A serum-free medium formulation supporting growth of human umbilical cord vein endothelial cells in long-term cultivation

Regina Labitzke & Peter Friedl*

Institut für Biochemie, Technische Universität Darmstadt, Petersenstraße 22, 64287 Darmstadt, Germany

(* Author for correspondence; E-mail: di58@pop.tu-darmstadt.de)

Received 20 January 2000; accepted 8 June 2000

Key words: cells, cultured, endothelium, serum-free medium

Abstract

A serum-free medium formulation – TUD-1 – was developed supporting growth of HUVEC in tissue culture. Special features of the basal medium formulation are highly elevated levels of glutamine and serine as well as the inclusion of N-acetylcysteine and phosphoascorbic acid. The cellular mitogenic needs are satisfied by bFGF, VEGF, EGF and liver growth factor. Further hormone supplementation consists of insulin and hydrocortisone. A protocol for serum-free passage of HUVEC was established for serum-free long-term cultivation of freshly isolated HUVEC for up to 20 cumulative population doublings without significant differences in final cell density compared to controls cultivated with serum.

Abbreviations: bFGF, basic fibroblast growth factor; EC, endothelial cells; EGF, epithelial growth factor; HUVEC, Human umbilical cord vein endothelial cells; PBS, phosphate-buffered saline without magnesium and calcium; 20x SSC, solution of 3 M NaCl and 0.3 M sodium citrate; SFM, serum-free medium; VEGF, vascular endothelial growth factor.

Introduction

Studies of human endothelial cells have mainly been accomplished with endothelial cells derived from the umbilical cord vein (HUVEC). The tissue was readily available, and endothelial cells could be easily isolated (Jaffe et al., 1973; Gimbrone et al., 1978). However, the long-term cultivation of HUVEC was quite demanding and had to be considerably improved by stepwise optimisation of culture conditions (Gospodarowicz et al., 1981; Vlodavsky et al., 1979; Maciag et al., 1981; Knedler and Ham, 1987; Friedl et al., 1989). For the establishment of a defined culture regimen serum has to be excluded from the medium formulation. Various publications claimed the development of a serum-free medium for vascular EC or even HUVEC (de Groot et al., 1983; Fujii et al., 1983; Hoshi and McKeehan, 1984; Weiss et al., 1990). However in our hands none of the above cited media was able to promote growth of HUVEC in a long-term cul-

tivation nor could we find further publications where the successful use of these media had been described. Recently the use of a serum-free medium for vascular EC was published, however with unknown composition of the medium (Gorfien et al., 1993). The medium promoted serum-free growth of BAEC and allowed survival of HUVEC. Here we describe the use of a serum-free medium for the long-term cultivation of HUVEC with a detailed description of its composition and handling.

Materials and methods

Materials

Trypsin (L 2133) – Biochrom KG seromed® (Berlin/Germany); EGF (19005) – Eurobio (Raunheim/Germany); β -mercaptoethanol (63690) – Fluka Chemie AG (Buchs/Switzerland); L-glutamine (21051-024), recombinant bFGF (13256-029) – GibcoBRL

Table 1. Medium composition of TUD-1

	(mg l ⁻¹)	(mol l ⁻¹)
Inorganic salts		
CaCl ₂ (anhydr.)	99.11000	8.93 E-4
CuSO ₄ ·5H ₂ O	0.00125	5.01 E-9
FeSO ₄ ·7H ₂ O	0.41700	1.50 E-6
KCl	276.80000	3.71 E-3
KNO ₃	0.03800	3.76 E-7
MgCl ₂ (anhydr.)	28.61000	3.01 E-4
MgSO ₄ (anhydr.)	289.60000	2.41 E-3
MnSO ₄	0.22000	1.46 E-6
NaCl	6052.00000	1.04 E-1
Na ₂ HPO ₄	71.02000	5.00 E-4
NaH ₂ PO ₄	54.30000	4.53 E-4
Na ₂ SeO ₃ ·5H ₂ O	0.01260	4.79 E-8
ZnSO ₄ ·7H ₂ O	3.88200	1.35 E-5
Vitamins		
Biotin	0.01015	4.15 E-8
D-Pantothenic acid (Ca salt)	2.24000	4.70 E-6
Choline chloride	8.98000	6.43 E-5
Folic acid	2.65000	6.00 E-6
i-Inositol	12.60000	6.99 E-5
Niacinamide	2.01850	1.65 E-5
Pyridoxal·HCl	2.00000	9.82 E-6
Pyridoxine·HCl	0.03100	1.51 E-7
Riboflavin	0.21900	5.82 E-7
Thiamine·HCl	2.17000	6.43 E-6
Vitamin B ₁₂	0.68650	5.06 E-7
Amino acids		
L-Alanine	22.45000	2.52 E-4
L-Arginine·HCl	219.50000	1.04 E-3
L-Asparagine·H ₂ O	40.71000	2.71 E-4
L-Aspartic acid	33.65000	2.53 E-4
L-Cysteine·HCl·H ₂ O	17.56000	1.00 E-4
L-Cystine·2HCl	45.62000	1.46 E-4
L-Glutamic acid	79.85000	5.43 E-4
L-Glutamine*	1314.90000	9.00 E-3
Glycine	18.75000	2.50 E-4
L-Histidine·HCl·H ₂ O	45.98000	2.19 E-4
L-Isoleucine	54.47000	4.15 E-4
L-Leucine	59.05000	4.50 E-4
L-Lysine·HCl	119.25000	6.53 E-4
L-Methionine	20.00000	1.34 E-4
L-Phenylalanine	77.48000	4.69 E-4
L-Proline	37.25000	3.24 E-4
L-Serine	76.25000	7.26 E-4

* These components are prepared as sterile stock solutions and are added to the sterile liquid basal medium.

Table 1. (continued)

	(mg l ⁻¹)	(mol l ⁻¹)
Amino acids		
L-Threonine	53.45000	4.49 E-4
L-Tryptophan	9.02000	4.42 E-5
L-Tyrosine (di-Na-salt)	55.99000	2.49 E-4
L-Valine	64.85000	5.54 E-4
Growth factors		
Epidermal Growth Factor (EGF), human*	0.01000	
Fibroblast Growth Factor (bFGF), human*	0.01000	
Liver Growth Factor (gly-his-lys)*	0.05000	
Vascular Endothelial Growth Factor (VEGF ₁₂₁)*	0.00500	
Other organic compounds		
N-Acetyl-L-Cysteine	40.80000	2.50 E-4
Ascorbic acid-phosphate (Mg salt, n H ₂ O)		1.00 E-3
BSA (Fraction V)/oleic acid*	200/1.60	
D-Glucose	3151.00000	1.75 E-2
Hydrocortisone*	1.00000	2.76 E-6
Hypoxanthine (Na salt)	3.68300	2.71 E-5
Insulin, bovine*	5.00000	
Linoleic acid	0.04200	1.50 E-7
Lipoic acid	0.10500	5.09 E-7
2-Mercaptoethanol*		1.25 E-5
Pyruvic acid (Na salt)	55.00000	5.00 E-4
Putrescine·2HCl	0.08050	5.00 E-7
Thymidine	0.36500	1.51 E-6
Transferrin, human*	5.00000	
Buffers and indicators		
HEPES	2979.00000	1.25 E-2
NaHCO ₃	2100.00000	2.50 E-2
Phenol red	4.30000	1.21 E-5
Carbon dioxide		5 %

* These components are prepared as sterile stock solutions and are added to the sterile liquid basal medium.

Life Technologies (Karlsruhe/Germany); transferrin, human (823431) – ICN (Meckenheim/Germany); EDTA disodium salt (8418) – Merck (Darmstadt/Germany); oleic acid (72131) – Roth (Karlsruhe/Germany); BSA, fraction V (47299), liver growth factor (51535) – SERVA (Heidelberg/Germany); VEGF – GBF (Braunschweig/Germany); gelatin (G

1890)), DAPI (D 1388), hydrocortisone (H 0888), insulin (I 5500), methylcellulose (M 0512), potassium chloride (P 5405), potassium phosphate monobasic (P 5655), sodium bicarbonate (S 5761), sodium chloride (S 5886), sodium phosphate dibasic (5136) – SIGMA (Deisenhofen/Germany); tissue culture water (Milli-Q UF Plus) – Millipore (Eschborn/Germany); tissue culture plates and flasks from Greiner (Frickenhofen/Germany) or Falcon® Becton Dickinson (Heidelberg/Germany); sterile filter (Sartolab P, 18053 D) – Sartorius (Göttingen/Germany); microscope (Nikon Diaphot TMD) – Nikon (Düsseldorf/Germany).

Solutions and buffers

PBS (phosphate buffered saline): 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄ dissolved in 1 l of tissue culture water and sterilized by autoclaving. **Trypsin/EDTA**: 0.05% (w/v) trypsin, 0.02% EDTA in PBS. **Methylcellulose**: 0.1% (w/v) in basal medium; the appropriate amount of methylcellulose is put in an empty glass bottle together with a magnet stirring bar and sterilized by autoclaving. At room temperature the corresponding volume of sterile basal medium is added and the methylcellulose dissolved by stirring at 4 °C for 1–2 days. **Gelatin**: 1% (w/v) in tissue culture water dissolved by autoclaving.

Preparation of serum-free medium

The serum-free medium TUD-1 is purchased as a customer's formulation from GIBCO except for those components marked in Table 1. The medium powder for 1 l of medium is dissolved in 800 ml of water (tissue culture quality), 2.1 g NaHCO₃ are added, the volume adjusted to 1 l and the medium passaged through a sterile filter (0.20 µm). After a control incubation at 37 °C for 3 days the medium is stored at 4 °C until completion with the different supplements. These are prepared as sterile stock solutions (see Table 2) and diluted into the basal medium to the final concentration according to Table 1. The complete medium is stored at 4 °C and shall be used up within 2 weeks. The stock solutions are stored at –20 °C.

Preparation of conditioned medium

Confluent monolayers of HUVEC are cultivated with 0.2 ml TUD-1 cm^{–2}. After 24 h the medium is aspirated, centrifuged at 800 × g for 5 min and stored at –20 °C.

Table 2.

Supplement	Stock solution
L-glutamine	200 mM in basal medium
EGF	10 µg ml ^{–1} in PBS
bFGF	40 µg ml ^{–1} in PBS
Liver growth factor	100 µg ml ^{–1} in PBS
VEGF	50 µg ml ^{–1} in 50 mM Na-phosphate buffer pH 6.0 + 100 µg BSA/ml
Hydrocortisone	1 mg ml ^{–1} PBS/ethanol (1:1) (dissolve in ethanol and dilute with PBS)
Insulin	10 mg ml ^{–1} in PBS (add dropwise 2 N HCl until dissolved)
Transferrin	5 mg ml ^{–1} in PBS
BSA/oleic acid	50 mg BSA + 0.4 mg oleic acid pro ml in PBS (Jäger et al., 1988)
β-Mercaptoethanol	purum

Coating of tissue culture flasks

The bottom surface of tissue culture flasks was covered with a solution of 1% [w/v] gelatin in tissue culture grade water for 1 h at room temperature. The culture vessel was once washed with PBS and immediately used.

Isolation and cultivation of HUVEC

Safety guidelines were applied to avoid a potential personnel contamination. All materials and liquids were sterilised. HUVEC were isolated and cultivated as described previously (Friedl et al., 1989). With serum-free medium cultures are not washed between medium exchanges.

Passaging of serum-free HUVEC cultures

The medium is aspirated and the cell layer once washed with 0.2 ml PBS per cm² of culture area. The cell layer is covered by 0.04 ml trypsin/EDTA per cm² of culture area, the solution immediately removed again, leaving a thin film of trypsin/EDTA upon the cells for 1–5 min until the cells detach. Trypsinisation is stopped by adding 0.12 ml methylcellulose per cm² of culture area. Methylcellulose is essential as a stabilisator for the cells; omission of methylcellulose leads to considerable loss of cells thus unabling expansion of the culture. The cells are carefully suspended, sedimented by centrifugation at 110 × g for 5 min, resuspended in SFM and seeded at a density of 20 000 cells per cm² into a gelatinized tissue culture flask.

Figure 1. Growth of HUVEC in TUD-1 and in serum-containing medium. HUVEC were seeded into a 24-well plate at $20\,000\text{ cells cm}^{-2}$ and cultivated with 0.5 ml medium TUD-1 (*), respectively serum-containing medium (Δ) per well with medium exchange after 2–3 days. At the indicated times cell growth was assayed by the DAPI-test. Each point represents the arithmetic mean of three individual wells; error bars depicts the SEM.

DAPI-test of cell growth

The test was stopped by aspirating the medium, washing once with PBS and adding $500\text{ }\mu\text{l}$ lysis solution (0.02% SDS, $1\times\text{ SSC}$). The plate was shaken for 15 min at ambient temperature, $500\text{ }\mu\text{l}$ of DAPI staining solution ($2\text{ }\mu\text{g ml}^{-1}$ DAPI in PBS) were added and the plate was again agitated for 5 min. The DNA-DAPI fluorescence of the cell lysates was measured in a FLUOSTAR (BMG LabTechnologies, Offenburg) fluorescence reader (excitation wavelength 355 nm, emission wavelength 460 nm, gain setting 30). It was assured that the fluorescence signal gave a linear response to the cell number for the range of 400 to 1×10^5 cells.

Results and discussion

Performance of the medium TUD-1

First of all it should be stressed that the below described performance of the presented medium is based on the use of gelatinised tissue culture plastics from selected manufacturers (see the Section Material and Methods, and Friedl et al., 1989). Figure 1 shows a growth curve for primary HUVEC in either TUD-1 or a previously described medium optimized for the growth of HUVEC (Friedl et al., 1989). HUVEC grew comparably well in the serum-containing medium and in the serum-free medium TUD-1. A picture

A

B

Figure 2. Confluent monolayers of HUVEC in TUD-1 and in serum-containing medium. HUVEC were seeded into tissue-culture flasks and grown to confluence in medium TUD-1 (A) respectively serum-containing medium (B).

of confluent HUVEC cultures is shown in Figure 2; there is a slight difference in morphology: whereas the serum-containing culture shows a prominent cobblestone pattern, the shape of HUVEC in the medium TUD-1 is a bit more elongated. However the final cell densities are in both cases satisfying: $0.8\text{--}1.2 \times 10^5\text{ cells cm}^{-2}$ for the serum-containing medium and $0.8\text{--}1.1 \times 10^5\text{ cells cm}^{-2}$ for TUD-1 (see also Figure 3). There is no difference in the appearance of typical endothelial markers as von Willebrand factor or *Ulex eur.* Lectin I (data not shown) indicating comparable differentiation status in both media formulations. Figure 3 compares the long-term cultivation of HUVEC in serum-free TUD-1, respectively, serum-containing medium: obviously the serum-free medium performs as well as the usual medium. Cultivation periods of

Figure 3. Long-term cultivation of HUVEC HUVEC in TUD-1 and in serum-containing medium. Two separate cultures are started with HUVEC from a cord preparation. One culture is cultivated in TUD-1 medium (black bars) the other in serum-containing medium (open bars). Cultures are passaged every 4–5 days with reseeding at a cell density of 20 000 cells cm⁻². The bars represent the final cell density before splitting of the culture as the arithmetic mean of three individual flasks; error bars depicts the SEM.

Figure 4. Effect of conditioning TUD-1 medium for growth at low cell densities. HUVEC are seeded at the indicated cell densities (cells cm⁻²) and cultivated either in pure TUD-1 (open bars) or in TUD-1 supplemented with 50% (v/v) conditioned medium (hatched bars). After 4 days cell growth is determined by the DAPI-method. The bars represent the arithmetic mean of three individual wells; error bars depicts the SEM.

51 days are reached with 21, respectively 23, cumulative population doublings in serum-free respectively serum-containing medium. The medium performance does not depend on a single cell donor. We used a great number of different cord preparations during the establishment of the medium. Figure 1 is one of 3 tests with cells from 3 different donors, the same holds true for Figure 4. Figure 3 is one of 4 long-term cultivation tests each performed with cells of a different donor.

We noticed only two functional differences between normal and serum-free medium. Whereas normal medium allows cultivation of HUVEC even at extremely low seeding densities – 50 cells cm⁻² – the serum-free cultivation with TUD-1 requires seeding densities of 10 000–20 000 cells cm⁻² for long-term cultivation. For individual assays seeding densities may be lowered to 5000 cells cm⁻². Figure 4 shows that supplementation of TUD-1 with conditioned TUD-1 medium improves growth at low cell densities indicating that an essential serum component has not been substituted by the formulation of TUD-1. This component seems to be secreted by HUVEC but the necessary concentrations for growth promoting activity are only achieved at higher cell densities. The other difference concerns the sensitivity of HUVEC cultures against stress. Once the culture has been stressed by some disadvantageous environmental events, TUD-1 is no longer sufficient for vigorous cell growth. For example commercial HUVEC suppliers initiate HUVEC cultures with 4–5 flasks (75 cm²) from just one cord preparation, so they can sell a higher number of lots as primary cultures. This extreme culture expansion excludes use of serum-free TUD-1. However the normal cell preparation starting with one 25 cm⁻² flask for the cells of a cord preparation allows satisfying culturing with serum-free TUD-1 as described above. In this context we want to stress that the correct handling of serum-free HUVEC cultures is extremely important and should be done exactly as described here; any ‘shortcuts’ and ‘simplifications’ are fatal!

Formulation of the basal medium. The medium TUD-1 is a further development of a previously described medium optimized for the growth of HUVEC with IF as basal medium (Friedl et al., 1989). The basal medium IF – a 1:1 mixture of Iscove’s MDM and Ham’s F12 – had already been shown to be useful for the formulation of serum-free media for technical purposes (Jäger V et al., 1988). The medium composition of TUD-1 is listed in Table 1. Extraordinary features are the highly elevated levels of glutamin – 9 mM – and serine – 0.726 mM – as well as the inclusion of acetylcysteine – 0.25 mM – and phosphoascorbic acid – 1 mM. The latter two components were already described to be beneficial for the sustaining confluent BAEC cultures with an appropriate morphology (Sunada et al., 1993). The high demand for glutamine and serine could be explained by an highly increased *de novo* synthesis of nucleotides if

one takes into account the considerable secretion of glutamate and glycine by HUVEC (Schrimpf et al., 1994) hinting at metabolic exploitation of glutamine by glutaminamidotransferases and serine by the glycinehydroxymethyltransferase.

Acknowledgements

We are grateful to the helpful midwives of the Städtischen Kliniken Darmstadt (Darmstadt/Germany) for providing human umbilical cord veins.

References

- de Groot PG, Willems C, Gonsalves MD, van Aken WG and van Mourik JA (1983) The proliferation of human umbilical vein endothelial cells in serum-free medium. *Thromb Res* 31: 623–634.
- Friedl P, Tatje D and Czapla R (1989) An optimized culture medium for human vascular endothelial cells from umbilical cord veins. *Cytotechnology* 2: 171–179.
- Fujii DK, Cheng J and Gospodarowicz D (1983) Phosphatidyl choline and the growth in serum-free medium of vascular endothelial and smooth muscle cells, and corneal endothelial cells. *J Cell Physiol* 114: 267–278.
- Gimbrone MA, Shefton EJ and Cruise SA (1978) Isolation and primary culture of endothelial cells from human umbilical vessels. *Tissue Culture Ass Man* 4: 813–817.
- Gorfien S, Spector A, DeLuca D and Weiss S (1993) Growth and physiological functions of vascular endothelial cells in a new serum-free medium (SFM). *Exp Cell Res* 206: 291–301.
- Gospodarowicz D, Vlodavsky I and Savion N (1981) The role of fibroblast growth factor and the extracellular matrix in the control of proliferation and differentiation of corneal endothelial cells. *Vision Res* 21: 87–103.
- Hoshi H and McKeethan WL (1984) Brain- and liver cell-derived factors are required for growth of human endothelial cells in serum-free culture. *Proc Natl Acad Sci USA* 81: 6413–6417.
- Jaffe EA, Nachman RL, Becker CG and Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745–2756.
- Jäger V, Lehmann J and Friedl P (1988) Serum-free growth medium for the cultivation of a wide spectrum of mammalian cells in a stirred bioreactor. *Cytotechnology* 1: 319–329.
- Knedler A and Ham RG (1987) Optimized medium for clonal growth of human microvascular endothelial cells with minimal serum. *In Vitro Cell Dev Biol* 23: 481–491.
- Maciag T, Hoover GA, Stemerman MB and Weinstein R (1981) Serial propagation of human endothelial cells in vitro. *J Cell Biol* 91: 420–426.
- Schrimpf G, Schröder M, Weitnauer E and Friedl P (1994) Metabolic rates of endothelial cells in vitro. *Cytotechnology* 16: 43–50.
- Sunada H, Masuda M and Fujiwara K (1993) Preservation of differentiated phenotypes in cultured aortic endothelial cells by malotilate and phosphoascorbic acid. *Eur J Cell Biol* 60: 48–56.
- Vlodavsky I, Johnson LK, Greenburg G and Gospodarowicz D (1979) Vascular endothelial cells maintained in the absence of fibroblast growth factor undergo structural and functional alterations that are incompatible with their in vivo differentiated properties. *J Cell Biol* 83: 468–486.
- Weiss TL, Selleck SE, Reusch M and Wintroub BU (1990) Serial subculture and relative transport of human endothelial cells in serum-free, defined conditions. *In Vitro Cell Dev Biol* 26: 759–768.