from 24 h of culture, was concentrated by the Vivaspin tube with a molecular weight cut off 100,000 Da (Sartorius, Germany), and spun down at 6,500 rpm for 10 min. The concentrated culture medium (Retentrate) was dissolved in 5 X SDS-PAGE sample buffers and then heated at 95 ° C for 5 min. The denatured sample was subjected to SDS-PAGE (15% polyacrylamide for separated gel) and sample proteins were separated at 100 Volt for 2 h. Following separation, gel was washed with water for a short time period, and then was fixed with the fixative enhancer solution for 20 min. After that, gel was rinsed again with distilled water for 10 min. To visualize protein bands, gel was stained with the silver staining solution for 20 min and then stopped with 5 % acetic acid solution. In other experiment gel was then transferred to the nitrocellulose membrane (GE Healthcare) by electroblotting at 100 Volt for 2 h. After blocking with diluted Tris-buffered saline containing 0.05 % Tween-20 (T-TBS) and 5 % skim milk for 1 h, monoclonal anti-collagen type I (mouse IgG1 isotype, Sigma) was added at the dilution ratio of 1:1,000-1:2,000 in TBS solution at 37 °C for 2 h. After washing with T-TBS, rabbit anti-mouse IgG conjugated with horseradish peroxidase (Dako, Denmark) as secondary antibody was added at the dilution ratio of 1:1,000 and incubated at 37 °C for 1 h. Following the incubation period, collagen type I was visualized after incubated with 3, 3-diaminobenzidine tetrahydrochloride hydrate solution for 2-5 min. A direct enzyme-linked immunosorbent assay (ELISA) was also performed according to the standard protocol.

Microcarrier culture

The attachment and growth of Vero cells on microcarriers in SFM01-M was studied. Cytodex^{TM} 1 microcarriers (GE Healthcare, 2005) were prepared according to the manufacturer's instructions. Briefly, a concentration of microcarriers at 2.0 g/L was added in a 500 mL siliconized

spinner flask (Techne, UK) containing 50-100 mL of PBS and then left for 3 h at room temperature with gentle agitation. After that, spent PBS was removed and replaced with fresh PBS. Microcarriers were autoclaved at 121 ^{o}C, 15 psi for 30 min. Before use in the experiment, microcarriers were washed once with serum-free MEM medium for 5-10 min. Starter cells were obtained from the SFM01-M cultures at passage 5 (P5). Cell inoculums at a 5 cells/one microcarrier were prepared in 100 mL of SFM01-M and then transferred to spinner flask. The effectiveness of Vero cells in attaching to microcarriers was observed during the first 3 h of culture, which the mixture of cells and microcarriers was stirred continuously at 10-20 rpm at 37 ^{o} C and 5 % CO_2. Following the study of cell attachment, the growth of microcarrier grown cells was followed continuously for 7 days at 37 ^{o} C, 5 % CO_2 and 10-20 rpm-agitation. Vero cell morphology was observed by a light microscope (Olympus CH30RF200, Japan) and recorded as photographs. To follow the consumption of free amino acids during the course of microcarrier culture, free amino acids at day 7 of culture were analyzed according to in-house method based on J. Assoc. Off Anal. Chem. Vol 72. No. 6 (1989) at Central Laboratory (Thailand) Co., Ltd).

Cell counting

The growth of viable cells was determined daily. In SFM01-M cultures, the trypsin-EDTA solution was replaced by TrypLE^{TM} Select (1X). Briefly, spent medium was removed and washed once with PBS, and 0.2 mL of TrypLE^{TM} Select (1X) was added to T-flask. Cells were detached at 37 ^{o} C for 2-5 min until cells were dislodged. Dislodged cells in T-flask were added with 1.0 mL of SFM01-M. Cells were mixed several times and then transferred to a 1.5 mL centrifuge tube. Cells were separated by centrifugation at 1,000 rpm for 5-10 min. Spent medium was removed and replaced with 1.0 mL of the same medium. Cell pellets were mixed several times before cell counting. For microcarrier culture, cell counting was carried out by using a mixture of 0.1 % (w/v) crystal violet and 0.1 M citric acid. Briefly, 1 mL of sample taken from the spinner flask was left for about 5 min until microcarriers were settled down to the bottom of centrifuge tube. The supernatant was removed and replaced with the mixture of 0.1 % (w/v) crystal violet and 0.1 M citric acid. Sample was left for 1 h at 37 ^{o} C. Viable cells were counted as cell nuclei by a haemocytometer.

Statistical analysis

Data were representative of two independent experiments unless otherwise stated and were represented as mean ± standard deviation (SD). Two-tailed Student's t test was used to compare mean and P-value < 0.05 was regarded as statistically significant.

Results

Subcultures of Vero cells in new and reused T-flasks

The results showed that the cell yields obtained from both types of T-flasks were not significantly different as shown in Table 1. The morphology of Vero cells obtained from P3 of both types of T-flasks was also found to be not different (Figure 1; C and D). Based on this result, the attachment and growth of Vero cells was independent on culture surfaces.

Table 1. Cell yields obtained from 3 successive subcultures of cells in new and reused T-flasks.

Type of flasks		Passage numbers and cell yields ($x10^6$ cells/T-flask)			
	Positive control	P0	P1	P2	P3
New	2.68 ± 0.11	2.32±0.19	2.22±0.19	2.28±0.17	2.20±0.21*
Reused			1.99±0.01	2.06±0.20	2.11±0.04*

Positive control was a SCM culture. A reused T-flask obtained from P0 was reused for 3 times at P1, P2, and P3. A comparison of the cell yields at P3 obtained from new and reused T-flask cultures was not significantly different (* P-value > 0.05).

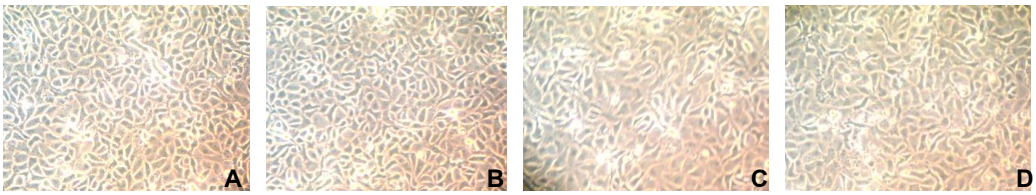

Figure 1 Vero cell morphology: A) a positive control that the cells were cultured in SCM in new T-flasks, B) the cells were obtained from P0 in new T-flasks where the cells were precultured in SCM at 37 ^{o} C for 3 h before replaced directly with SFM01-M, C) the cells were obtained from P3 of new T-flask cultures, and D) the cells were obtained from P3 of reused T-flask cultures. A magnification of photographs was 100X.

Effect of vitamin C on Vero cell growth and on the secretion of extracellular matrix proteins - collagens

The results showed that vitamin C had much effect on Vero cell growth (Table 2). The cell yields significantly decreased when vitamin C was higher than 10 mg/L. The morphology of Vero cells (Figure 2) at a low concentration of vitamin C (0–10 mg/L) was found to be similar to those obtained from higher ones (25–50 mg/L). Therefore, the optimal concentration of vitamin C was 0-10 mg/L.

Table 2. Effect of vitamin C concentration supplemented in SFM01-M on Vero cell growth

Culture conditions	Cell yields (x 10^6 cells/T-flask)
Positive control	2.68 ± 0.11
1) SFM01-M+ 0 mg/L vitamin C	2.10 ± 0.21*'**
2) SFM01-M+10 mg/L vitamin C	2.13 ± 0.12*
3) SFM01-M+25 mg/L vitamin C	1.24 ± 0.16**
4) SFM01-M+50 mg/L vitamin C	1.16 ± 0.13

Positive control was a SCM culture. A comparison of the cell yields obtained between culture condition 1) and 2) was not significantly different (* P-value > 0.05), whereas a comparison of the cell yields obtained from culture condition 1) or 2) with both of culture conditions 3) and 4) was significantly different (** P-value <0.05).

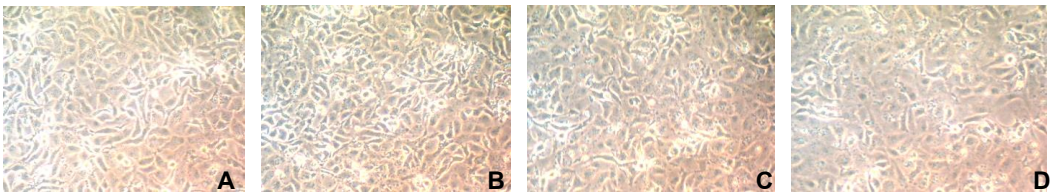

Figure 2 Vero cell morphology; A) SFM01-M+0 mg/L vitamin C, B) SFM01-M+10 mg/L vitamin C, C) SFM01-M+25 mg/L vitamin C, D) SFM01-M+50 mg/L vitamin C. A magnification of photographs was 100X.

Generally, the attachment and spreading of animal cells on a solid substratum can occur during 24 h of culture: 1-2 h for attachment and 24 h for spreading (Butler, 1996). In this study, SFM01-M had no adhesion factors and new T-flasks were not coated with adhesion factors. Therefore, the interaction between the cell membrane and the solid substratum is critical. In serum-containing medium (SCM), the attachment of cells can occur when the solid substratum is coated with serum-derived glycoproteins (e.g. fibronectin) and conditioning factors released by cells and divalent cations (e.g. Ca^{2+}). Therefore, the secretion of extracellular matrix proteins, especially collagens, in SFM01-M supplemented with different concentrations of vitamin C was analyzed using SDS-PAGE during 24 h of cultures. The results demonstrated that SFM01-M supplemented with all concentrations of vitamin C could give the similar protein bands with a molecular weight (MW) of approximately 146 kDa (Figure 3). However, the result obtained from western blotting showed that these protein bands could not react specifically to monoclonal anti-collagen type I (data not shown). On the other hand, collagen type I was detected when a direct

enzyme-linked immunosorbent assay (ELISA) was applied in all cultured conditions as shown in Figure 4.

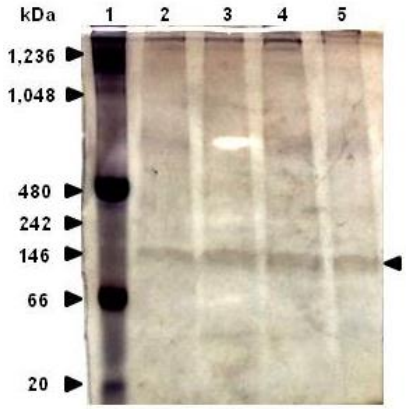

Figure 3 SDS-PAGE of extracellular matrix proteins; Lane 1) Marker proteins (NativeMark™ Unstained Protein Standard, Gibco) developed with silver staining, Lane 2) SFM01-M+0 mg/L vitamin C, Lane 3) SFM01-M+10 mg/L vitamin C, Lane 4) SFM01-M+25 mg/L vitamin C, Land 5) SFM01-M+50 mg/L vitamin C. An arrow head on the right side of SDS-PAGE gel marks the position of protein bands.

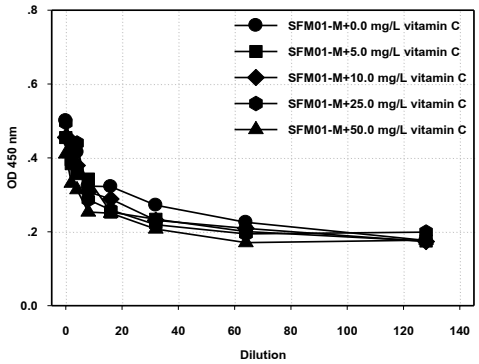

Figure 4 Enzyme-linked immunosorbent assay for analyzing collagen type I.

Microcarrier culture

As shown in Figure 5B, Vero cells adapted to growth in SFM01-M for at least 3 passages could attach well on Cytodex™ 1 microcarriers during the first 3 h of culture. Cells could grow well (Figure 5C). The maximum cell concentration was 14.90 ± 0.56 x 10^4 cells/mL at day 6 of culture (Figure 5A). The concentrations of free amino acids obtained from day 7 of microcarrier culture were analyzed. As indicated in Figure 6, the concentrations of proline and lysine decreased to 20.85 % and 21.02 % of the initial concentrations, respectively. However, the consumption of both may depend on the cell concentration in culture. Additionally, it was found that leucine, valine, serine, aspartic acid concentrations also decreased, especially serine. Serine was consumed more than 50 % of initial concentration in the serum-free culture of Vero cells as reported by Quesney et al. (2003).

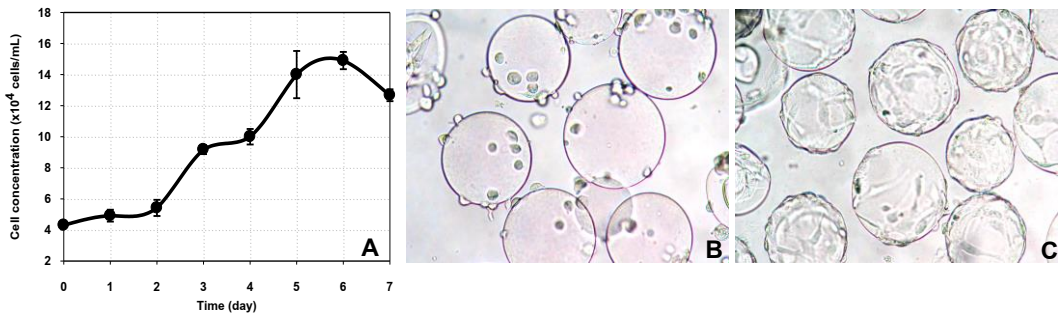

Figure 5 Characteristics of Vero cells cultured in SFM01-M on Cytodex™ 1 microcarriers in a spinner flask containing 100 mL of medium and a 5 cells/one microcarrier. A) Cell growth curve, B) Attachment of Vero cells at the first 3 h of culture, and C) Cell growth at day 6 of culture. A magnification of photographs for Figure 5B and Figure 5C was 100X.

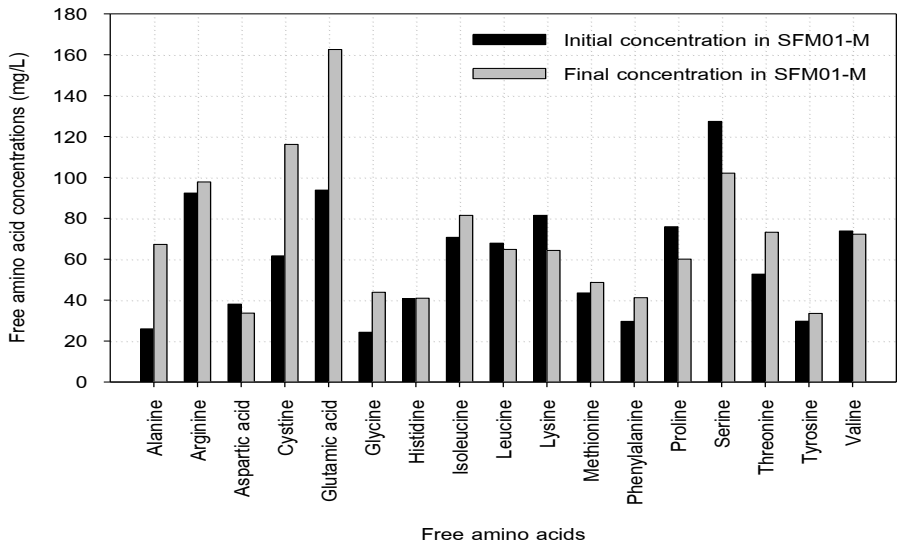

Figure 6 Free amino acid concentrations in SFM01-M after Vero cell culture on Cytodex™ 1 microcarriers in a spinner flask for 7 days.

Discussion

In general, serum is normally added to culture media at a concentration of 5-20 % (v/v) to promote cell growth. For anchorage-dependent cells, serum-containing medium (SCM) can also provide adhesion factors (e.g. fibronectin) that facilitate the suitable surface condition for the attachment of cells on a solid substratum. Additionally, the maximum cell attachment is also dependent on the density of the electrostatic charge on the solid substratum. At present, glass tissue culture ware is replaced by plastic one, which is made of sulphonated polystyrene with a

surface charge of 2-5 negatively charged groups per nm^2 (Butler, 1996). In addition, to connect cells in tissues, intercellular spaces are filled with extracellular matrix (ECM), whose component is dependent mainly on cell types and the composition of the ECM also regulates the cell phenotype. ECM is comprised of collagen, laminin, fibronectin, hyaluronan, and proteoglycans such as betaglycan, decorin, perlecan, and syndecan-1, some of which bind to growth factors or cytokines (Freshney, 2010). Vero cells, derived from African green monkey kidney, used in this study were those (Vero ATCC® CCL81™) which cell morphology is epithelial or maybe fibroblast (Butler, 1996). In cultures, only few articles were reported about the production of ECM (e.g. type IV collagen, fibronectin) by Vero cells; however, the production of such ECM was conducted directly in the serum-containing condition (Santos et al., 2001, 2004). Although serum-free media (SFM) for Vero cells are commercially available and have been used widely in biopharmaceutical industries, most of them are still supplemented with some of adhesion factors (e.g. fibronectin) to help cell attachment. Currently, the matrix materials are commercially available and adopted extensively in animal cell cultures (Freshney, 2010). Based on the literature reviews (Kao et al., 1990; Arigony et al., 2013), the hypothesis that the ECM-collagens could be produced and secreted into culture medium when supplementing with vitamin C was proven; therefore, the experiment was conducted in this study by using a modified serum-free medium (SFM01-M) without the addition of any adhesion factors. In the first experiment, Vero cells in which cells were subcultured in SCM for at least 3 passages were used as starter cells for P0. The cell yields obtained from a positive control ($2.68 \pm 0.11 \times 10^6$ cells/T-flask) and from P0 ($2.32 \pm 0.19 \times 10^6$ cells/T-flask) was found to be not significantly different (P-value > 0.05). This no difference of cell yields may involve directly with the residue of serum components coating on the cell surface during which cells were grown at P0 (Hewlett, 1991). At P3, the comparison of cell yields between new T-flask ($2.20 \pm 0.21 \times 10^6$ cells/T-flask) and reused T-flask ($2.11 \pm 0.04 \times 10^6$ cells/T-flask) cultures was also not significantly different (P-value > 0.05). This no difference of cell yields may occur from both of cultures free of the residual serum components or from both of cultures were in the certainly serum-free condition. However, the cell yields of both cultures were found to be significantly different (P-value < 0.05) and be not significantly different (P-value > 0.05) when compared with that of a positive control and P0, respectively. During the successive subcultures of cells at P1, P2, and P3, the morphology of growing cells were found to be not different. From this first experiment, the culture surface of new and reused T-flask can give the similar cell yields, although the use of reused T-flask may be limited, at least 3 times, to provide a constant growth. Consequently, this result indicates that SFM01-M can stimulate the attachment of Vero cells regardless of culture surface. Actually, in this study T-flask (Nunc™

EasYFlask™ 25 cm^2) is made of polystyrene with Nunclon™ Delta surface coating, which can offer maximum adhesion for a broad range of cell types; however, this plastic ware is commonly used as a disposable device. In the large scale production of viral vaccines (e.g. influenza, enterovirus 71), the total cost of an investment in a whole process can be reduced when reused T-flasks or roller bottles are applied. In the second experiment, the different concentrations of vitamin C (0, 10, 25, 50 mg/L) were added in SFM01-M before cell cultures. Only two concentrations of vitamin C (0, 10 mg/L) gave the cell yields (> 2.0 x 10^6 cells/T-flask), whereas other two could not. The cell yields of SFM01-M supplemented with vitamin C at 25 and 50 mg/L were found to be lower significantly (P-value < 0.05) than those of SFM01-M supplemented with vitamin C at 0 and 10 mg/L. This result indicated that cell growth may be inhibited when a concentration of vitamin C was higher than 10 mg/L in SFM01-M. However, these four concentrations of vitamin C did not show a significant difference of cell yields obtained from an eye tissue-trabecular meskwork (Yue et al., 1990). Therefore, the optimal concentration of vitamin C in SFM01-M for Vero cell cultures should be less than 25 mg/L. Basically, vitamin c is a micronutrient required for several biological functions not only as a cofactor of certain enzymes, especially prolyl and lysyl hydroxylases, which play a key role in the collagen synthesis, but also as an antioxidant against reactive oxygen species (ROS) (Kishimoto et al., 2013). Also, several biological functions of vitamin C have been described elsewhere by Arigony et al., 2013. In this study, although few articles showed that Vero cells could produce type IV collagen and fibronectin; however, those cultures were still performed in serum-containing medium (SCM) (Santos et al., 2001, 2004). For western blotting analysis, it was found that the protein bands found in SDS-PAGE gel did not react specifically with monoclonal anti-collagen type I (mouse IgG1 isotype, Sigma, Product Number C2456). This mistake may occur by heating sample at 95 ^{o}C before loading sample on SDS-PAGE. Heating may destroy a native form of proteins that is not suitable for reacting with monoclonal anti-collagen type I while it was detected by using a direct enzyme-linked immunosorbent assay (ELISA). However, all culture conditions showed the similar protein bands with molecular weight of approximately 146 kDa on SDS-PAGE gels (Figure 3), which were similar to other protein bands as reported by Ohkura et al (1990). Therefore, all types of collagens should be further examined by means of using specific monoclonal anti-collagens, enzyme-linked immunosorbent assay (ELISA) or N-terminal sequencing. Generally, all of the ECM components will primarily contribute to produce a basal lamina, which facilitates the cells to attach to culture surface or in tissue (Lodish et al., 2008; Freshney, 2010).

In the third experiment, the microcarrier cell culture was performed to characterize the attachment and growth of Vero cells in SFM01-M in a spinner flask. The result showed that free

cells (unattached cells) less than 10 % of total amounts of cells used could not attach on Cytodex™ 1 microcarriers during the first 3 h of culture. Since the surface of Cytodex™ 1 microcarriers are normally covered with positively charged groups (GE Healthcare, 2005), the interaction between the cell membrane and microcariers might probably stimulate the rapid attachment of cells on microcarriers. After the attachment of cells, Vero cells could well spread and grow on microcarriers and could give the maximum cell concentration of 14.90 ± 0.56 x 10^4 cells/mL or 3.46 times higher than that of the initial cell concentration used. When free amino acids were analyzed, proline and lysine were respectively reduced to 20.85 % and 21.02 % of the initial concentrations at the end of culture (Figure 6). This result was corresponding to a report of Quesney et al. (2003) during the bioreactor cultures of Vero cells on Cytodex™1 microcarriers with serum-free medium containing about 2.25 mM glutamine. Also, Petiot et al. (2010) reported the consumption of lysine during the culture of Vero cells in a complex serum-free medium (reference medium) containing 4 mM glutamine and 22 mM glucose. However, these reports did not pay attention to the importance of both amino acids in collagen synthesis but only focused on the metabolism of Vero cells under different serum-free cultures. Actually, these two amino acids are the precursors of hydroxyproline and hydroxylysine mostly found in the structure of collagen during the collagen synthesis by prolyl and lysyl hydroxylases (Kishimoto et al., 2013).

Conclusion

A modified serum-free medium (SFM01-M) developed for Vero cells was used to study the attachment and growth of Vero cells in new and reused tissue culture flasks (T-flasks) without precoating with adhesion factors (e.g. fibronectin, laminin, and collagen). Cell yields obtained from new and reused T-flask cultures were not significantly different during subcultures for at least 3 passages (P1, P2, and P3). All concentrations of vitamin C supplemented in SFM01-M could give the same protein bands with a molecular weight of approximately 146 kDa. These protein bands could react specifically with monoclonal anti-collagen type I using a direct enzyme-linked immunosorbent assay (ELISA). The analysis of free amino acids in SFM01-M in the microcarrier culture showed that proline and lysine, the precursors of (pro) collagen synthesis, were decreased to 20.85 % and 21.02 % of the initial concentrations, respectively. Therefore, the reduction of both amino acids may involve with the synthesis of collagen. From this study, the attachment and growth of Vero cells on T-flasks and on Cytodex™ 1 microcarriers may occur from the secretion of collagen in conjunction with the difference of electrostatic charges between the cell membrane and a solid substratum.

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