Ex Vivo Expansion of Conjunctival and Limbal Epithelial Cells Using Cord Blood Serum–Supplemented Culture Medium

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PURPOSE. Conventional cell culture methods use fetal bovine serum (FBS) as a growth supplement. The purpose of this study was to develop a xenobiotic-free culture system using umbilical cord blood serum (CBS) as an alternative growth supplement for the cultivation of human conjunctival and limbal epithelial cells.

METHODS. Human conjunctival and limbal epithelial cells were cultivated in varying concentrations of CBS-supplemented medium and compared with FBS-supplemented medium. Bromodeoxyuridine (BrdU) ELISA proliferation assay, colony-forming efficiency (CFE), and a number of cell generations were analyzed. Cytokeratin expression of cultured cells was evaluated (K3, K4, K12, K13, K14, K15, K19, and PanCK). The authors compared the cytokine and growth factor levels in CBS, FBS, and adult serum using antibody array assays.

RESULTS. Conjunctival and limbal cells cultivated in 0.25% CBS- and 0.5% CBS-supplemented culture media demonstrated the highest proliferative capacity in terms of BrdU proliferation assay, CFE, and number of cell generations. These results were comparable to FBS-supplemented medium. Cultured epithelial cells retained their normal cytokeratin expression. Cytokines brain-derived neurotrophic factor, growth-related oncogene, and leptin and growth factors EGF, HGF, FGF-6, IGF-1, PDGF, and IGFBP were present in higher concentrations in CBS than in FBS and adult serum.

CONCLUSIONS. CBS-supplemented culture medium supported the proliferation and differentiation of conjunctival and limbal epithelial cells. CBS contained a higher concentration of growth factors and cytokines than FBS and adult serum. CBS may be a viable and safer alternative to FBS as a growth supplement in the culture medium for culturing epithelial cells, which may have important clinical implications when bioengineering tissues for clinical use. (Invest Ophthalmol Vis Sci. 2011;52:6138–6147) DOI:10.1167/iovs.10-6527

The most widely used method for cultivating epithelial cells requires the use of fetal bovine serum (FBS) as a growth supplement in the culture medium, often in combination with a 3T3 murine feeder layer.1–5 Cultivation of ocular surface epithelial cells (i.e., cornea, limbal, and conjunctival epithelial cells) uses similar FBS-containing methods.6–10 Corneal stem cells and oral epithelial cells cultured in these culture systems have been used in the treatment of severe ocular surface disorders such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and chemical injury.11–17 However, the use of xenobiotic material in the cultivation of epithelial cells is associated with the risks of xenograft rejection and the transmission of zoonotic infections such as bovine spongiform encephalopathy.

In the development of tissue equivalents for clinical transplantation, the ideal culture condition is one that is safe from disease transmission and is able to support the proliferation and differentiation of cells. The elimination of bovine serum in the development of bioengineered tissue equivalents for clinical transplantation would be significantly advantageous because of the reduced risk for disease transmission and xenograft rejection.

Various serum-free culture media have been developed to avoid the use of FBS.18–24 However, most of these serum-free culture systems still require the use of bovine pituitary extract to promote the proliferation of epithelial cells.25–29 We have previously demonstrated the use of serum-free medium.25–29 Human adult serum30 and autologous serum31–33 for the cultivation of conjunctival, corneal, and oral epithelial cells. However, these culture systems were less effective than FBS-supplemented medium for serially passing epithelial cells.30,31

We hypothesized that umbilical cord blood serum could be an alternative to FBS as a growth supplement for culturing epithelial cells for research or clinical purposes. Umbilical cord blood is the blood left in the placenta and in the umbilical cord after the birth of an infant. Cord blood, which contains stem cells, has been used effectively for treating hematopoietic and genetic disorders. Removing the umbilical cord blood is not harmful to the infant, and the blood would normally be thrown away as medical waste. Cord blood serum (CBS) would be the human equivalent of FBS, and CBS is likely to have more growth factors than adult serum to promote cell proliferation. The growth-promoting properties of CBS have been demonstrated in some clinical applications, and CBS eyedrops have been shown to promote faster healing of persistent epithelial defects than autologous serum eyedrops.34,35

We evaluated the use of CBS as a growth supplement for the cultivation of corneal and conjunctival epithelial cells and analyzed the level of growth factors and cytokines in CBS. We demonstrated that CBS supported the proliferation and differentiation of conjunctival and limbal cells, comparable to the FBS-supplemented culture medium. To our knowledge, a study...
of this nature has not been previously described. CBS may be a useful and safer alternative to FBS as a growth supplement in culture media and could potentially be extended for culturing other cell lines. This may have important clinical implications when bioengineering tissues for clinical use because it provides a safer and xenobiotic-free culture system.

MATERIALS AND METHODS

Chemical Reagents and Cell Culture Media

Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F12, keratinocyte serum-free medium, bovine pituitary extract, human epidermal growth factor, penicillin, streptomycin, amphotericin B, dispase, and trypsin-EDTA (EDTA) were purchased from Invitrogen-Gibco (Grand Island, NY), insulin, hydrocortisone, adenine, and cholera toxin were purchased from Sigma-Aldrich (St. Louis, MO); FBS was purchased from Hyclone (Logan, UT). Bromodeoxyuridine (BrdU)-ELISA cell proliferation assay kit was purchased from Amersham Biosciences (Freiburg, Germany). Primary antibodies used in this study and their sources are listed in Table 1. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse and rabbit anti-goat IgG secondary antibodies were purchased from DakoCytomation (Carpinteria, CA). Propidium iodide, optimal cutting temperature (OCT) freezing compound (Tissue-Tek), and mounting medium were purchased from Chemicon (Temecula, CA), Sakura Finetek (Torrance, CA), and DakoCytomation, respectively.

Preparation of Human Samples

Human conjunctival and limbal biopsy samples and cord blood were obtained from prospective surgery patients after they provided proper informed consent and approval, according to the statutes of the Institutional Review Board of the Singapore National Eye Center and Singapore General Hospital. All experimental procedures used here complied with the guidelines of the Declaration of Helsinki in Biomedical Research Involving Human Subjects.

Preparation of Cord Blood Serum

CBS was obtained from patients undergoing routine obstetric delivery by cesarean section. Exclusion criteria were an infectious disease in either the mother or the fetus or a medical history in which maternal cord blood was used for therapeutic purpose. Cord blood was collected at the time of delivery under aseptic conditions. After clamping and cutting of the umbilical cord, the blood from the placental side of the umbilical cord was collected in a sterile collection bag (250-mL Transfer Pack; JMS, Singapore). The blood was then allowed to clot at 4°C for at least 4 hours. Then the serum was decanted away from the clot and was processed by centrifugation at 1200g for 20 minutes to separate the serum from remaining cells. The supernatant was collected and sterilized with a 0.2-μm filter. Serum was then stored in sterile tubes at −80°C and was used for the experiments within 6 months of storage.

Isolation and Cultivation of Primary Conjunctival Epithelial Cells

Conjunctival biopsy specimens were obtained from consenting patients undergoing routine surgery for nasal pterygium or cataract. A small (1 × 3 mm) piece of normal conjunctiva was removed from the superior bulbar region, 10 to 15 mm away from the limbus. Patients with extensive pterygia or with any other ocular surface disorders that might involve the area of biopsy were excluded from the study. The biopsied conjunctival tissue was transported to the laboratory in the medium (Leibovitz L-15; Invitrogen-Gibco). The conjunctiva was washed in phosphate-buffered saline (PBS) three times, 5 minutes each, and then washed in the antibiotics solution containing penicillin 200 IU/mL, streptomycin 200 ng/mL, and amphotericin B 200 ng/mL.

For cell suspension monolayer cultures, the tissues were placed in 1.2 U/mL dispase and incubated at 37°C for 2 hours. The epithelial sheet was removed by gentle scraping and separated into single cells by 0.25% trypsin and 0.02% EDTA for 8 minutes. The cell pellets collected after centrifugation were plated onto culture dishes containing a mitomycin C-treated 3T3 feeder layer at a density of 10^4 cells/cm² in cell culture dishes. For explant cultures, the tissue was cut into 1-mm² pieces and was cultivated as explants on 35-mm tissue culture dishes.

The cultures were initiated in media consisting of a 1:1 mixture of DMEM and Ham’s F12 supplemented with 5% FBS, 10 ng/mL human epidermal growth factor, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.1 mM cholaer toxin, 50 IU/μL penicillin, 50 μg/μL streptomycin, and 50 ng/mL amphotericin B. After the second day, the culture medium was changed to serum-free medium (keratinocyte serum-free medium supplemented with 5 ng/mL human epidermal growth factor, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 50 IU/μL penicillin, 50 μg/mL streptomycin, and 50 ng/mL amphotericin B) to wash out the FBS for at least 6 days. On reaching 70% to 80% confluence, the 3T3 feeder layer was removed with 0.02% EDTA for 5 minutes, and the epithelial cells were subcultured by enzymatic disaggregation with 0.25% trypsin and 0.02% EDTA.

The cells were subsequently subcultured in the various culture conditions investigated at a density of 3 to 4 × 10^4 cells/cm² on a mitomycin C-treated 3T3 feeder layer. The culture conditions were as follows: (1) Conventional FBS-supplemented culture medium (positive control) consisting of a basal medium containing a 1:1 mixture of DMEM and Ham’s F12 supplemented with 5% FBS, 10 ng/mL human epidermal growth factor, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 50 IU/μL penicillin, 50 μg/mL streptomycin, and 50 ng/mL amphotericin B. (2) CBS-supplemented culture media, consisting of a basal medium containing a 1:1 mixture of DMEM and Ham’s F12 supplemented with different concentrations of CBS (0.05%, 0.1%, 0.25%, 0.5%, 1%, and 2.5%). 10 ng/mL human epidermal growth factor, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 50 IU/mL of penicillin, 50 μg/mL streptomycin, and 50 ng/mL amphotericin B. (3) Plain basal

### Table 1. Antibodies and Source

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medium (negative control) consisting of a 1:1 mixture of DMEM and Ham’s F-12.

The cells were incubated at 37°C under 5% CO₂ and 95% air, with a medium change every 2 days. Cultures were monitored under an inverted phase-contrast microscope (Axiovert; Carl Zeiss Meditec, Inc., Oberkochen, Germany). All experiments were carried out in triplicate.

Isolation and Cultivation of Primary Limbal Epithelial Cells

Human limbal rims were obtained from the Singapore Eye Bank after the central corneal button was used for corneal transplantation. The tissues were washed as described. After careful removal of the corneal endothelium, iris, excessive sclera, conjunctiva, and subconjunctival tissue, the limbal rings were exposed to 1.2 U/mL dispase and incubated at 37°C for 2 hours. The epithelial sheet was removed by gentle scraping and separated into single cells by 0.25% trypsin and 0.02% EDTA for 8 minutes. Cells were plated at 3 to 4 × 10⁴ cells/cm² in cell culture dishes containing a mitomycin C-treated 3T3 feeder layer as described. On reaching 70% to 80% confluence, the 3T3 feeder layer was removed with 0.02% EDTA for 5 minutes, and the epithelial cells were subcultured by enzymatic disaggregation with 0.25% trypsin and 0.02% EDTA.

The cells were subsequently subcultured in conventional FBS-supplemented medium (positive control), CBS-supplemented media at various concentrations (0.05%, 0.1%, 0.25%, 0.5%, 1%, and 2.5%), and plain basal media (negative control), as described.

Preparation of 3T3 Feeder Layers

The 3T3 cell line was maintained using DMEM and 10% FBS. At 70% to 80% confluence, 3T3 cells were treated with 4 μg/mL mitomycin C for 2 hours at 37°C under 5% O₂ and 95% CO₂ to arrest cell growth. After incubation, the cells were washed with PBS three times for 5 minutes each, then trypsinized using 0.25% trypsin and 0.02% EDTA for 5 minutes, and replated at a density of 2.4 × 10⁴ cells/cm². These cells were used within 24 hours of preparation.

FIGURE 1. Representative phase-contrast appearance of passage 1 human conjunctival (A–D) and limbal (E–H) epithelial cells in culture. (A, C) Conjunctival epithelial cells cultivated in 0.5% CBS-supplemented medium on day 4 (A) and day 6 (C) after plating. (B, D) Conjunctival epithelial cells cultivated in FBS-supplemented medium on day 4 (B) and day 6 (D). (E, G) Limbal epithelial cells cultivated in 0.25% CBS-supplemented medium on day 3 (E) and day 6 (G). (F, H) Limbal epithelial cells in FBS-supplemented medium on day 3 (F) and day 6 (H). Scale bar, 100 μm.
Quantitation of Growth and Proliferative Capacity of Cells

Several proliferation assays were used to assess the proliferative capacity of cells cultured under the various culture conditions.

**BrdU ELISA Cell Proliferation Assay.** The proliferative capacity of passage 2 human conjunctival and limbal epithelial cells was determined by a BrdU-ELISA cell proliferation assay. The epithelial cells were seeded in 96-well plates at a density of 1600 cells/well with 100 μL culture media. Cultured cells were incubated with 10 μM BrdU labeling solution (Amersham Biosciences, Freiburg, Germany) for 20 hours at 37°C, followed by washing with 200 μL PBS containing 1% serum. Then the cells were fixed and incubated with 100 μL monoclonal antibody against BrdU for 2 hours, followed by 100 μL peroxidase substrate per well. The BrdU absorbance in each well was measured directly using a spectrophotometric microplate reader (Tecan, Grodgr, Austria) at a test wavelength of 450 nm and a reference wavelength of 490 nm. The respective plain basal media without cells were used as negative controls. The optical density (OD) reading for the negative control was subtracted from the sample readings. This gave us a measure of the degree of cell proliferation, which we termed the proliferation index.

**Colony-Forming Efficiency Assay.** The clonal growth ability of cultured cells was determined by the colony-forming efficiency. Subcultured cells were plated at a density of 1000 cells in 60-mm culture dishes already containing a feeder layer of ST3 cells. A colony was defined as a group of eight or more contiguous cells. The cells were found attached from day 2, and the colonies were fixed on day 10 using 2% formaldehyde, stained with rhodamine B, and counted under a dissecting microscope. The colony-forming efficiency was determined as follows:

\[
\text{Colony-forming efficiency} \% = \frac{\text{Colonies formed at end of growth period}}{\text{Total number of viable cells seeded}} \times 100\%
\]

**Number of Cell Generations.** Cells were subcultured when they achieved 70% to 80% confluence, as described. They were plated onto tissue culture dishes at a seeding density of 3 to 4 × 10^4 cells/cm². The number of cell generations, N, was calculated as follows:

\[
x = \log_{N_0}/N_0\times \log_2(N/N_0),
\]

where N is the total number of cells harvested at subculture, and N₀ is the number of viable cells seeded. We analyzed the number of cell generations for passage 1 and 2 cells.

**Immunocytochemistry**

To induce the differentiation of conjunctival and limbal epithelia, the basal culture media were changed from 1:1 DMEM/Ham’s F-12 ratio to 3:1 DMEM / Ham’s F-12 ratio with a resultant increase in calcium concentration from 0.9 mM to 1.3 mM. The cells were incubated in the differentiation media for 6 days.

To characterize the cells, cultivated conjunctival and limbal epithelial cells were fixed with 90% methanol and incubated for 1 hour with monoclonal antibodies to keratins K3, K4, K12, K13, K14, K19, and PanCK, listed in Table 1. We also evaluated the presence of the limbal and conjunctival basal cell marker Keratin 15 (Leica Biosystems, Newcastle, UK). Pan-cytokeratin was used as a positive control, and IgG was used as a negative control. The staining of keratin 12 and goat IgG were detected by incubation with FITC-conjugated rabbit anti-goat IgG secondary antibody and the rest with FITC-conjugated donkey anti-mouse IgG secondary antibody. This was counterstained with propidium iodide or DAPI and mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

**Antibody Array**

Three different pooled samples of CBS, FBS, and adult serum were analyzed for the level of cytokines and growth factors (RayBio Human Cytokine Antibody Array V and RayBio Human Growth Antibody Array I; RayBiotech Inc., Norcross, GA). We analyzed the following growth factors: fibroblast growth factor 6 (FGF-6), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein (IGFBP-1, IGFBP-2, IGFBP-4), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF-AA, PDGF-AB, PDGF-BB). The following cytokines were analyzed: brain-derived neurotrophic factor (BDNF), growth-related oncogene (GRO), and leptin.

The various serum samples were used to measure the cytokine and growth factor levels using the array membranes according to the manufacturer’s instructions. The total amount of serum for each membrane was 50 μg. Each experiment was conducted in triplicate. The intensities of signals on the x-ray films exposed to the membranes were quantified, and the total densities of the signals were normalized according to the signal densities of positive controls in each membrane.

**Statistical Analysis**

The Student’s unpaired t-test was used to compare the means of the various culture conditions for BrdU cell proliferation index, colony-forming efficiency, and cell generations as well as the cytokine and growth factor levels (Microsoft Excel, XP Professional Version; Microsoft, Redmond, WA). Test results were reported as two-tailed P values, and P < 0.05 was considered statistically significant.
RESULTS

Morphology of Epithelial Cells in Different Culture Conditions

Conjunctival epithelial cells cultured in 0.25% and 0.5% CBS formed colonies consisting of 20 to 40 cells on day 3, and these cells continued to proliferate and formed cells that were tightly arranged (Fig. 1A). Limbal epithelial cells grown in 0.25% and 0.5% CBS-supplemented media also formed colonies with 15 to 30 tightly arranged cells on day 3 (Fig. 1E). By day 6 to 8, both conjunctival and limbal cells achieved 70% to 80% confluence, and the cell colonies exhibited a cobble-stone morphology with small, ovoid, or round cells (Figs. 1C, 1G). Cells cultured in 2.5% and 5% CBS proliferated more slowly and formed only small colonies with elongated cells. Epithelial cells cultivated in 5% FBS-containing medium also formed colonies from day 3 and achieved 70% to 80% confluence by day 6 to 8 (Figs. 1B, 1D, 1F, 1H). However, these epithelial cells appeared more elongated than the cells cultured in CBS.

Growth and Proliferative Capacity

BrdU ELISA Cell Proliferation. Conjunctival cells cultured in 0.25% and 0.5% CBS-supplemented media had proliferation indices of 1.23 ± 0.16 and 1.17 ± 0.14, respectively, whereas 5% FBS-supplemented cultures had a proliferation index of 1.3 ± 0.21 (Fig. 2A). These differences were not statistically significant (t-test; P > 0.05). Cells cultured without serum had a proliferation index of 0.4 ± 0.13, which was significantly lower than cells cultured in CBS- and FBS-supplemented media (t-test; P < 0.05).

Limbal cells cultured in 0.25% and 0.5% CBS-supplemented cultures had proliferation indices of 1.3 ± 0.184 and 1.45 ± 0.24, respectively, whereas 5% FBS-supplemented culture had a proliferation index of 1.46 ± 0.14 (Fig. 2B). These differences were not statistically significant. Limbal cells cultured without serum had a proliferation index of 0.49 ± 0.15, which was significantly lower than cells cultured in CBS- and FBS-supplemented media (t-test; P < 0.05).

For both conjunctival and limbal cells, increasing or decreasing the concentration of CBS beyond 0.25% and 0.5% resulted in a reduction in the proliferative capacity of cells and a drop in proliferation indices (Figs. 2A, 2B).

Clonal Growth Assay of Cultivated Epithelial Cells.

Conjunctival cells cultivated in 0.25% and 0.5% CBS-supplemented media had CFEs (24.2% ± 3.5% and 27.4 ± 3.2%, respectively) that were similar to those of cells cultured in 5% FBS-supplemented media (27.5% ± 4.1%; Fig. 3A). A similar result was observed with limbal cells; cells cultivated with 0.25% and 5% CBS-supplemented media had CFEs (12% ± 1.6% and 10.6% ± 1.8%, respectively) that were comparable to 5% FBS-supplemented cultures (CFE, 11.9% ± 1.7%; Fig. 3B).

For both conjunctival and limbal cells, increasing or decreasing the concentration of CBS beyond 0.25% and 0.5% resulted in a drop in CFE. Conjunctival and limbal cells cul-

Figure 3. Colony-forming efficiency of cells cultivated in various conditions. The graphs show the percentages of passage 1 (A) conjunctival and (B) limbal epithelial cells forming colonies after 10 days of plating. Cells cultivated in 0.25% and 0.5% CBS-supplemented medium had the highest CFEs. This was comparable to the 5% FBS-supplemented medium (n = 9).

Figure 4. The number of generations for passage 1 and passage 2 (A) conjunctival and (B) limbal epithelial cells in various culture conditions. Cells cultivated in 0.25% and 0.5% CBS-supplemented media had highest number of cell generations, comparable to that of 5% FBS-supplemented medium (n = 11).
tured without serum had CFEs that were significantly lower than serum-supplemented cultures.

**Total Number of Cell Generations**

The number of cell generations achieved with passage 1 conjunctival cells in 0.25% or 0.5% CBS-supplemented cultures was 5.9 ± 0.7 or 6.3 ± 0.4 respectively, which was comparable to that of 5% FBS-supplemented cultures (6.9 ± 0.5; Fig. 4A). Passage 2 conjunctival cells cultivated in 0.25% and 0.5% CBS-supplemented media had similar numbers of cell generations (4.6 ± 1.0 and 4.6 ± 0.4, respectively) compared with 5% FBS-supplemented culture (4.7 ± 0.9). These differences were not statistically significant (t-test, P > 0.05).

The number of cell generations achieved with passage 1 limbal cells in 0.25% or 0.5% CBS-supplemented conditions was 6.18 ± 0.59 and 6.06 ± 0.5 respectively (Fig. 4B), which was similar to that of 5% FBS-supplemented media (6.66 ± 0.4). These differences were not statistically significant (t-test, P > 0.05). Passage 2 limbal cells cultured in 0.25% or 0.5% CBS-supplemented media had results similar to those of 5% FBS-supplemented cultures, for which the numbers of cell generations were 4.3 ± 0.5, 3.9 ± 0.1, and 3.9 ± 0.8, respectively. These differences were not statistically significant (t-test, P > 0.05). CBS concentrations that were higher or lower than 0.25% and 0.5% resulted in a decline in number of cell generations.

**Immunocytochemistry of Cultivated Epithelial Cells**

Conjunctival epithelial cells cultured in CBS- and FBS-supplemented media expressed the normal conjunctival phenotype with positive expression of keratins K4, K13, K19, and Pan-CK (Fig. 5). The corneal-specific keratins K3 and K12 were not expressed by the conjunctival cells. Limbal epithelial cells cultivated in CBS- and FBS-supplemented media expressed keratins K3, K12, K14, and Pan-CK (Fig. 6), which is consistent with the normal

**Figure 5.** Immunocytochemistry of cultivated conjunctival epithelial cells in CBS-supplemented (A, C, E, G) and FBS-supplemented (B, D, F, H) media. (G, H) Antibody against Pan-CK was used as a positive control. Conjunctival cells cultivated in both CBS- and FBS-supplemented media stained positively for keratins (A, B) K4, (C, D) K13, and (E, F) K19. Scale bar, 500 μm.
limbal epithelium. Expression of the limbal basal cell marker keratin 15 was similar in CBS- and FBS-supplemented media (Figs. 6I, 6J).

Growth Factors and Cytokine Array

Analyzing cytokine levels in CBS revealed that BDNF, GRO, and Leptin were present in significantly higher concentrations in CBS compared with FBS (Fig. 7A). An analysis growth factor levels showed that growth factors such as FGF-6, HGF, IGF-1, IGFBP-1, IGFBP-2, IGFBP-4, EGF, and PDGF were also found in much higher concentrations in CBS than in FBS (Figs. 7B, 7C).

We found that cytokine levels (BDNF, GRO, and Leptin) in CBS were present in higher concentrations in CBS than in adult serum (Fig. 8A). In addition, CBS had much higher concentra-
DISCUSSION

The preferred method for cultivating ocular surface epithelial cells requires the use of bovine serum in the culture system. However, the use of bovine material in the culture media, such as FBS or bovine pituitary extract, is a major concern throughout Europe because certain infectious diseases, such as bovine spongiform encephalopathy, cannot be detected by any known in vitro tests. As such, European regulatory authorities prefer that cells cultured for clinical use avoid the use of bovine products so as to reduce the risk of disease transmission and rejection.

Various attempts have been made to improve the cell culture system to one that is free of bovine and animal products. A serum-free culture system has been used for ex vivo expansion of conjunctival epithelial cells for clinical transplantation, but it still requires bovine pituitary extract to promote epithelial proliferation and this has a lower capacity to promote the serial propagation than FBS-containing medium. Although the use of serum-free formulations without bovine pituitary extract has been reported for corneal epithelial cell cultivation, serial transfer and stratified epithelial organization were not demonstrated, and cell cultures underwent altered differentiation and developed cornified envelopes. We previously investigated the use of human serum as an alternative to FBS. However, cells cultivated in human serum had limited proliferative capacity and were contaminated with fibroblasts.

In this study, we evaluated the use of CBS as an alternative growth supplement for FBS in a cell culture system. The ability of the cell culture medium to support the proliferation of cells is shown in Figure 7A. CBS contained higher levels of growth factors FGF-6, HGF, IGF-1, IGFBP-1, IGFBP-2, IGFBP-4, EGF, and PDGF than did FBS (B, C).

FIGURE 7. Comparison of cytokine and growth factor profiles of CBS and FBS. The levels of BDNF, GRO, and leptin were higher in CBS than in FBS (A). CBS contained higher levels of growth factors FGF-6, HGF, IGF-1, IGFBP-1, IGFBP-2, IGFBP-4, EGF, and PDGF than did FBS (B, C).

FIGURE 8. Comparison of cytokine and growth factor profiles of CBS and adult serum (AS). The levels of BDNF, GRO, and leptin in CBS were higher than those in adult serum (A). CBS contained higher levels of growth factors FGF-6, HGF, IGF-1, IGFBP-1, IGFBP-2, IGFBP-4, EGF, and PDGF than did adult serum (B, C).
is a critical factor when analyzing the usefulness and efficacy of the culture medium, especially if it is for clinical transplantation, in which continuous tissue regeneration is important. We demonstrated that limbal and conjunctival epithelial cells cultivated in CBS had a proliferative capacity comparable to that in FBS in terms of BrdU proliferation assay, colony-forming efficiency, and number of cell generations. The cell cultures system maintained the normal phenotypic expression of cytokerines for each epithelial cell type; the cultivated conjunctival epithelial cells expressed keratins K4, K13, and K19 and the cultivated limbal cells expressed keratins K3, K12, and K14.

CBS may be viewed as the human equivalent of FBS because both are derived from blood taken at the highly proliferative phase of early development. Growth factors in CBS are likely to be present in higher concentrations than in adult serum so as to facilitate the growth and development of the fetus. We demonstrated that CBS had a significantly higher concentration of growth factors (EGF, HGF, FGF-6, PDGF, IGFBP-1, IGFBP-2, and IGFBP-6) and cytokines (BDNF, GRO, and Leptin) than FBS. In addition, CBS contained a significantly higher concentration of growth factors (EGF, HGF, FGF-6, PDGF, IGFBP-1, IGFBP-2, and IGFBP-6) and cytokines (BDNF, GRO, and Leptin) than adult serum. Growth factors EGF, HGF, FGF-6, PDGF, IGFBP-1, IGFBP-2, and IGFBP-6 have been shown to promote the proliferation and differentiation of different types of cells.\textsuperscript{36–40} EGF and FGF have been reported as mitogenic factors for both limbal and corneal epithelia.\textsuperscript{40,41} Cytokines BDNF and GRO have been demonstrated to have growth-regulatory properties.\textsuperscript{42,43}

The presence of higher concentrations of these growth factors and chemokines in CBS may explain why CBS is more efficacious in supporting cell proliferation than adult serum or FBS. As such, a relatively low concentration of CBS (0.25%–0.5%) was found to be optimal in enhancing the in vitro proliferation of human ocular surface epithelial cells. In contrast, previous studies on human serum and autologous serum required the use of 5% to 10% concentrations of human serum to promote cell proliferation.\textsuperscript{30–33} Previous studies have shown that the interaction and effects of these growth factors in modulating cell growth and differentiation is dynamic and complex.\textsuperscript{44,45} Depending on the relative concentrations of these components, cell proliferation or differentiation may be preferentially induced. Cells that are exposed to a higher concentration or a longer duration of certain growth factors may become less proliferative or more differentiated.\textsuperscript{46} In our study, higher concentrations of CBS were associated with a drop in cell proliferative capacity, probably caused by the interaction of the various growth factors and cytokines that could have preferentially induced cell differentiation.

Requiring only a low concentration of CBS to promote cell proliferation has advantages. The low concentration of CBS helps to minimize the amount of allogeneic material in the culture medium. As such, this supports the practical and economic viability of using CBS as a growth supplement because a single collection of cord blood serum would be sufficient for supporting the ex vivo cell expansion of ocular surface cells that may be used in more than 20 to 30 clinical transplantations. In addition, umbilical cord blood serum is easily obtainable from the routinely discarded afterbirth, and there are no ethical concerns related to its use.

In summary, our study is the first to demonstrate the effective use of CBS in supporting the proliferation and differentiation of both conjunctival and limbal epithelial cells. CBS may be a viable and safer alternative to FBS as a growth supplement in culture media and could potentially be used for culturing other epithelial cell lines. The elimination of animal and nonhuman material from the culture system offers significant advantages over existing FBS-supplemented culture procedures when culturing cells for clinical use because it reduces the risk for transmission of zoonotic infection and xenograft rejection. These findings bring us one step closer to the development of a safe and effective xenobiotic-free bioengineered tissue equivalent for clinical transplantation.

References

Conjunctival and Limbal Cell Culture with Cord Blood Serum 6147


