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Scale up cultivation of primary human umbilical vein endothelial cells on microcarriers from spinner vessels to bioreactor fermentation

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Abstract

Five types of dextran-based microcarriers (Dormacell™, Pfeifer and Langen) with different concentrations of dimeric DEAE anion-exchange groups (nitrogen contents from 1.2 up to 2.9%) were tested as growth substrates for the cultivation of human umbilical vein endothelial cells (HUVECs). All microcarriers were gelatinized before use to improve cell adhesion. The one with the highest DEAE-group density was found to be most suitable for HUVEC propagation reaching final cell densities of 8×10^5 viable cells ml^{-1} (95% viability) using microcarrier concentrations of 3 g l^{-1}. Furthermore, metabolic data of glucose/lactate and amino acid metabolism are presented in this study. The concentrations of 18 amino acids were monitored throughout cultivation. A considerable decrease of glutamine and inverse increase of glutamate was observed. Cultivation with initial glucose concentration of 16.5 mmol l^{-1} resulted in high glutamine consumption rates, whereas high glucose-supplemented starting culture medium (30 mmol l^{-1}) gave considerably lowered rates, indicating altered glutamine metabolism due to different glucose feeding. The glucose consumption and lactate production rates increased 2.6 fold and 3.5 fold, respectively, due to switch over from low to high glucose supplemented cultures. The rate of glucose metabolism was found not to be directly related to cell growth, because almost identical growth rates and doubling times were obtained. Considering the remaining 16 amino acids measured, serine concentrations considerably declined and glycine as well as alanine concentrations raised strongly. Most amino acid values were found insignificantly altered during 14 days of cultivation. Spinner vessel cultures served as inoculum for up scale propagation of HUVECs in membrane stirred 2 liter bioreactors. About 5×10^9 HUVECs were produced, which were used for the isolation and structural characterization of glycosphingolipids, cell membrane compounds, which are suggested to be involved in e.g. selectin-carbohydrate interaction (cell-cell adhesion), carcinogenesis and atherogenesis.

Abbreviations: HUVECs, human umbilical vein endothelial cells; PBS, phosphate buffered saline

Introduction

Endothelial cells play an important role in the regulation of blood coagulation, inflammation, and immune response (Jaffe, 1987; Fajardo, 1989). Model systems for *in vitro* studies of vascular function, physiology and biochemistry have been developed (Audus *et al.*, 1990). Due to the huge biosynthetic capacity of endothelial cells and their involvement in various inflammatory reactions in the vascular system (Pober and Cotran, 1990), there is an increasing medical and biotechnological interest in products from these cells. Human umbilical vein endothelial cells (HUVECs) are an appropriate source of primary cells which like all normal human diploid cell types show a finite life span *in vitro* and require growth factors for proliferation and

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long term cultivation (Hoshi and McKeehan, 1984). Optimized media formulation for the maintenance of HUVECs (Friedl *et al.*, 1989) and successful propagation of endothelial cells on various types of microcarriers (Schrimpf and Friedl, 1993) have been reported. In the latter study, various solid and macroporous microcarriers were investigated as growth support for the cultivation of HUVECs. In our study dextran based microcarriers carrying different amounts of dimeric DEAE-groups on their surface (Dormacell™, Pfeifer and Langen) were compared for their ability to enable growth of HUVECs. The one with the highest anion-exchanger concentration was found to be optimal and therefore used for production of HUVECs from spinner up to 2 liter bioreactor scale. Glycosphingolipids, predominantly expressed on the plasma membrane of animal cells, are suggested to play an important role e.g. in the pathogenesis of immuno suppression and atherosclerosis (Bergelson, 1995). Although the importance of glycosphingolipids as cell surface antigens and receptors has received increasing attention in recent years, only little information is available about these membrane components of endothelial cells (Gillard *et al.*, 1987), most likely due to the lack of sufficient cell material for their detailed structural characterization. In this study we report on the large scale cultivation of HUVECs on Dormacell™ microcarriers, the physiological parameters raised throughout spinner and bioreactor cultivation and explain the biological relevance of the glycosphingolipids isolated from HUVECs (Duvar *et al.*, 1995).

Materials and methods

Cells and culture conditions

Primary human umbilical vein endothelial cells (HUVECs) were isolated from one umbilical cord vein according to the procedure described by Gimbrone *et al.* (1974) and the modifications of Friedl *et al.* (1989). HUVECs were cultivated in a 1:1 mixture of Iscove's MDM and Ham's F12 basal medium (Gibco BRL, Eggenstein, Germany), and the medium was supplemented with 20% (v/v) human serum (German Red Cross Blood Transfusion Service, Springe, Germany), 1.25 $\mu g\,l^{-1}$ human basic fibroblast growth factor (FGF, Sigma), sodium heparin (Serva, Germany), 2 $mmol\,l^{-1}$ glutamine, 12.5 $\mu mol\,l^{-1}$ ß-mercaptoethanol, 5 $mg\,l^{-1}$ iron saturated human transferrin (Behring Werke AG, Marburg,Germany), 2.1 $g\,l^{-1}$ $NaHCO_3$, 65 $mg\,l^{-1}$ benzylpenicillin and 100 $mg\,l^{-1}$ streptomycin sulfate (= culture medium). The culture medium for the Superspinner cultures was additionally supplemented with 2 $mmol\,l^{-1}$ glutamine and 200 $\mu mol\,l^{-1}$ serine. The bioreactor culture medium contained enhanced glucose concentrations of 30 $mmol\,l^{-1}$. All tissue culture flasks and microcarriers (see below) were coated with an aqueous 0.1% (w/v) gelatine solution for 30 minutes at room temperature; the gelatine solution was aspirated and the flasks or microcarriers were once washed with phosphate buffered saline (PBS). The cells were routinely passaged by trypsinization with 0.05% trypsin and 0.02% EDTA in Puck's saline A (Gibco; w/w/v). Cell numbers were determined in duplicate by trypan blue exclusion with a hemocytometer.

The cultures in conventional culture flasks and spinner vessels were incubated at 37 °C in a humidified 5% CO_2-air atmosphere. The same gas mixture (5% CO_2, 95% air) was used for the aeration of the Superspinner cultures. In the bioreactor the temperature was set to 37 °C, pH to 7.2 and pO_2 to 30% air saturation.

Analytical methods

Glucose and lactic acid concentrations were determined with an automatic analyzer system based on enzymatic and electrochemical reactions (YSI 2700, Yellow Springs Instruments, OH, USA).

Amino acids of cell-free culture supernatants were analyzed with an automated reversed phase high performance liquid chromatography system (RP-HPLC) with precolumn derivatization using the o-phthaldialdehyde method (Büntemeyer *et al.*, 1991, Müthing *et al.*, 1996).

All data given in the figures represent mean values of double measurements.

Equations for the calculation of cell specific parameters

Specific parameters for cell growth, substrate utilization and product formation were calculated following the equations according to Pirt (1985) and as described by Büntemeyer *et al.* (1991).
Cell growth

$$\mu = \frac{1}{x}\frac{dx}{dt}\ [d^{-1}]$$

Doubling time

$$t_d = \frac{\ln 2}{\mu}\ [d]$$

Specific substrate utilization

$$q_s = -\frac{1}{x}\frac{dc_s}{dt}\ [\text{mol cells}^{-1}\ \text{d}^{-1}]$$

Specific production

$$q_p = \frac{1}{x}\frac{dp}{dt}\ [\text{mol cells}^{-1}\ \text{d}^{-1}]$$

Growth yield

$$Y_{x/s} = \frac{\mu}{q_s}\ [\text{cells mol}^{-1}]$$

Symbols

x	viable cell density	[cells ml^{-1}]
t	time	[d]
μ	specific growth rate	[d^{-1}]
c_s	substrate concentration	[mol l^{-1}]
q_s	specific substrate utilization rate	[mol $cells^{-1}$ d^{-1}]
p	product concentration	[mol l^{-1}]
q_p	specific production rate	[mol $cells^{-1}$ d^{-1}]
$Y_{x/s}$	growth yield	[cells mol^{-1}]

Microcarriers

All glass ware was silanized with a 4% (v/v) solution of dimethyldichlorosilane in carbon tetrachloride to prevent sticking of cells and beads to the walls.

Dormacell™ microcarriers (Pfeifer & Langen, Dormagen, Germany), consisting of a solid neutral cross-linked dextran matrix and substituted with N,N-diethylaminoethyl (DEAE)-groups, were used. The positively charged dimeric DEAE-groups are covalently linked to the surface of the beads. Overall charge capacity of this type of microcarriers is reduced compared to other commercially available beads (Butler, 1988). Five different types of microcarriers, differing in their respective nitrogen contents and anion exchange capacities, were employed in this study. Their main physical properties have been recently explained (Müthing *et al.*, 1996). The density in PBS amounts to 1.05 g ml^{-1} for all microcarrier types. 1 g of dry beads corresponds to about 6.2×10^6 microcarriers with a surface of 7000 cm^2 (Butler, 1988) which relates to the surface of 40 culture flasks of 175 cm^2, each.

The microcarriers, obtained in a dry state, were swollen in sterile PBS (pH 7.2) overnight at room temperature. Thereafter, the preswollen beads were washed three times with PBS and autoclaved (121 °C, 20 min). Before use, the microcarriers were washed twice with prewarmed culture medium containing 10% (v/v) human serum.

Microcarrier cultures in conventional spinner vessels

Five hundred ml (total volume) spinner vessels (Techne, Duxford, Cambridge, UK) were used with working volumes between 100 and 300 ml. The impeller of this type of spinner vessel consists of a bulb-shaped rod with an integrated magnet (Hirtenstein *et al.*, 1982). Agitation is attained on common magnet spinner stations and optimal culture mixing is achieved in vessels with a rounded, indented base. Spinner vessels were supplied with 35 ml of culture medium and placed overnight in a CO_2 incubator to allow gas and temperature equilibration. The inoculum was obtained by trypsinizing exponentially grown HUVECs from culture flasks (see above). Enzyme incubation was stopped by adding excess culture medium with 20% (v/v) human serum, and cells were seeded in a ratio of ca. 4 to 5 viable cells per microcarrier. During the first 6 h the spinner vessels were stirred for 2 min in intervals of 30 min. After this initial phase, the spinner cultures were agitated continuously at 30 rpm, filled up with fresh culture medium to the final volume of 100 ml 24 h after inoculation, and continued with 3 g l^{-1} microcarrier concentrations.

For monitoring cell growth on microcarriers, 1 ml samples were withdrawn from a well-mixed spinner vessel and colonized beads were washed twice with PBS. The sediment was then taken up in 1 ml trypsin-EDTA solution (see above) and incubated for 15 min at 37 °C in a waterbath with continuous shaking. The enzyme incubation was stopped by addition of culture medium containing 20% (v/v) human serum. Cells and beads were separated by sedimentation at unit gravity. Free cells were recovered in the supernatant and washed with culture medium before counting by trypan blue exclusion.

Microcarrier culture in a Superspinner vessel

A Superspinner is composed of a 1 liter Duran flask with integrated membrane stirrer for bubble free aeration which consists of 2 m polypropylene hollow fiber membrane tubing and a membrane pump for the gas supply (Heidemann *et al.*, 1994). The Superspinner was used alternatively to the above described culti-

vation in conventional spinner vessels for inoculum production of bioreactors (see below). The Superspinner flask was provided with 200 ml culture medium and 3 g of microcarriers. Inoculation was performed with *ca.* 5 viable cells per bead. Within the first 12 h, the Superspinner was stirred for 2 min in intervals of 20 min. After the initial phase, the suspension was agitated continuously at 30 rpm. At 24 h cultivation time the Superspinner was filled up to the final volume of 1 liter with a final density of 3 g beads per liter and the stirring speed was increesed to 40 rpm to prevent settling of beads and to maintain homogeneous distribution.

Bioreactor inoculum

To provide an appropriate and sufficient inoculum for large scale propagation of HUVECs in a bioreactor, at least 3 conventional spinner cultures with subconfluent microcarriers (3 g l^{-1}) were employed for a 2 liter bioreactor with a desired microcarrier concentration of 3 g l^{-1}. First, the medium was removed from the spinner cultures by low speed centrifugation (5 min at 111xg). The beads were then washed with PBS, followed by low speed centrifugation and replacement of PBS by trypsin-EDTA solution. After 5–10 min incubation at 37 °C with occasional gentle shaking and using microscopic examination to monitor detachment of cells from microcarriers, enzyme incubation was stopped by addition of culture medium. The beads were allowed to settle and the supernatant with free cells was withdrawn, centrifuged (5 min at 111xg) and resuspended in fresh culture medium. Such cell preparation was used as inoculum for cultivation in a 2 liter bioreactor described below.

Microcarrier bioreactor culture

Cultivation was carried out in a Biostat BF bioreactor with a 2 liter culture vessel (B. Braun International, Melsungen, Germany) equipped with a membrane stirrer for bubble free aeration which consists of 5 m polypropylene hollow fiber membrane tubing (Lehmann *et al.*, 1987; Lehmann *et al.*, 1988).

The bioreactor vessel was provided with 500 ml culture medium and 6 g of microcarriers which corresponded to a final density of 3 g beads per liter after replenishment of the bioreactor (see below). Inoculation was performed with ca. 7 viable cells per bead; exact cell numbers depended on cell yields from spinner vessels, which served as inoculum cultures. Within the first 12 h, the bioreactor culture was stirred for 2 min in intervals of 20 min. After this initial phase, the suspension was agitated continuously at 30 rpm. At 48 h cultivation time the bioreactor was filled up to its final volume of 2 liters and the stirring speed was increased to 50 rpm to prevent settling of beads and to maintain homogeneous distribution.

Results

Growth of HUVECs on Dormacell^{TM} microcarriers with differing nitrogen content

In a first set of experiments five types of solid dextran-based microcarriers, all differing in their respective nitrogen content, i. e. anion exchange capacity, were tested as a growth support for the cultivation of HUVECs in conventional spinner vessels. The physical properties of the different microcarriers (manufacturers data) have been described elsewhere (Müthing *et al.*, 1996). Cells were seeded in densities of 2×10^5 cells ml^{-1} together with 3 g l^{-1} of microcarriers, both calculated as final concentrations and corresponding to ca. 5 cells per bead. After 24 h the spinner vessels were filled up to the final volume of 100 ml with complete culture medium. The propagation on microcarriers is characterized by a short period of bead colonization, growth phase and the stage of confluence as illustrated for all five types of microcarriers in Fig. 1. A maximum final cell density of 8×10^5 cells ml^{-1} was obtained with the type of microcarriers with the highest nitrogen content. Notable microheterogeneity was observed within spinner cultures by light microscopy visual control showing that all five types of microcarriers had approximately 10% beads without cells.

Growth and metabolic parameters of HUVECs propagated on various types of microcarriers

Cultivation parameters of conventional spinner cultures with different types of microcarriers from Fig. 1 are summarized in Table 1. Calculations were performed for the periods of early exponential growth within time intervals from t = 24 to t = 72 h. Deduced from growth curves of Fig. 1, microcarriers with highest density of dimeric DEAE-groups (type 2.9% nitrogen) were chosen for further upscale cultivation experiments of HUVECs.

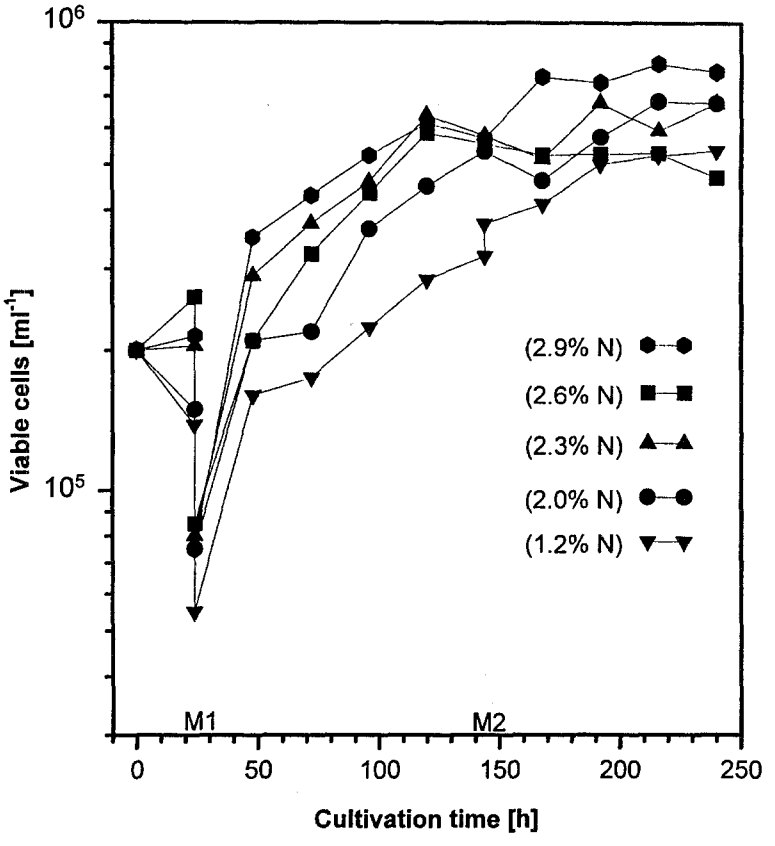

Figure 1. Growth of HUVECs on various types of Dormacell™ microcarriers with differing nitrogen content. Cells were cultivated in spinner vessels with microarrier concentrations of 3 g l^{-1}, respectively. 24 h after initial cultivation in volumes of 35 ml, vessels were completed to the final volumes of 100 ml, each. M1: medium fill up at t = 24 h; M2: medium replenishment to 100 ml at t = 144 h.

Table 1. Growth and metabolic parameters of HUVECs propagated on Dormacell™ microcarriers with differing nitrogen content[a]

Microcarrier type (nitrogen content in %)		1.2	2.0	2.3	2.6	2.9
Growth rate μ	[d^{-1}]	0.53	0.50	0.65	0.58	0.70
Doubling time t_d	[d]	1.31	1.39	1.07	1.20	0.99
Glucose utilization q_s	[μmole 10^{-4}cells d^{-1}]	0.07	0.04	0.03	0.04	0.03
Lactate production q_p	[μmole 10^{-4}cells d^{-1}]	0.06	0.06	0.04	0.05	0.04
Growth yield $Y_{X/S}$	[10^4cells μmole^{-1}]	8.15	14.29	25	15.68	26.92

[a] Calculated within intervals of exponential growth from t = 24 h to t = 72 h; cultivations were performed in conventional spinner vessels with microcarrier concentrations of 3 g l^{-1}.

Growth and metabolism of HUVECs propagated on Dormacell™ microcarriers (type 2.9% nitrogen) in a Superspinner

As an example for a Superspinner cultivation, amounts of viable and dead cells as well as glucose and lactate concentrations throughout a 10 day Superspinner culture are shown in Figs. 2A and 2B, respectively. Cells were grown on microcarriers (type 2.9% nitrogen) at a concentration of 3 g l^{-1}. In this experiment, final densities of about 8×10^5 viable cells ml^{-1} (95%) and 4×10^4 dead cells ml^{-1} (5%) were obtained (Fig. 2A). After 10 d cultivation the glucose concentration dropped down from 16.5 to 4.5 mmol

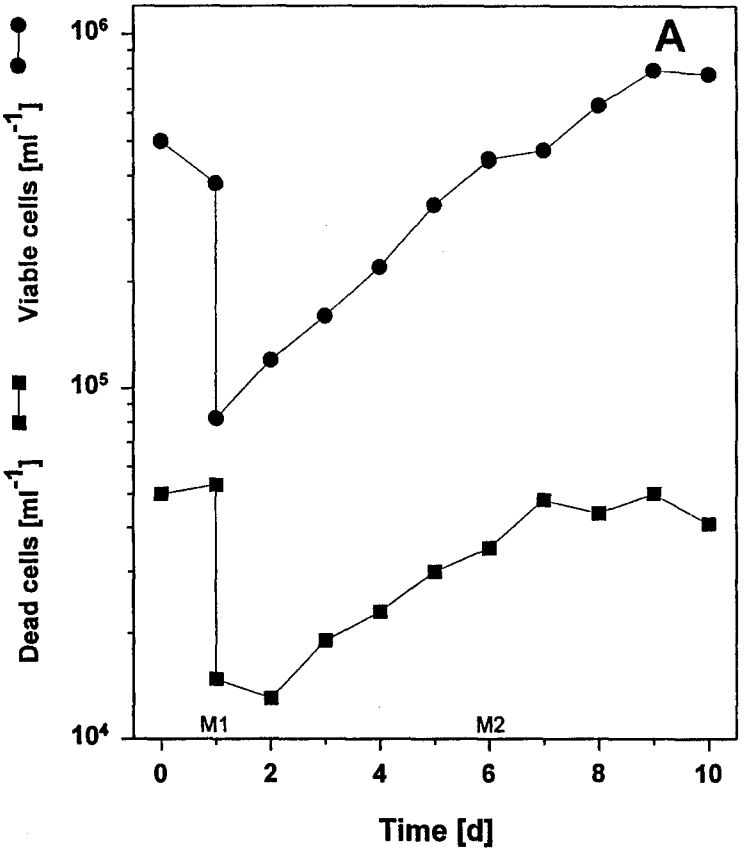

Figure 2a. Growth (A) and metabolite concentrations (B) of HUVECs propagated on 3 g l^{-1} Dormacell^{TM} microcarriers with 2.9% nitrogen content in a Superspinner vessel. The culture was started with 200 ml and filled up to the final volume of 1 liter 24 h after initiation. M1: medium fill up at t = 24 h; M2: medium replenishment to 1 liter at t = 144 h.

Table 2. Synopsis of metabolic data of HUVECs grown on Dormacell^{TM} microcarriers (type 2.9% nitrogen) in membrane stirred Superspinner and bioreactor vessels

Vessel		Superspinner[a]	Bioreactor[b]
Volume	[ml]	1000	2000
Microcarrier	[g l^{-1}]	3	3
Growth rate μ	[d^{-1}]	0.16	0.13
Doubling time t_d	[d]	4.33	5.33
Glucose consumption q_s	[μmol 10^{-6} cells d^{-1}]	0.68	1.80
Lactate production q_p	[μmol 10^{-6} cells d^{-1}]	0.73	2.58
Glutamine consumption q_{s*}	[μmol 10^{-6} cells d^{-1}]	0.496	0.162
Glutamate production q_{p*}	[μmol 10^{-6} cells d^{-1}]	0.028	0.146

[a] Low glucose culture with initial 16.5 mmol l^{-1}; values were calculated within intervals of late exponential growth from t = 7 d to t = 10 d.
[b] High glucose culture with initial 30 mmol l^{-1}; calculation was performed during late exponential growth from t = 9 d to t = 14 d.

l^{-1} and lactate concentrations inversely increased from 0.5 to 9 mmol l^{-1}. The metabolic data of this low glucose Superspinner culture with initial 16.5 mmol l^{-1}, obtained during late exponential growth within time intervals from t = 7 d to t = 10 d, are summarized in Table 2. Considering the concentrations of 18 amino

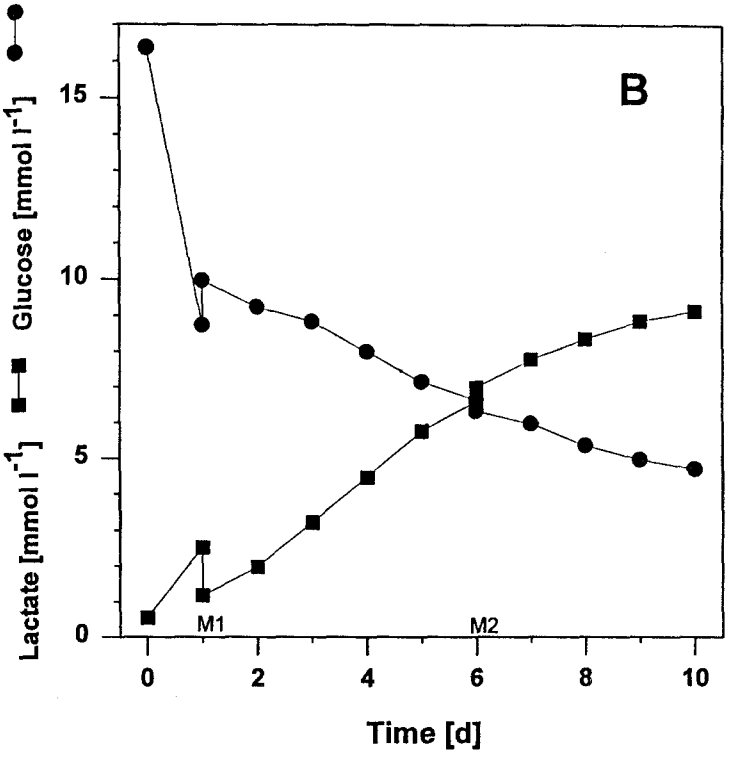

Figure 2b.

acids which were monitored throughout the 10 d cultivation, a considerable decrease in glutamine from 2.9 to 1.2 mmol l^{-1} and inverse increase of glutamate from 250 to 750 μmol l^{-1} were observed (Fig. 3). Absolute amounts of glutamate production (0.5 mmol l^{-1}) and glutamine consumption (1.7 mmol l^{-1}) were calculated as a glutamate/glutamine – quotient and gave a value of 0.3. This value indicates high glutamine consumption and low glutamate production rates. Glutamine consumption and glutamate production were specified and calculated from t = 7 d to t = 10 d as shown in Table 2. Beside glutamine, serine was found to be the second most consumed amino acid. The concentration of serine dropped from 340 μmol l^{-1} persisting at a low level of approximately 100 μmol l^{-1} (Fig. 3). Alanine was produced to a maximum of 300 μmol l^{-1} from initial amounts of 220 μmol l^{-1} after 10 days, indicating a reciprocal metabolic course compared to serine decrease. The glycine content also increased from about 200 μmol l^{-1} to more than 500 μmol l^{-1}. Most amino acid values were found to be insignificantly altered during 10 days cultivation and the cells maintained almost constant levels throughout cultivation. Some amino acids showed low but trivial decreasing rates.

Large scale production of HUVECs on Dormacell^{TM} microcarriers (type 2.9% nitrogen) in a 2 liter bioreactor

The production of large quantities of anchorage dependent cells is limited by the surface area available as support for cell monolayers. The surface area/volume ratios obtained by use of 3 g l^{-1} of microcarriers in spinner vessels and bioreactors were recently displayed by Müthing *et al.* (1996). One batch with a volume of 2 liters containing 6 g of microcarriers equals the surface area of 240 conventional culture flasks of 175 cm^2, each. Deduced from spinner culture experiments presented in this study (see above), microcarriers with the highest nitrogen content (2.9%) in a concentration of 3 g l^{-1} were selected for large scale production of HUVECs in a 2 liter bioreactor. Inoculation and colonization of the beads was carried out in 500 ml initial volume with interval stirring for 2 min at 30 rpm every 20 min for 24 h. After this colonization phase the suspension was continuously agitated for 24 h. Thereafter,

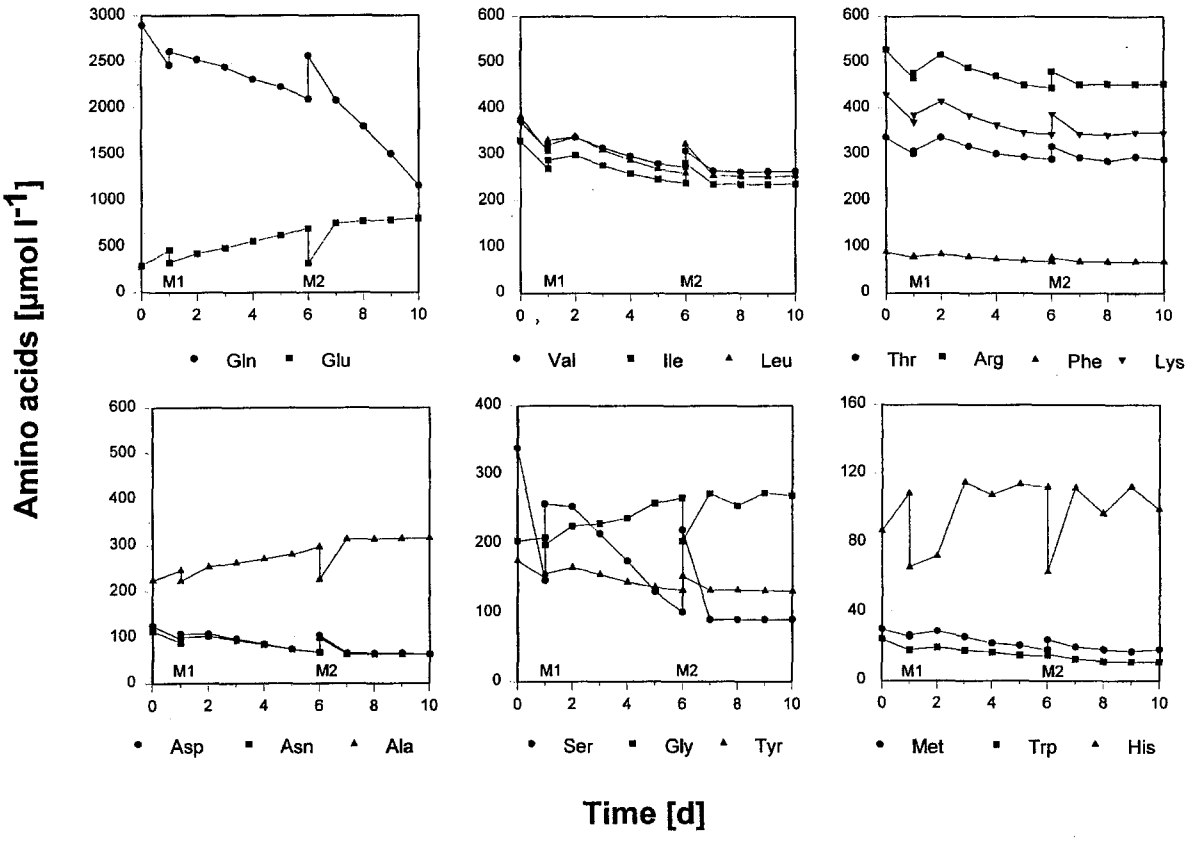

Figure 3. Amino acid concentrations during cultivation of HUVECs on 3 g l^{-1} Dormacell™ microcarriers with 2.9% nitrogen content in a Superspinner vessel. The culture was started with 200 ml and filled up to the final volume of 1 liter 24 h after initiation (see Fig. 2). M1: medium fill up at t = 24 h; M2: medium replenishment to 1 liter at t = 144 h.

the bioreactor was filled up to 2 liter working volume and further stirred at 50 rpm.

Growth and metabolism of HUVECs propagated on Dormacell™ microcarriers (type 2.9% nitrogen) in a bioreactor

As an example, one bioreactor cultivation out of three almost identical experiments is presented. In Fig. 4 growth (A) and metabolite concentrations (B) of HUVECs propagated on beads in a 2 liter bioreactor (3 g l^{-1}, type 2.9% nitrogen) are shown. The culture was maintained over a period of 14 d and reached a final density of 8×10^5 viable cells ml^{-1} (95%) and 5×10^4 dead cells ml^{-1} (5%). Glucose concentrations decreased from 30 mmol l^{-1} to 11.5 mmol l^{-1} and lactate concentrations inversely increased from 1.5 to 19 mmol l^{-1} as expected from previous spinner cultivations (see above). The metabolic data of this high glucose bioreactor culture with initial 30 mmol l^{-1}, obtained during late exponential growth within time intervals from t = 9 d and t = 14 d, are summarized in Table 2. Amino acid monitoring indicated considerable decrease of glutamine from 2.3 mmol l^{-1} to 1.5 mmol l^{-1} and inverse increase of glutamate from 250 to 1400 µmol l^{-1} throughout 14 d cultivation (Fig. 5). A quotient of 1.4 was calculated from amounts of absolute glutamate production (1.15 mmol l^{-1}) and glutamine consumption (0.8 mmol l^{-1}). This value indicates low glutamine consumption and high glutamate production rates. According to the Superspinner cultivation (see above), glutamine consumption and glutamate production were specified from t = 9 d and t = 14 d as shown in Table 2. Considering the remaining 16 amino acids measured throughout bioreactor cultivations, serine concentrations considerably decreased and glycine as well as alanine concentrations strongly increased according to the results obtained by Super-

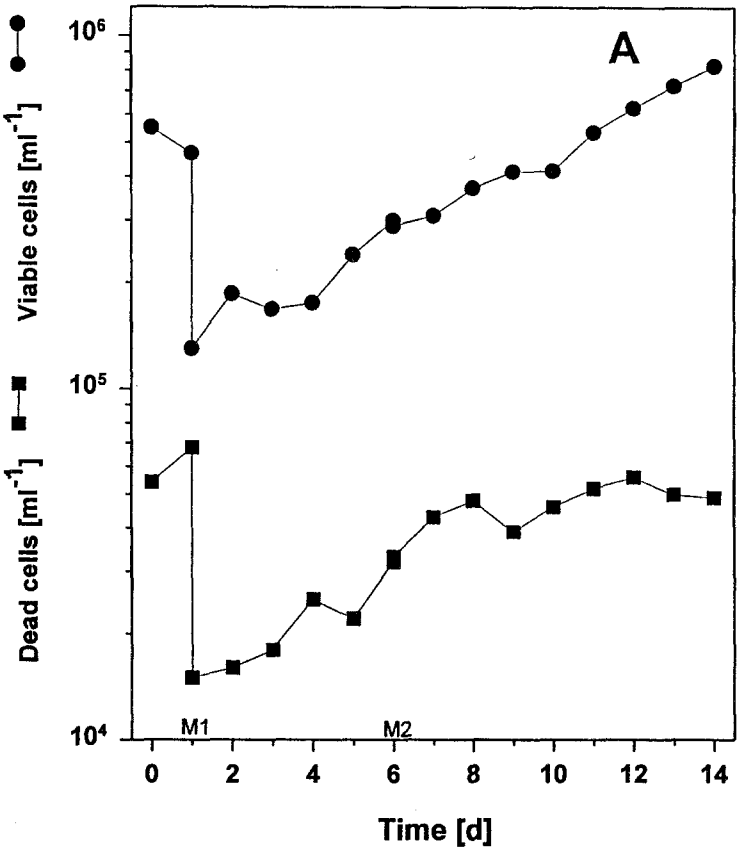

Figure 4a. Growth (A) and metabolite concentrations (B) of HUVECs cultivated on 3 g l^{-1} Dormacell^{TM} microcarriers with 2.9% nitrogen content in a 2 liter bioreactor. M1: medium fill up at t = 24 h; M2: medium replenishment to 2 liter at t = 144 h.

spinner cultivations (see above). Again, most amino acid values were found only insignificantly altered during 14 d cultivation. However, some amino acids showed low but trivial decreasing values.

In summary, 4.85×10^9 cells were produced with 3 bioreactor batch cultivations and the produced endothelial cells were used for isolation and structural characterization of their glycosphingolipid composition (Duvar *et al.*, 1995).

Discussion

Optimal attachment and growth of HUVECs were obtained with beads of highest nitrogen content (2.9% nitrogen) and maximum final cell densities of 8×10^5 viable cells ml^{-1} (95% viable cells) were acquired using microcarrier concentrations of 3 g l^{-1}. This type has been previously reported for successful amplification of CHO-cells (Jacobs *et al.*, 1991) and for propagation of bovine aortic endothelial cells (Müthing *et al.*, 1996). Details concerning physical properties, handling *etc.* of Dormacell^{TM} microcarriers have been reported in the latter reference. Various types of microcarriers have been tested by Schrimpf and Friedl (1993) as growth substrate for the cultivation of HUVECs and an immortalized cell line of endothelial origin. Solid (Cytodex type I, II, III, Gelibeads, Mica) and macroporous (Polyhipe, CultiSpher GL, PolyporE type I) microcarriers were probed for their capability of providing support for endothelial cells. To complement these investigations, in this study we provide growth data achieved with five kinds of microcarriers varying in their respective density of dimeric DEAE-groups on their surface (from 1.2% up to 2.9% nitrogen content, see Müthing *et al.*, 1996).

Concerning the metabolic data of HUVECs presented in this work, hypoglycemia rapidly develops in cultures of HUVECs as reported earlier by Jaffe (1992). To avoid glucose deprivation bioreactor cul-

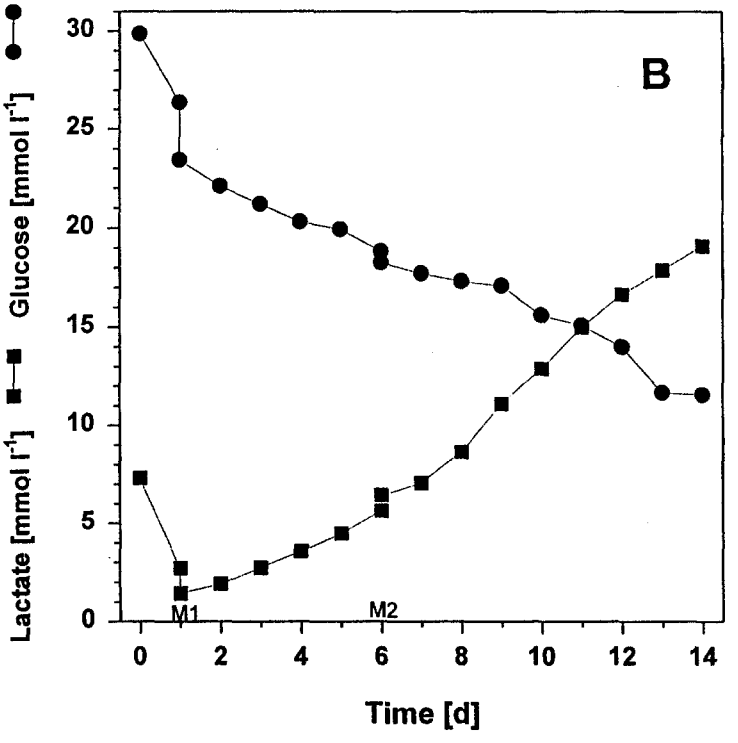

Figure 4b.

tures were supplemented to initial concentrations of about 30 mmol l^{-1} glucose compared to Superspinner cultures initiated with 16.5 mmol l^{-1} glucose. A concomitant 2.6 fold and 3.5 fold glucose consumption and lactate production rate, respectively, were the consequences of this additional carbohydrate feeding (see Table 2). High consumption rates of glutamine were observed in HUVEC cultures reflecting its assumed role as a major energy source, also reported by others in earlier studies with serveral different kinds of mammalian cells (Reitzer *et al.*, 1979; Zielke *et al.*, 1984a; Zielke *et al.*, 1984b). Interestingly, the glutamine consumption decreased about 3 fold from 0.496 to 0.162 [μmol 10^{-6} cells d^{-1}] whereas the glutamate production increased about 5fold from 0.028 to 0.146 [μmol 10^{-6} cells d^{-1}] switching from low to high glucose supplemented cultures (see Table 2). These data confirm earlier investigations of Zielke *et al.* (1984a, 1984b) who explored the glutamine metabolism in cultured human fibroblasts. The rate of glutamine oxidation was found to be regulated by the presence of glucose, i.e. as the glucose concentration is decreased, the rate of glutamine oxidation increases (Zielke *et al.*, 1984a). The rate of glucose metabolism was found not to be directly related to cell growth, since almost identical growth rates and doubling times were obtained in low and high glucose supplemented endothelial cultures despite strongly differing glucose consumption and lactate production rates (see Table 2). This is also in good agreement to earlier fibroblast investigations (Zielke *et al.*, 1984b). The glutamate production was found in our study to be inversely correlated to the glutamine consumption (see Table 2). The first endogenous enzyme in the utilization of glutamine is glutaminase to form glutamate. It seems unlikely that glutamate is secreted from the cells and it is assumed, that the 'glutamate production' is due to chemical and/or enzymatical glutamine desamination in the 20% (v/v) human serum containing culture fluid, however a speculative interpretation at this stage of research. Beside glutamine, serine was found to be the second most consumed amino acid, whereas alanine and glycine were produced in considerable quantities according to previous data obtained from bovine aortic endothelial cells and leukocyte derived cell lines (Müthing *et al.*, 1996; Büntemeyer *et al.*, 1991). None of the 18 amino acids monitored throughout HUVEC cultivation ran

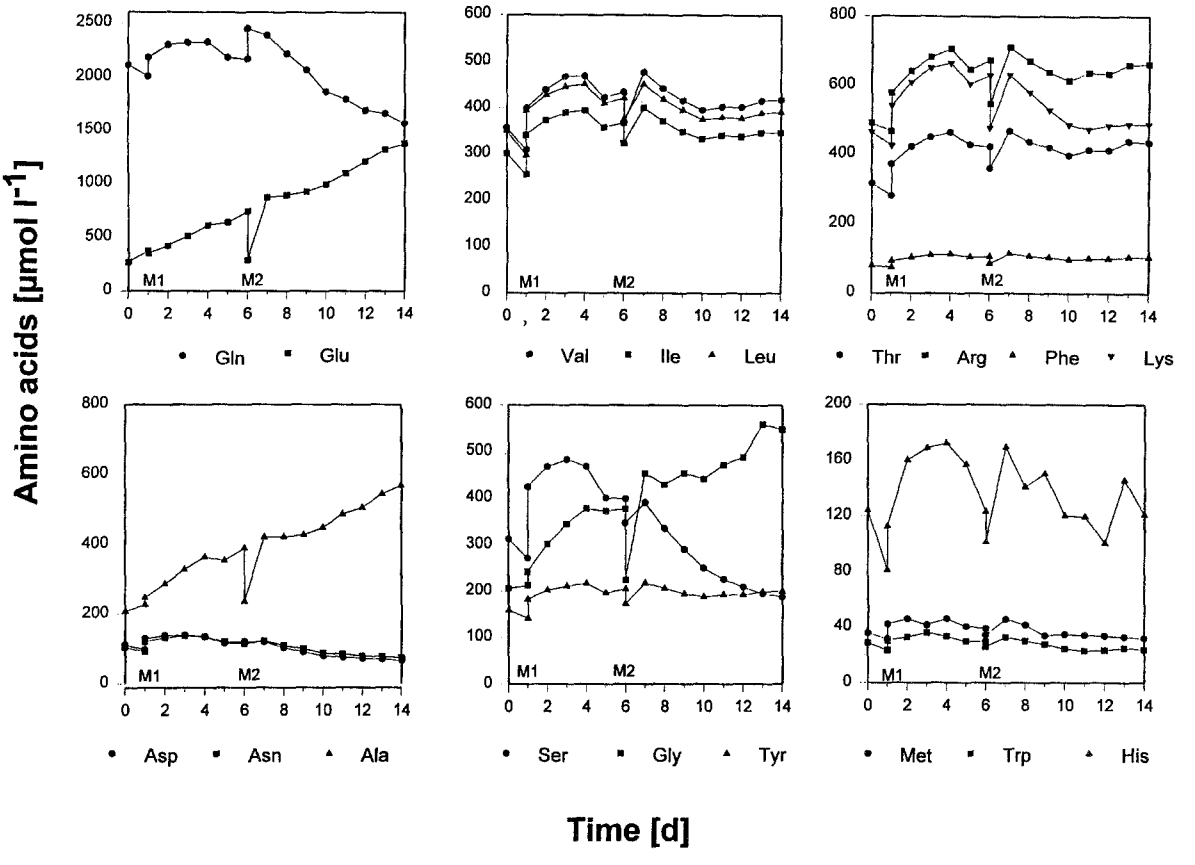

Figure 5. Amino acid concentrations during cultivation of HUVECs on 3 g l^{-1} Dormacell^{TM} microcarriers with 2.9% nitrogen content in a 2 liter bioreactor. M1: medium fill up at t = 24 h to 2 liter volume; M2: medium replenishment up to 2 liter at t = 144 h.

into limitation using the 1:1 mixture of Iscove's MDM and Ham's F12 basal medium.

Mass cultivation of endothelial cells is the only way to get adequate amounts of desired products, which can be isolated from the culture fluid (soluble products) and/or from cells themselves (e.g. membraneous compounds). For example, the fermentation of endothelial cell-surface heparan sulphates has been reported by Jerg *et al.* (1990). Due to the discovery of carbohydrate – carbohydrate (Kojima *et al.*, 1992) and carbohydrate – selectin interactions of leukocytes and endothelial cells (Lasky, 1992) there is a strong demand for membraneous compounds, particularly of endothelial cells, to elucidate structures of proteins, glycoproteins and glycosphingolipids, which are involved in cell–cell adhesion phenomena. For example, the E-selectin expressed on endothelial cells upon TNFα, IL 1 or LPS stimulation binds to various extent to Lewis^a, Lewis^x and sialyl Lewis^x carbohydrates presented by e.g. granulocytes (Rosen and Bertozzi, 1994) and provide the initial leukocyte 'rolling' on activated endothelial cells (Zimmerman *et al.*, 1992). On the other hand, there is lack of data of glycoconjugates from primary endothelial cells most likely due to limited availability of these cells (Gillard *et al.*, 1987). Thus, one aspect of our work was to produce large numbers of HUVECs to isolate and analyse their glycosphingolipid expression. We finally succeeded in the structural characterization of these cell surface antigens (Duvar *et al.*, 1995), which have also been reported to play a role in tumour metastasis (Kojima *et al.*, 1992) and in the pathogenesis of atherosclerosis (Bergelson, 1995).

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